The role of magnesium in egg shell formation and function

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THE ROLE OF MAGNESIUM IN EGG SHELL FORMATION AND FUNCTION

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ABSTRACT

The effects on egg production of feeding experimental diets low in either magnesium, manganese or copper were studied in two separate flocks of domestic hens. All experimental diets were based upon a single formulation (the basal mixture) designed to contain the lowest possible levels of the three elements.

There were no effects on group mean egg number, egg weight or shell thickness as a result of feeding diets low in either copper or manganese. In contrast, birds receiving the low-magnesium diets became hypomagnesaemic within a week and, within 3 weeks, group mean egg number and egg weight were reduced. In the same birds there was a marked decline in group mean egg shell thickness which could not be accounted for by the gradual decline associated with increasing egg size observed in other groups. Administration of low-magnesium diets also caused a reduction in the magnesium content of the egg shell, as measured by atomic absorption spectroscopy (AAS), the effect occurring within a week. There was no appreciable difference in the mean interval between successive ovipositions in groups receiving either inadequate (132, 207 ppm) or adequate (1323, 1522 ppm) amounts of magnesium in the diet. It was concluded that magnesium plays a major role in the provision of calcium during shell formation in the domestic hen. Possible consequences of this are discussed in the context of the widely reported resistance of Galliforme birds to the shell-thinning effects of DDE.

In order to study more precisely the effects of magnesium deficiency on egg shell formation, selected shells were examined

using scanning electron microscopy (SEM). Although magnesium deficiency had no detectable effect on cone numbers, sphere-like mineral deposits occurred at the cones and outer membrane fibres of a small proportion of shells produced by magnesium-depleted birds. Examination of acid-etched shells showed that there was a reduction in egg shell pore number per unit surface area in birds receiving low-magnesium diets.

The radial distributions of shell calcium and magnesium were examined using electron probe microanalysis (EPMA). In all cases, the amount of calcium remained substantially constant across the thickness of the shell. There were, however, two peaks in the small quantity of magnesium (< 0.4 wt %) present in the egg shells of birds receiving diets containing adequate amounts of this element. One peak occurred within the cone layer and the second towards the outer shell surface. The progressive rise in magnesium content across the column layer, giving rise to the second peak, was virtually absent in the shells of birds receiving the low-magnesium diets.

A precise correspondence was observed betweeen the distribution of shell magnesium and the known distribution of shell organic material. It is suggested that the characteristic distribution of magnesium in the hen's egg shell is associated with the incorporation of shell organic material.

In the context of avian reproduction, the presence of magnesium in the cone layer is probably of major importance. It was notable that the peak in cone layer magnesium content was absent from the eroded cones of successfully incubated eggs.

INTRODUCTION

The three elements, magnesium, manganese and copper all play an important role in the formation of the hen's egg shell. The involvement of magnesium in particular has received considerable attention (Staller and Sunde, 1964; Mehring and Johnson, 1965; Cox and Sell, 1967; Sell et al., 1967; Stafford and Edwards, 1973; Atteh and Leeson, 1983a). The observation that magnesium-depleted hens lay thin-shelled eggs has been taken as evidence of the element's role in the parathyroid hormone-induced resorption of medullary bone calcium which normally occurs towards the end of shell formation (Stafford and Edwards, 1973). The precise relationship between magnesium metabolism and shell formation, even in the domestic fowl, requires further study.

The laying bird's requirement for manganese has been recognised since the observation by Lyons (1939) that a deficiency of this element led to the production of rough, translucent egg shells. Hill and Mathers (1968) reported that laying birds were not susceptible to the effects of this deficiency unless a low-manganese diet was first administered pre-lay. Egg shells produced by manganese-depleted birds are often thinner than those of normal eggs (Mathers et al., 1971). This raises the question of the precise involvement of this element in egg shell deposition. Although manganese is known to be important in the synthesis of egg shell mucopolysaccharides (Longstaff and Hill, 1972; Leach and Gross, 1983) the relationship between the deposition of shell organic and mineral phases may not be a simple one in view of the

fact that different shell regions contain different amounts of organic material (Terepka, 1963b; Cooke and Balch, 1970b; Krampitz and Witt, 1979).

The essentiality of copper in the laying bird results from the element's role as a co-factor in the lysyl oxidase-induced cross-linking associated with the normal development of the shell membrane fibres (Schraer and Schraer, 1965; Baumgartner et al., 1978; Harris et al., 1980; Crombie et al., 1981). Birds fed on copper-deficient diets produce eggs with characteristically thin and wrinkled shells (Baumgartner et al., 1978). There have been few studies carried out, however, on copper-depleted hens due perhaps to the very small amount of this element constituting a normal dietary level (3.5 ppm). The effects of feeding copper-deficient diets can be exacerbated both by feeding the diets pre-lay and by increasing the level of dietary zinc (Baumgartner et al., 1978).

Clearly, magnesium, manganese and copper are all important in the synthesis and provision of major shell and shell membrane components. In Part II of the present study the changes in egg production occurring as a consequence of feeding experimental diets low in each of the three elements were studied in selected laying birds of Flock 1. Attention was then focused on the role of magnesium in egg production in a second flock of birds (Flock 2).

In Part III, egg shells from each flock were examined using scanning electron microscopy (SEM) to determine whether or not the feeding of various levels of dietary magnesium had any effect on shell ultrastructure. In addition to its role in the provision of calcium, magnesium is itself a shell constituent and accounts for

about 0.25 wt % of the hen's egg shell (Romanoff and Romanoff, 1949). Selected shells in the present study were therefore analysed by electron probe microanalysis (EPMA) in order to determine the precise changes in shell magnesium content occurring as a consequence of feeding diets low in this element. Because the presence of cone layer magnesium is a feature common to the egg shells of all birds (Board and Love, 1980, 1983), the potential importance of this element in shell function was also considered.

PART I: LITERATURE REVIEW

FORMATION OF THE HEN'S EGG SHELL

The structure of the avian egg shell is determined by the physiological and physico-chemical processes which lead to its development. In turn, the form and composition of the shell will determine the role it serves in reproduction. In this way egg shell structure links adult reproductive physiology to the requirements of successful embryo development. Although there have been numerous reviews concerning shell formation and structure (Richardson, 1935; Romanoff and Romanoff, 1949; Simkiss, 1968; Wilbur and Simkiss, 1968; Tyler, 1969b; Simkiss and Taylor, 1971; Simons, 1971; Mueller and Leach, 1974; Becking, 1975; Bond, 1980; Tullett, 1987) a complete understanding of the process has proved elusive. The vast majority of research into this subject has been carried out on the domestic hen. Although many of the findings are applicable to other species, it must be recognised that egg shells are often highly adapted to the reproductive strategies of particular bird groups. Nevertheless, the study of shell formation in domestic birds will lead to a better understanding of the mechanisms whereby evolutionary pressures can influence shell structure in the wild. In the present review emphasis has therefore been placed on how the behaviour and physiology of the female bird can affect the course of shell formation. Initially this must involve the ability of the hen to provide sufficient calcium for egg shell production though the ultimate form of the shell will depend upon much more subtle biological influences.

1. The provision of egg shell calcium in the laying hen

a Dietary calcium

The amount of calcium available for egg shell formation is determined initially by the availability of this element in the diet and by the bird's ability to regulate its intake from this source. Previous attempts to influence shell quality by altering the intake of calcium can conveniently be divided on this basis.

In the natural environment, wild birds can obtain dietary calcium from limestone grit, the shells of molluscs and the skeletons of prey organisms (Hurwitz, 1978). In the domestic fowl, dietary calcium is commonly supplied as both dicalcium phosphate and calcium carbonate. The requirement for the former is approximately the same in growing chicks and adult birds. Due to the demands of shell formation, however, laying birds require a four fold increase in the level of calcium carbonate which results in a typical dietary calcium content of 3-4 wt %. According to Gilbert (1979), layers' diets which contain between 0.5 and 2.5 wt % calcium are potentially harmful being neither sufficient for general body maintenance nor deficient enough to stop egg production. As Taylor (1972) pointed out, the extent of the response is strain-dependent. Some high-production birds will continue laying on a calcium-deficient diet until they collapse due to acute osteoporosis while other strains will cease laying almost immediately. In less extreme conditions birds will adapt to a restriction in dietary calcium intake by regulating the number of eggs produced. Gilbert et al. (1981) administered diets containing between 0.048 and 3.68 wt % calcium and concluded that the major

mechanism for controlling calcium loss was a reduction in the rate of ovulation and hence of the number of eggs produced. It had previously been shown (Luck and Scanes, 1979b) that plasma ionic calcium values of less than 1.0 mM led to the suppression of luteinising hormone-release hormone (LH-RH) thus providing a mechanism whereby very low dietary calcium levels could cause cessation of lay. Between the extremes of calcium deficiency and calcium excess, however, the relationship between egg production and dietary calcium level is more complex (Gilbert et al., 1981). A surgical thread placed in the shell gland results in the production of thin-shelled or shell-less eggs (Lake and Gilbert, 1964; Ogasawara et al., 1974; Ogasawara and Koga, 1977a). Birds which are obliged to lay such poorly shelled eggs exhibit a higher ovulation rate than controls (Lake and Gilbert, 1964) suggesting that the demands of shell formation limit the rate of ovulation. It may be expected from this that a reduction in dietary calcium would lead to the production of shell-less eggs as a means of conserving available calcium (Gilbert et al., 1981). Moreover, if calcium was a limiting factor in egg production then reducing the dietary content of this element might be expected to cause a marked increase in food consumption. Although both egg size and shell calcium content decrease with increasing dietary calcium level neither effect contributes greatly to the conservation of available calcium (Gilbert et al., 1981). Furthermore, food consumption has been found to decrease as dietary calcium is reduced (Gilbert et al., 1981) or to increase only slightly (Mongin and Sauveur, 1974). These adaptations are consistent with the production of fewer,

normally shelled eggs, reflecting perhaps the development of an optimum reproductive strategy in bird evolution (Gilbert et al., 1981). The maintenance of high egg production is of paramount importance only within the poultry industry and it appears that intense selection towards high egg production has led to a loss of ability to regulate calcium output, at least in some strains (Taylor, 1972).

It has been demonstrated that voluntary food consumption rises by about 25% on days when egg formation takes place (Morris and Taylor, 1967). Such a rise does not occur when a low-calcium diet is given or when a free-choice calcium supplement is offered. It seems, moreover, that the regulation of food intake is adversely affected when coupled to the bird's requirement for calcium (Taylor, 1972; Mongin and Sauveur, 1974; Sauveur and Mongin, 1974). According to Taher et al. (1984) some of the earlier assumptions that birds cannot regulate the consumption of grit according to their requirements arose because of a sudden switch from diet-incorporated to free-choice calcium. Birds offered a separate calcium source have been found to produce more eggs with better quality shells (Sauveur and Mongin, 1974).

The intermediate step of providing a particulate calcium source within the main diet seems also to promote shell quality especially in high-production birds (Scott et al., 1971; Meyer et al., 1973). This effect is reduced if the level of available dietary calcium is sufficient (Roland, 1981) and Hamilton et al. (1985) concluded that the improvement in shell quality obtained with an integrated particulate calcium source may be countered by a fall in the

efficiency of egg production. Nevertheless, a recent review of the subject has indicated that the incorporation of particulate calcium carbonate into the diet would result in increased egg shell quality under most commercial conditions (Roland, 1986).

Mongin and Sauveur (1974) demonstrated that the intake of free-choice oyster shell was related to the egg-forming cycle. It had previously been shown that a peak in food consumption occurs during shell formation shortly before the onset of the dark period (Morris and Taylor, 1967). By uncoupling the regulation of food intake from the calcium requirement of laying birds, Mongin and Sauveur (1974) were able to show that the regulation of calcium intake was diurnal in nature and that the demands of shell production amplified the peak in calcium consumption observed prior to the dark period. It was subsequently shown that egg shell quality was maintained even if the provision of oyster shell was restricted to a 3.5 h period starting 6 h before lights-off (Sauveur and Mongin, 1974). Reichmann and Connor (1979) attempted to resolve the contribution of the major dietary components by administering a high-energy diet in the morning and a high-protein, high-calcium diet in the afternoon. Egg production was adversely affected suggesting that the relative proportion of dietary metabolites is crucially important and determines their absorption from the small intestine. More recently Chah and Moran (1985) observed an increase in shell and albumen production in hens which had 'cafeteria access' to energy, protein and calcium. Given the choice birds preferentially consumed protein early in the day and then switched to calcium later on.

The availability of calcium is also influenced by the presence of other minerals in the diet (Portsmouth, 1970; Holder and Huntley, 1978; Atteh and Leeson, 1983a). Magnesium will compete with calcium at the sites of uptake from the gut and there is some evidence that excess dietary magnesium adversely affects both calcium uptake and shell production (MacWard, 1967; Stillmak and Sunde, 1971; Atteh and Leeson, 1983a). There is some dispute as to what level of magnesium constitutes an excess. Stafford and Edwards (1973) found that although hens receiving diets containing 2176 ppm magnesium became hypermagnesaemic they still maintained a high rate of egg production. Mehring and Johnson (1965) found that dietary magnesium levels of up to 8380 ppm had no adverse effect on egg production and McWard (1967) observed a reduction in egg size only when the diet contained in excess of 12000 ppm. Staller and Sunde (1964) reported no reduction in either egg production or egg weight even when the diet contained 13300 ppm. At high concentrations, however, magnesium in the gastrointestinal tract can have a cathartic effect thereby adversely affecting the retention of all minerals (Atteh and Leeson, 1983a). The efficiency of active calcium uptake also decreases with increasing dietary calcium content, though this is offset by the fact that the intestinal retention of calcium is mainly passive (Gilbert, 1983). Whereas several workers observed detrimental effects on egg production at 4 wt % calcium (Scott *et al.*, 1971) others have reported little or no effects of feeding diets containing up to 5.69 wt % calcium (Reichmann and Connor, 1979). The antagonistic relationship between calcium and magnesium and its effect on certain blood, bone and

shell parameters has been investigated by Atteh and Leeson (1983 a,b). The main effects of excess dietary magnesium seem to be decreased bone and shell calcium contents and a related fall in shell quality, as assessed by deformation.

Although older hens can absorb and mobilise calcium as well as younger hens (Roland, 1980a), there is still a decline in shell quality associated with ageing. The main cause of this seems to be a continuous increase in egg size occurring throughout the laying phase causing a constant amount of shell to be spread progressively thinner. Roland (1980a) attempted to prolong shell quality in aged hens by reducing the protein content of the diet. Although egg weight, and hence size, decreased there was also a decrease in the amount of shell deposited. When protein intake was reduced during the first 5 months of lay, however, a significant reduction in the rate of increase in egg size resulted in the maintenance of shell quality (Roland, 1980b). Concomitant with the increase in egg size in older birds is a decline in the renal production of 1,25-dihydroxycholecalciferol (1,25-DHCC) from its precursor cholecalciferol (Abe et al., 1982). Although the intestinal absorption of calcium is mainly passive (Gilbert, 1983), the process is enhanced by an active uptake mechanism dependent upon 1,25-DHCC and calcium-binding protein (CaBP). A cyclical variation in the circulating level of 1,25-DHCC during egg production has been taken as evidence of the hormone's involvement in shell formation (Abe et al., 1979). The enhancement of calcium uptake from the intestine may not be the major role of cholecalciferol (vitamin D_3) in shell formation however (El-Boushy, 1966). Despite an increase in the level of

vitamin D_3-dependent CaBP in the intestinal mucosa observed at the onset of lay, the intestinal absorption of calcium is not correlated with any changes in the level of CaBP during egg formation (Bar and Hurwitz, 1975).

The specific gravity of eggs laid in the late afternoon has been found to be greater than that of those laid in the morning (McDaniel and Roland, 1977; Farmer and Roland, 1983). This has been taken as evidence that shell quality is improved if the bird has access to dietary calcium during the later stages of shell formation. There have been at least two attempts to improve shell quality by feeding birds during the dark period (see Belyavin and Boorman, 1981). Many more workers have recognised that the provision of a particulate calcium source, either incorporated into the diet or presented separately, leads to a more graded release of calcium from the intestine during the dark period and hence to less reliance on skeletal reserves (Scott et al., 1971; Meyer et al., 1973; Mongin and Sauveur, 1979; Roland, 1986). Farmer and Roland (1983) suggested that the provision of calcium by the skeletal system may be less effective than the direct uptake of this element from the intestine, thus providing a basis for the superior shell quality of eggs laid in the afternoon. Despite this, most commercial diets are formulated to contain approximately 3.5 wt % calcium in the form of pulverised limestone flour. It therefore seems likely that under these conditions the skeleton plays at least some part in shell formation. Comar and Driggers (1949), using radioactively labelled calcium, reported that 60 to 75% of the calcium in the egg came directly from the diet and van de Velde

and Vermeiden (1984) stated that 60% of shell calcium was derived from this source with 40% coming from the skeleton. The results of Bragg et al. (1971) indicated that less than 40% of shell calcium came directly from the feed though this low value was attributed to the procedures used in the administration and collection of the isotope.

b Role of medullary bone

The relationship between bone physiology and egg shell production has received considerable attention and much of the earlier work has been reviewed by Simkiss (1961, 1967) and by Taylor and Stringer (1965). In particular, the role of medullary bone in shell formation has long been appreciated. The deposition of this unique material is under the control of the sex hormones and its appearance coincides with the development of the ovary and its secretory activity (Simkiss, 1967; Hurwitz, 1978). Moreover, the formation of medullary bone is controlled by the synergistic action of both oestrogens and androgens and is thus distinct from the oestrogenically-induced hypercalcaemia observed prior to the onset of lay (Simkiss, 1967). Immature male chicks treated with both types of sex hormones will deposit medullary bone only in the presence of vitamin D_3 (Takahashi et al., 1983). Moreover, in the laying quail both the extent of medullary bone formation and its calcium content are reduced if the birds are deficient in this vitamin (Takahashi et al., 1983). This confirms that vitamin D plays an important role not only in bone resorption but also in bone mineralization (Simkiss, 1967; Hurwitz, 1978).

Medullary bone is composed mainly of amorphous calcium phosphate deposited around a loosely organised matrix of collagen fibrils. Although medullary bone is far less crystalline than cortical bone, it is better mineralized in terms of its mineral: collagen ratio (Simkiss, 1967) and contains more osteocytes (Simkiss, 1961). According to Hurwitz (1978) medullary bone is characteristic of immature bone elements, the formation of cortical bone being normally caused by the maturation of an amorphous calcium phosphate phase (Termine and Posner, 1970; Termine et al., 1970; Bachra, 1972; Termine, 1972). Moreover, the deposition of medullary bone is dependent upon the extent of the immediate blood supply. The better vascularised bones of the skeleton are also the sites where deposition of this secondary bone is most evident (Simkiss, 1967).

The composition of medullary bone and the conditions under which it is deposited provide evidence of its high lability which in turn is the key to its role in shell formation. Contrary to earlier beliefs, the medullary bone does not act as a buffer against the depletion of cortical bone. During chronic calcium deficiency it is cortical bone which is depleted to provide egg shell calcium while the amount of medullary bone remains constant (Simkiss, 1967; Candlish, 1971; Bar and Hurwitz, 1984). Medullary bone thus acts as a readily available source of calcium during those times when the output of this element due to shell formation exceeds its input from the diet. In most cases this state will be reached during the latter half of the dark period when the availability of calcium from the intestine is minimal. Candlish

(1971) has shown that the resorption of medullary bone is intensive during shell calcification but that there is little change in the absolute amount present due to rapid resynthesis. Cortical bone was reported to be relatively inert during the course of shell deposition though it probably provides some calcium for medullary bone synthesis in the early stages. Although the amount of medullary bone shows little change with time there is a net loss of calcium from this source as calcification proceeds (Van de Velde and Vermeiden, 1984). As a result, there is a fall in the calcium : phosphorus ratio of this material. Analysis of other bone minerals confirms that the composition of medullary bone changes in accordance with the changes in plasma composition attendant upon shell formation (Simkiss, 1967).

The resorption of medullary bone mineral during egg shell calcification is only one aspect of the hormonal regulation of plasma calcium. Both Hurwitz (1978) and Dacke (1979) have reviewed the endocrinology of calcium metabolism in birds. It is presently assumed that calcitonin (CT) serves to limit the hypercalcaemia which normally occurs at the end of shell formation (Baimbridge and Taylor, 1981). The amount of this hormone in hen plasma is very small and this has led to difficulties in determining its precise role (Dacke et al., 1972). Even with the advent of more sensitive techniques the presence of this hormone could only be detected at around the time of oviposition (Baimbridge and Taylor, 1981). When the shell gland is active the resorption of medullary bone minerals, under the influence of parathyroid hormone (PTH), predominates and injections of CT during this time have failed to

reduce the level of plasma calcium (Luck et al., 1980). There is some evidence that 1,25-DHCC acts to potentiate the response of PTH to changes in plasma ionic calcium level (Singh et al., 1986). Below a certain minimum concentration of plasma 1,25-DHCC the parathyroid glands appear unable to initiate the release of PTH above the basal level. Normally the amount of circulating PTH is reciprocally related to plasma ionic calcium content (Luck et al., 1980; Singh et al., 1986). The PTH level rises during active shell calcification reflecting the increasing demand for shell calcium. McManus et al. (1971) observed that PTH-induced resorption of bone in the rat was dependent upon magnesium acting as a co-factor. In magnesium-depleted laying birds there is an increase in the mineral content of the skeleton resulting mainly from a decreased resorption of medullary bone and it has been suggested that this is the cause of the thin shells produced by such birds (Stafford and Edwards, 1973).

2 Changes in plasma composition during egg shell formation

a Calcium

The demands of shell formation place considerable strain on the homeostatic mechanisms of laying birds (Hurwitz, 1978). According to Hertelendy and Taylor (1961) an amount of calcium equivalent to that circulating in the plasma at any one instant is removed every 15 min during shell formation. Winget and Smith (1958) observed a decrease in total plasma calcium associated with shell calcification. This decrease was almost entirely due to a fall in the level of bound calcium since the level of ionised

calcium remained relatively constant. Polin and Sturkie (1957) had previously reported a reduction in ionised plasma calcium level during shell calcification with little change in the level of total calcium. Hertelendy and Taylor (1961) recognised that variations between individual birds may have masked the significance of the earlier works. These authors demonstrated that during shell calcification there is a fall in the level of total plasma calcium in individual birds which is only reversed when shell formation ends. In most birds there was an associated rise in plasma calcium content in the early morning, at a time when dietary calcium intake was minimal (Hertelendy and Taylor, 1961). The reduction in total calcium during shell formation was subsequently shown to be due to a fall in the ionic or diffusible calcium level (Taylor and Hertelendy, 1961). Moreover, decreases in total calcium greater than 2 mg % were associated with a noticeable reduction in the level of bound or non-diffusible calcium indicating that the bound and unbound fractions were in equilibrium (Taylor and Hertelendy, 1961). Hodges (1969) reported that total plasma calcium fell by up to 25% during shell formation but that this was not significant due to large variations between individual birds. The decline in plasma calcium was most pronounced in blood taken from the shell gland veins, the difference between arterial and shell gland venous blood quickly becoming significant. This difference persisted until 80% of the shell-forming period had elapsed (Hodges, 1969). The radiographic studies of Bradfield (1951) indicated that the rate of shell formation decreased during the final hours of calcification though this has been disputed (Talbot and Tyler, 1974c).

The ionised calcium fraction of the blood accounts for only 20% of the total in laying hens due to the presence of calcium-binding yolk proteins (Luck, 1979). Moreover, the equilibrium between the bound and unbound calcium fractions is pH-dependent (Taylor and Hertelendy, 1961) and consequently the measurement of either diffusible or ionic plasma calcium is problematic. Recently, both Luck and Scanes (1979a) and Singh et al. (1986), using a calcium-specific electrode, were able to measure directly the changes in ionic plasma calcium during egg formation. They observed that the level of ionised calcium varied sigmoidally during the ovulation cycle reaching a minimum value 3 to 6 h before oviposition. This is despite the suggestion that changes in the ionised plasma calcium level are buffered by the high calcium-binding capacity, and low binding affinity, of the blood proteins, principally vitellogenin (Wideman and Buss, 1985). The demands of shell formation may overwhelm the buffering ability of the bound calcium fraction with the result that both bound and unbound fractions are depleted during shell formation. Nevertheless, Luck and Scanes (1979a) were unable to demonstrate any change in the level of total plasma calcium during shell formation.

Although the buffering ability of the protein-bound calcium fraction may be overwhelmed during shell formation, it seems likely that this is the mechanism which allows the calcium-carrying capacity of the blood to increase dramatically in laying hens (Wideman and Buss, 1985). The level of ionised plasma calcium is of crucial importance in many regulatory and sensory systems and the oestrogen-induced synthesis of blood lipophosphoproteins in the

liver of laying birds (Hurwitz, 1978) protects the ionised calcium level against disruption due to yolk synthesis (Wideman and Buss, 1985). Nevertheless, any increase in bound calcium-carrying capacity in the blood will cause some increase in the level of ionised calcium due to the dynamic equilibrium which exists between the two fractions. This will result in an increase in the amount of ionised calcium available for shell formation. Wideman and Buss (1985) reported that selecting for thick or thin egg shells in three different strains of domestic hens resulted in co-selection for mean total plasma calcium level. These authors concluded that the difference between birds producing thick and those producing thin shells resulted from lower plasma calcium values in the latter, as neither egg size nor the ability of the shell gland to secrete calcium differed between the two groups. Despite the clear correlation between group mean total calcium and shell thickness, the predictive value of blood calcium level in individual birds was found to be poor. Lennards et al. (1981) had previously found no relationship between serum calcium and egg shell weight and Hamilton et al. (1981) concluded that no relationship existed between plasma and shell calcium.

b Phosphorus

The level of inorganic phosphorus in the blood of laying hens also varies during the ovulation cycle (Miller et al., 1977a, b; Mongin and Sauveur, 1979; Sauveur and Mongin, 1983; Miles et al., 1984). In general, the level of plasma inorganic phosphorus rises sharply following the egg's entry into the shell gland,

reaching a peak value towards the end of shell formation, before returning to the pre-shelling level shortly before oviposition. Several theories attempting to account for the cyclic variations in plasma phosphorus have been suggested. All invoke some relationship between the intestinal absorption and urinary excretion of this element and its role in bone physiology (Tullett et al., 1976; Mongin and Sauveur, 1979; Sauveur and Mongin, 1983).

Miles et al. (1984) suggested that the increased shell quality of eggs laid in the afternoon was due to a reduced resorption of skeletal calcium occurring towards the end of shell formation. As the ratio of calcium to phosphorus in the skeleton is only about one third of the ratio in the diet (Miles et al., 1984), it was surmised that the increase in bone resorption necessary to maintain shell calcification would normally liberate large amounts of phosphorus into the blood. Excess levels of plasma phosphorus have been shown to have a detrimental effect both on shell specific gravity (Miles et al., 1983) and shell weight (Sauveur and Mongin, 1983). Mongin and Sauveur (1979) found that the provision of a particulate calcium source, presented separately from the diet, reduced the mobilisation of bone during the dark period and led to a marked reduction in the maximum level of plasma phosphorus. It now seems that both the onset and degree of bone mobilisation occurring at the end of shell formation depend upon the calcium status of the intestine. This in turn will depend both upon the form of the dietary calcium and upon the regulation of its uptake by the hen (Mongin and Sauveur, 1979; Sauveur and Mongin, 1983).

c Magnesium

The changes in plasma magnesium content associated with egg laying have not been widely investigated. Taylor and Hertelendy (1961) reported a small, though consistent, decrease in plasma magnesium level associated with egg shell formation but considered that this was unlikely to be significant. Misra and Kémeny (1964) noted that the plasma magnesium content of non-laying Hungarian Yellow hens was only 65% of that found in laying birds during shell formation. A similar result was obtained for plasma calcium content. These authors also noted a rise in plasma calcium, magnesium and phosphorus content associated with the presence of the egg in the shell gland though the exact stage of shell development was not determined. In the hen, Solomon (1971) found that the magnesium content of the venous blood draining the shell gland decreased immediately prior to the onset of shell calcification, suggesting an increase in the retention of this element. There was, moreover, a concurrent decrease observed in the amount of calcium leaving the shell gland, indicating an increasing demand for both elements during shell calcification.

3 Changes in shell gland fluid composition during egg shell formation

The formation of the calcitic shell of the hen's egg accounts for approximately 20 h of a typical 25 h laying cycle (Talbot and Tyler, 1974c) and takes place in the highly specialised environment of the shell gland lumen. During this time the outer surface of the egg is continually bathed in a gel-like secretion, the uterine or

shell gland fluid, from which the inorganic constituents of the shell are formed. It has been shown that the composition of the shell gland fluid varies during the course of shell deposition (El-Jack and Lake, 1967; Edwards, 1977). Indeed, changes in the composition of biomineralizing fluids can have profound effects both upon the duration of mineral deposition and upon the form and composition of the finished structure (Simkiss, 1964a; Ogasawara et al., 1975; Tullett et al., 1976; Lorens and Bender, 1977; Ogasawara and Koga, 1977a; Mann, 1983; Board et al., 1984).

a Calcium

The hen's shell gland actively transports calcium from the plasma during bulk egg shell mineralization. Evidence for this comes from the observations that the excised organ can move calcium against a concentration gradient and that the movement of radioactively labelled calcium into the shell gland lumen is 2.5 times greater than its transfer in the opposite direction (Simkiss, 1968). This removal of calcium from the blood, especially during late calcification, leads to the depletion of this element in the plasma as previously outlined (Hertelendy and Taylor, 1961; Luck and Scanes, 1979a; Singh et al., 1986). Simkiss (1968) suggested that the movement of calcium into the shell gland resulted from a 'facilitated permeability' though with a strontium : calcium discrimination factor of only 0.927 the transfer was not considered to result from a specific calcium pump. On the other hand, Coty and McConkey (1982) have identified a highly specific ATP-dependent calcium transport system in shell gland epithelial tissue. The

subcellular fraction responsible for the transfer of calcium into the lumen is apparently sensitive to DDE poisoning in some birds (Miller et al., 1976; Lundholm, 1980, 1982, 1984a-c, 1985). Although the uptake of calcium from the blood appears to be unaffected by this metabolite, the secretion of the element into the shell gland lumen is impaired, resulting in the retention of calcium by the gland mucosa and a reduction in shell thickness (Lundholm, 1984a-c). DDT and its metabolites have previously been implicated in the decline of shell thickness in certain populations of wild birds (Ratcliffe, 1967; Cooke, 1973). The transfer of calcium seems to be mediated by the microsomal fraction of gland cell homogenates, the main effect of DDE being the inhibition of calcium secretion from membrane-bound vesicles within the cell (Coty and McConkey, 1982; Lundholm, 1984a). In the normal case, the ionic composition of the intravesicular fluid will be determined by the selective permeability of the surrounding membrane (Loewy and Siekevitz, 1978). Mann (1983) has proposed that the ionic composition of large volumes of extracellular biomineralizing fluids may be determined by the biological control of vesicle membrane permeability.

The secretion of calcium by the shell gland mucosa is dependent, at least partly, upon the distension of the shell gland caused by entry of the egg (Eastin and Spaziani, 1978a). There is an increase in the rate of secretion of shell gland calcium at this time with the maximum rate occurring some 5 to 6 h later. This high rate is maintained until 2 h before oviposition at which time it falls to only one fifth of its maximum value (Eastin and Spaziani,

1978b). The increase in calcium secretion by the shell gland corresponds to the time at which the individual mammillae on the surface of the egg fuse to form the foundation of the column layer (Talbot and Tyler, 1974c). During the 5 to 6 h prior to this, water, glucose and potassium from the shell gland fluid pass freely into the egg resulting in a doubling of the volume of albumen (plumping) and a change in the ionic content of the egg (Edwards, 1977).

Edwards (1977) has suggested that the secretion of calcium by the folded uterine mucosa is largely confined to those regions in close contact with the developing shell. This would explain the increase in calcium secretion following fusion of the mammillary layer, at which point a larger area of shell will press against the shell gland lining. The localized secretion of calcium within the shell gland will complicate the determination of shell gland fluid composition. Edwards (1977) distinguished between the true calcification fluid at the site of shell growth and the general bulk of shell gland fluid. In addition, the presence of the egg will modify the primary secretion of the shell gland due to the ionic exchanges which occur during shell formation (Edwards, 1977). Regions of the oviduct other than the shell gland may also contribute to the composition of the calcifying fluid. The removal of blood calcium, magnesium and potassium by the magnum during shell formation has been taken as evidence of the region's involvement in supplying calcifying fluid to the shell gland (Moynihan and Edwards, 1975).

b Phosphorus

Although the inorganic phosphorus level of the blood is elevated during most of the shell-forming period (Mongin and Sauveur, 1979; Sauveur and Mongin, 1983), there is no corresponding rise in the amount of this element in shell gland fluid until shortly before oviposition (Ogasawara et al., 1974). The exclusion of phosphorus from the lumen of the active shell gland is a necessary condition for the development of shell calcite due to the element's role as a crystal poison (Simkiss, 1964b; Tullett et al., 1976). It has, in fact, been suggested that the sudden increase in the phosphorus content of shell gland fluid, observed immediately prior to oviposition (Ogasawara et al., 1974), contributes to the termination of shell calcification.

Orthophosphate solutions injected into the lumen of the active shell gland have been found to induce premature oviposition (Ogasawara et al., 1975). Moreover, the proportion of premature ovipositions induced in this way increased with increasing orthophosphate concentration (Ogasawara et al., 1975). Ogasawara and Koga (1978) reported that the production of a prostaglandin-like substance by the shell gland during normal and phosphate-induced oviposition was higher than at all other times. Injections of prostaglandin into the shell gland also induce premature oviposition and the hormone has been implicated in the muscular contractions of the shell gland which lead to oviposition (Ogasawara and Koga, 1978). The termination of shell growth by high levels of shell gland phosphates and the contractions of the shell gland leading to oviposition, although linked, probably represent

quite distinct processes (Ogasawara and Koga, 1977a, b, 1978).

The presence of a surgical loop or thread in the shell gland leads to a low rate of secretion of shell gland fluid and to a reduced rate of shell formation (Lake and Gilbert, 1964; Ogasawara et al., 1974). Under these conditions the calcifying fluid also contains high levels of phosphorus and this often leads to the expulsion of soft-shelled eggs (Ogasawara et al., 1974).

Naturally occurring changes in the phosphorus content of shell gland fluid during shell formation have been implicated in the deposition of vaterite, as opposed to calcite, on the outer egg shell surface of several species of sea bird (Tullett et al., 1976). Vaterite also constitutes part of the outer shell surface of two species of non-parasitic cuckoos (Board and Perrott, 1979). Although limitations in the amount of medullary bone available from the strengthened skeleton of diving sea birds may lead to an increased dependence upon high-phosphorus cortical bone (Tullett et al., 1976) there would seem to be no such physiological constraints in land-living birds. This has led to the suggestion that the organic constituents of shell gland fluid may also play a part in determining the crystal form deposited (Board and Perrott, 1979). Studies of several molluscan species support this notion (Watabe and Wilbur, 1960). Nevertheless, the relative contributions of the organic shell matrix and of the ionic environment of the avian shell gland to shell formation have not been fully resolved. If the deposition of vaterite does indeed confer adaptive advantages to certain shells, either in terms of increased strength (Tyler, 1969a) or by preventing the occlusion of pore canals (Board

et al., 1984) then the biological regulation of the phosphorus content of the shell gland lumen may well be the mechanism whereby this is achieved. The finding that the vaterite covering the egg shells of certain sea birds contains levels of phosphorus (0.33 to 0.55 wt %) which are intermediate between those required for calcite deposition (< 0.1 wt %) and those required to terminate calcium carbonate growth entirely (> 2.5 wt %) supports this possibility (Tullett et al., 1976). The presence of amorphous calcium phosphate on the outer surface of flamingo, mallee fowl and grebe egg shells (Board, 1981; Board et al., 1982; Board et al., 1984) may represent a further adaptation dependent upon the phosphate content of the shelling environment.

c Magnesium

During egg shell calcification in the domestic hen there is an increased retention of magnesium analogous to that seen for calcium (Solomon, 1971). Moreover, the amount of magnesium present in shell gland fluid has been found to exceed its concentration in the blood during shell formation indicating that the element is actively taken up by this organ (Solomon, 1971). Edwards et al. (1962) reported that over 40% of injected Mg^{28} appeared in the shell of an egg-forming bird whereas in non egg-forming ones there was an increased excretion of the isotope, indicating an uptake of this element by the active shell gland. In addition, the magnum region of the oviduct also contributes plasma-derived magnesium to the shelling fluid (Moynihan and Edwards, 1975). Solomon (1971) found no appreciable change in the magnesium content of shell gland

fluid following the onset of shell formation. El-Jack and Lake (1967), however, reported that the amount of magnesium in 'oviposition' fluid was over seven times that observed in 'plumping' fluid, though the former was probably obtained at a time when the rate of shell calcification was not maximal, ie. within 2 h of oviposition. Nevertheless, Itoh and Hatano (1964) also considered that the concentration of magnesium in shell gland fluid varied during shell formation giving rise to the increasing amount of this element seen in the outer layers of the shell. Although magnesium has been recognised as a constituent of avian egg shells for over 100 years (see Romanoff and Romanoff, 1949), it is only recently that attention has been given to its role in shell formation and function. Romanoff and Romanoff (1949) considered that the element accounted for only around 0.25 wt % of the hen's egg shell. It is therefore unlikely that magnesium plays a direct role in the process of shell formation and probably occurs as a contaminant of the egg shell calcite. Consequently, the distribution of the element across the shell can be regarded as a potential 'marker' for some of the changes occurring in the environment of the shell gland lumen during shell calcification. Despite the small amounts present, Brooks and Hale (1955) demonstrated, by chemical analysis of successively dissolved shell layers, that magnesium was distributed unevenly across the shell with the highest level occurring towards the outer surface. This was confirmed by Itoh and Hatano (1964) who despite a similarly crude chemical technique also detected a second increase in magnesium content in the shell layer corresponding to the individual cones. Using both electron probe

microanalysis (EPMA) and secondary ion mass spectroscopy (SIMS), Quintana and Sandoz (1978) and Quintana et al. (1980) demonstrated a similar pattern of magnesium distribution in the quail shell. Recently, the distribution of egg shell magnesium in twenty orders of birds has been described, using EPMA, by Board and Love (1980). In most species of Galliformes, including the domestic hen, there were two peaks in shell magnesium content, one in the cone layer and a second at the outer surface of the shell. In the majority of species belonging to orders other than the Galliformes the second peak, at the outer surface, was absent and the suggestion has been made that the pattern of egg shell magnesium distribution may be of phylogenetic importance (Board and Love, 1983).

In the hen the first stages of shell calcification take place at the junction between the isthmus and shell gland (Wyburn et al., 1983), in a region termed the tubular shell gland or red region. According to El-Jack and Lake (1967) the 'plumping' fluid from which initial calcification of the cores occurs contains relatively low levels of magnesium. Because the level of this element in the cone layer was found to be comparable to that observed at the outer surface of the shell it has been suggested that the cone layer has an 'avidity' for this element (Board and Love, 1980). The finding that the cone layer invariably contains detectable amounts of magnesium, even in those shells which do not exhibit the second, outermost peak (Board and Love, 1980, 1983) points to the importance of this element in the reproductive strategies of all birds.

It is evident from this part of the present review that the levels of calcium, phosphorus and magnesium in shell gland fluid

change during shell formation. In reality, these changes occur in conjunction with a complex shift in the ionic composition of the shelling fluid, a proportion of which may be unrelated to shell calcification. The secretion of organic material into the shell gland lumen during plumping and shell formation will further complicate the environment in which shell material is formed (Salevsky and Leach, 1980). Despite this complexity the deposition of the shell will proceed in accordance with the thermodynamic and physico-chemical conditions obtaining in the shell gland fluid. An insight into at least some of these conditions is provided by the sequence of events occurring during shell formation and it is this sequence which forms the final part of the present review.

4 The course of egg shell formation

a The nucleation sites

(i) Formation

The inner layer of the true shell is composed of a profusion of knob-like processes known as cones or mammillae. According to Schmidt (1965) this characteristic appearance results from the spherulitic development, during early calcification, of numerous seeding sites distributed over the egg surface. Earlier, von Nathusius (see Tyler, 1964) had noted the existence of an optically dark area at the centre of each mammilla and Stewart (1935) stated that these cores were primarily organic in nature. Subsequent observations on decalcified shell preparations confirmed that this was the case (Simkiss and Tyler, 1957; Bellairs and Boyde, 1969). Under the scanning electron microscope the

decalcified mammillary cores give the appearance of having flowed onto and around the outer shell membrane fibres (Bellairs and Boyde, 1969). This impression is reinforced by a remarkable series of transmission electron micrographs (Simons and Wiertz, 1963) in which contact between the membrane fibres and core material seems to cause a characteristic transformation of the former. The mantle material, which normally encases the membrane fibres, branches dendritically and forms a 'belt' of material around the membrane fibre cores which pass further into the mammillary matrix where they too assume a tree-like or lattice appearance (Simons and Wiertz, 1963). This lattice may involve a number of fibre cores and is often superimposed upon the finer mantle lattice with the interstitial divide intact (Wyburn et al., 1973). In undecalcified preparations of 'shell-less' eggs, the fibres of the core lattice are coarsely serrated along their edges (Wyburn et al., 1973). Despite the contention that the transformed shell membrane fibres act as nucleation sites for the first calcite crystals (Wyburn et al., 1973), the true initiation site may be the mammillary core surface (Terepka, 1963a; Bunk and Balloun, 1977). Nonetheless, the characteristic tree-like appearance of the membrane fibre cores in decalcified preparations is evidence of shell crystal growth in this region. There is moreover, an apparent continuity between the mantles of the transformed membrane fibres and the radially oriented material of the mammillary core (Wyburn et al., 1973). In cross-section the fibrils of the dense mammillary matrix fan out from both the membrane core and mantle lattices and are delimited by a membrane-like condensation approximately 0.14 μm wide (Simons

and Wiertz, 1963). Beyond this membrane the organic material of the mammilla becomes less reticular and the region is characterised by numerous vesicles and coarse granular material (Simons and Wiertz, 1963). The interface between the organic material of the mammillary core and the matrix of the shell is therefore considered to be a potentially weak region with a sparse and poorly organised organic content (Simons and Wiertz, 1963).

Schmidt (1965) envisaged the mammillary core as a membranous structure surrounding a solid inner core of crystalline material (the 'primary spherite'). Simon and Wiertz (1963) pointed out the relationship between the orientation of the optical axes of this 'elementary unit' (Schmidt, 1957) and the radial ordering of the mammillary matrix fibrils. Erben (1970) supported Schmidt's interpretation of the organic-inorganic relationship but suggested that the two dimensional thin sections observed in the earlier work had not been obtained from the centre of the mammilla thus illustrating the difficulty associated with two dimensional studies of three dimensional objects. Erben (1970) stated that the centres of the primary spherites were in fact hollow, a view shared by Quintana and Sandoz (1978) in their study of quail eggshells. Bunk and Combs (1979) used resin casts to illustrate the outline of the mammillary core remaining after the removal of organic material, though these were not capable of reproducing the detailed microstructure of the mammillary matrix.

Tyler (1969b) observed that prematurely laid or surgically removed eggs were either free of core material or else exhibited rudimentary calcification. The inability to observe uncalcified

cores suggested that either they were deposited extremely quickly or were laid down simultaneously with the first stages of calcification. Terepka (1963a) had indicated that the calcification of the outer surface of the mammillary core may be qualitatively distinct from the crystallization which is initiated nearer the tip of the mammillae. The appearance of the mammillae during early development has been described as amorphous (Masshoff and Stolpmann, 1961) though it is clear that true egg shell crystal growth begins prior to the egg's entry into the shell gland (Fujii and Tamura, 1970). Nevertheless, the possibility remains that the transformation of the nucleating membrane fibres occurs subsequent to the deposition of the tubular shell gland secretions and that the calcification seen on the egg surface at the earliest stages is the result of more rapidly initiated shell crystal growth. Terepka (1963a) distinguished between the 'organic matrix cores' positioned at the tip of each mammilla (Simkiss and Tyler, 1957) and the 'dark mineral cores' observed nearer the centre (Romanoff and Romanoff, 1949). These latter regions may correspond to the edges of the enveloping membrane of the mammillary core which may itself act as a site of mineral deposition.

In the simplest case the morphogenesis of the mammillary cores can be regarded as being initiated by the secretions of the tubular shell gland (red region) coming into contact with the fibres of the outer shell membrane. As the deposition of the core material appears to be independent of local membrane topography (Fujii et al., 1970; Bellairs and Boyde, 1969) then the distribution of the cores and hence the mammillae will reflect the spatial

organisation of the secretory cells of the tubular shell gland (Wyburn et al., 1973; Tullett, 1976). An alternative account of mammillary core formation was given by Stemberger et al. (1977). These authors noted that small granular concretions occurred on the surface of developing inner shell membrane fibres. As shell membrane formation proceeded the number of grains increased, reaching a maximum value immediately before the egg's entry into the tubular shell gland. The marked reduction in the number of surface structures observed on eggs leaving this region was taken as evidence of the agglomeration of the small granular units to form the nucleating centres. Small secretions adhering to the inner shell membrane had previously been described by Fujii et al., (1970) who suggested that they may arise from the same mucin-like material which forms the mantle of mature shell membrane fibres. Furthermore, the beginning of shell calcification was marked by the appearance of numerous 'sand-like' granules which obscured the outlines of the ribbon-like outer membrane fibres (Fujii and Tamura, 1970). Thereafter, larger concretions arose over the entire outer surface and these were shown to be organic in nature. According to Baumgartner et al. (1978), the maturation of shell membrane fibres is crucial to normal shell development and this process appears to be dependent upon the production of lysine-derived cross-linking within the membrane fibres. In copper-depleted hens the shell membranes are immature in appearance and do not exhibit the profusion of knob-like organic processes typical of normal membranes (Baumgartner et al., 1978). Although the egg shells of copper-depleted birds are abnormal it seems likely

that this is due to the altered mechanical properties of the membrane (Baumgartner et al., 1978) and not to any disruption in core development arising from the changes in the number of membrane protrusions. Moreover, if these concretions are composed of the same substance as the mantle of membrane fibres (Fujii et al., 1970) then they cannot act as calcite nucleation sites themselves for the same reason that the outer membrane fibres do not normally give rise to indiscriminate calcification. Although the grains give a negative reaction for carbonate (Stemberger et al., 1977) they may act as centres of calcium deposition, perhaps in the form of the citrate salt. The presence of large quantities of both calcium (Taylor and Hertelendy, 1960; Schraer and Schraer, 1965) and citrate (Taylor and Hertelendy, 1960) in the isthmus has been adduced as evidence of the initiation of shell formation in this region (Simkiss, 1968). It may be that the grains attached to the developing membranes cause a local co-precipitation of the two ions. Furthermore, if these small, membrane-derived units act as physical contours for the secretions of the tubular shell gland (Stemberger et al., 1977) then the spatial organisation of the cells of this region can exert little influence on the ultimate morphology of the shell. Although it is true that mammillae often appear multinucleate (Stemberger et al., 1977) this could equally result from the secretions of the tubular shell gland coming into contact with, and transforming, several membrane fibre regions (see Simons and Wiertz, 1963).

Whatever the precise mechanism of mammillary core development, it is clear that during the 15 to 30 min that the egg spends in the

tubular shell gland the maturation of the nucleation sites is completed. The egg enters the shell gland proper with the mammillae already firmly anchored to the outer membrane by the rudimentary calcification which has already taken place (Wyburn et al., 1973).

(ii) Chemical composition

Simkiss and Tyler (1957) considered that the organic material of the mammillae was similar in composition to the organic matrix of the true shell being composed predominantly of acid mucopolysaccharides. Despite the presence of fat and a higher protein content, the mammillary cores were considered to arise from the condensation of acid mucopolysaccharide material around the keratin-like protein of the outermost shell membrane fibres. The difference in protein content between the mammillary core and shell matrix fractions was explained in terms of the more compact structure of the former and by a lack of polymerisation of the innermost core regions normally caused by shell calcification. However, Robinson and King (1968) believed that the mammillary core and outermost shell membrane fibres were composed mainly of neutral mucopolysaccharides. Moreover, they considered that the cores were merely a specialised form of the ground material of outer membrane fibres and were precipitated from a solution of membrane fibre precursors by means of intrachain bonding. Membrane fibres are known to contain both non-covalent and disulphide bonds (Masshoff and Stolpmann, 1961) and it was postulated that the structure of the mammillary core was also maintained in this way. However, the possibility that the organic cores were distinct from the material

of the membranes and were merely bound to them by interchain disulphide bonds was not excluded. Masshoff and Stolpmann (1961) had previously demonstrated that shell membrane fibres were composed of a keratin-like protein core surrounded by a mucopolysaccharide sheath. Moreover, the sheath could be enzymatically induced to undergo structural changes which the authors believed normally led to the development of a 'micronet' which permeated the entire shell. Although this latter claim has subsequently been refuted (Simons and Wiertz, 1963), it may be that the transformed membrane sheath is the organisational centre of the radiating fibrils of the mammillary core. The branching of the keratin-like core of the outer membrane fibres (Simons and Wiertz, 1963; Wyburn et al., 1973) which terminates nearer the tip of the mammilla may be the result of early crystal growth and may explain why the mammillary core contains more protein than the subsequently deposited shell matrix (Simkiss and Tyler, 1957).

It was originally thought that the high sialic acid content of mammillary core preparations was due to contamination by shell matrix material (Robinson and King, 1968). Cooke and Balch (1970a) reported, however, that mammillary cores with a high sialic acid content could be prepared free of the uronic acid typical of shell matrix. Sialic acid readily forms complexes with calcium and it was suggested that its presence in the core caused a local increase in calcium concentration sufficient to exceed the solubility product of calcium carbonate thus initiating calcification in this region (Cooke and Balch, 1970a). Ossifying cartilage typically contains three times the amount of sialic acid found in non-

ossifying cartilage and the presence of this substance in the inner levels of shell matrix (Cooke and Balch, 1970a) suggests that it has a role to play in maintaining shell calcification. On the other hand, the loosely bound sialomucin complex is situated on the surface of the mammillary core and thus corresponds to an area where there is weak connection between the organic and inorganic fractions (Stemberger et al., 1977). As previously suggested, initial calcification of the surface of the mammillary core may not be typical of the general development of the shell. Carbonic anhydrase has also been observed in mammillary core preparations (Robinson and King, 1963) and it too may be involved in the initiation or maintenance of shell crystal growth.

b Shell crystal growth

Calcium carbonate, in the form of calcite, constitutes approximately 95 wt % of the hen's egg shell. As a result, shell formation is essentially a process of crystal growth, the exact nature of which has been the cause of some disagreement during the past 30 years. The polarized light studies of Schmidt (1957, 1965, 1966) provided detailed evidence of the spherulitic development of shell crystals from numerous nucleating sites on the outer shell membrane. As envisaged by Schmidt the first crystals to develop were needle-like and radiated from the centre of these sites, or cores, to form a primary spherulite. As the crystals developed they gave rise to larger secondary spherulites, the inward growth of which was greatly inhibited by the shell membranes. The outward growth of the crystals would, however, be unimpeded and this would

result in the fusion of the individual spherulites to form the cone or mammillary layer characteristic of the inner shell surface (see also Erben and Kriesten, 1973). The column layer was considered to arise from the relatively few crystal units whose outward growth was approximately normal to the shell surface. The findings of Terepka (1963a) supported this concept in as much as the crystals of the column layer appeared to originate from the mammillae and to extinguish as single units under polarized light. Under closer examination, however, the crystals were seen to consist of smaller optically-aligned sub-units, some 10-15 µm wide. Masshoff and Stolpmann (1961) had previously suggested that the shell was composed of numerous small crystals arranged within a micronet of organic material. Heyn (1962, 1963a, b) concluded from an electron microscopical study of the hen's egg shell that the cone layer consisted of randomly oriented calcite microcrystals. Moreover, the same author believed that the crystals of the column layer developed dendritically in response to the presence of impurities such as magnesium or matrix material. The regularly distributed pits or vesicles observed within the crystal phase were considered to be 'dendritic voids' formed at the junctions of growing crystal limbs (Heyn, 1962, 1963a). In opposition to this,Schmidt (1965) argued that the branching of large crystals in the column layer, and the sectioning of their jagged edges, would explain the apparently dendritic growth of the shell mineral. The presence of concentric rings of gas inclusions radiating outwards from the mammillae was taken as evidence of the synchronous growth of the shell crystals by Schmidt (1965) who also believed that these

inclusions were responsible for the opacity of the finished shell.

Terepka (1963a), using polarized light, stated that the c-axes of the calcite crystals in the column layer were primarily oriented normal to the shell surface. Subsequent X-ray diffraction studies suggested that the crystal c-axes were either inclined at between 12 and 44° (Cain and Heyn, 1964) or else were poorly oriented with respect to the shell surface (Perrott et al., 1981). These last authors found that although there was little preferred orientation between the crystal columns, the individual crystallites of which they were composed exhibited preferred orientations (12 to 48°) similar to those reported in the earlier work. Quintana and Sandoz (1978) observed that in quail shell preparations the c-axes of crystals from the external cone layer were inclined at between 45 and 60° to the surface normal. Silyn-Roberts and Sharp (1985) considered that for most of its width the crystals of the quail shell exhibited only weak preferred orientation. There seems to be some agreement, however, concerning the orientation of crystals within the surface layer of the shell (Favejee et al., 1965; Perrot et al., 1981; Sharp and Silyn-Roberts, 1984). Sharp and Silyn-Roberts (1984) and Silyn-Roberts and Sharp (1986a, b) concluded that the orientation of the surface crystal layer arises from a progressive development of preferred crystal orientation across the shell from the randomly arranged crystals of the mammillae. Much of the dispute concerning the orientation of the shell crystals arises from the difficulty in extrapolating information from very small regions of the whole structure.

c Role of shell organic material

Although the organic component of the hen's egg shell accounts for only about 2-4 wt % its presence has a profound influence on shell morphology. Masshoff and Stolpmann (1961) believed that a 'micronet' of organic material, continuous with the outer shell membrane fibres, permeated the entire shell. Schmidt (1957) had previously suggested that the developing calcite columns were in contact with a keratinous shell gland secretion which became incorporated into the shell. Simons and Wiertz (1963) provided electron micrographic evidence of a clear demarcation between the organic material of the cone and column layers. They observed that the matrix of the column layer consisted mainly of fibrils, approximately 10 μm long and 0.01 μm wide, running parallel to the shell surface. Fujii and Tamura (1969) considered that the matrix network was composed of fibrils 1 to 2 μm in diameter. A fine network arising from vertical projections of the main fibrils has also been described (Simons and Wiertz, 1963; Fujii and Tamura, 1969). Fujii (1974) has proposed that the column layer is initiated from organic material which passes out of the egg through the numerous pore canals which remain open following fusion of the cones. Terepka (1963b) noted that the 'herring-bone' pattern characteristic of the fractured column layer was also evident in decalcified preparations suggesting a close and precise relationship between the deposition of the organic and inorganic phases. Pooley (1979) considered that the mineral phase contains and surrounds the matrix as opposed to being enveloped by it as in bone tissue. Bellairs and Boyde (1969) referred to lines of

matrix in the column layer which they considered lay between the borders of adjacent crystallites.

The distribution of organic material across the hen's egg shell is highly organised and results from a characteristic deposition of mineral and organic phases. The organic cores at the tip of each mammilla are firmly bound to the outer membrane fibres and are separated from the column layer by a region of sparse and poorly organised organic content (Simons and Wiertz, 1963). Beginning at the point where the cones fuse is a band of organic material separated from the remainder of the shell matrix by a second layer of low organic content (Terepka, 1963b; Talbot and Tyler, 1974c). Thereafter, the well ordered shell matrix continues towards the outer edge of the shell, the final 2 μm of which is characterised by a more extensive vertical branching of the matrix fibrils (Simons and Wiertz, 1963).

The role of the mammillary cores in initiating shell formation has already been discussed (Section 4a). The high organic content of these nucleation sites is not typical of the remainder of the mammillary region, the development of which appears to be highly crystalline (Simons and Wiertz, 1963; Creger et al., 1976; Stemberger et al., 1977). Terepka (1963b) and Quintana and Sandoz (1978) have proposed that there is an inverse relationship between organic content and crystal size, the latter authors suggesting that this is true for a range of biominerals. Creger et al. (1976) observed an amorphous calcium deposit at the point of fusion of the cone layer, corresponding to the innermost band of high organic content (Terepka, 1963b). The extrusion of egg organic

material by the developing cones is a possible source of this narrow, organic-rich layer and electron micrographs supporting this interpretation have been presented for both the hen (Fujii, 1974) and the duck (Sparks, 1985).

The initial deposition of the column layer is associated with a marked rise in the rate of shell formation (Talbot and Tyler, 1974c). Although the organic content varies across the width of the shell, the rate of calcite deposition was considered by Talbot and Tyler (1974c) to remain constant until termination. The second region of low organic content, between the cone and column layers, is laid down 7 to 9 h after the egg's entry into the shell gland (Talbot and Tyler, 1974c). This region is thought to be responsible for the opacity of normal eggs because its presence allows the matrix of the column layer to dry out following oviposition. Naturally translucent shells lack this boundary region and water passes continually from the egg contents across the shell membrane (Talbot and Tyler, 1974a). Moreover, translucent streaks often appear on shells which are cracked at their inner surface, presumably because water from the egg can by-pass the organic-poor layer (Talbot and Tyler, 1974b). Eggs which are prematurely removed from the shell gland only become opaque if calcification has proceeded for at least 12 h. At any earlier stage the organic-rich layer is assumed to be too thin to appear opaque (Talbot and Tyler, 1974c).

In most of the column layer, however, there is a well ordered relationship between mineral and matrix deposition (Terepka, 1963b). The herring-bone type striations characteristic of this

layer have been attributed to the cleavage of the calcite crystals (Silyn-Roberts and Sharp, 1986b). Terepka (1963b) observed that the pattern persisted in decalcified preparations and implicated the matrix as the organising component. There is currently some disagreement as to whether the organic matrix acts as a framework for shell development or is merely incorporated into the growing mineral phase.

The concept of an organising shell matrix arose from histochemical and histological studies of its composition (Simkiss and Tyler, 1957, 1958; Krampitz and Witt, 1979). These studies suggested that the matrix was a protein : acid mucopolysaccharide complex and that a fraction of the acid mucopolysaccharide was present as mucoitin sulphuric acid. Terepka (1963b) considered that the matrix was not fibrous in nature but was composed of a highly hydrated mucoid-like material. Baker and Balch (1962) found that the matrix consisted of 70% protein and 11% polysaccharide of which 35% was chondroitin sulphuric acid. Amino acid analyses revealed that the matrix protein was not present as collagen, as in bone, but resembled the non-collagenous protein typical of certain animal cartilage (Baker and Balch, 1962; Leach, 1982). The finding that matrix preparations act as chelating agents (Simkiss and Tyler, 1958) suggested a mechanism whereby the spatial ordering of constituent ions necessary for shell development could be achieved.

Support for the importance of the organic matrix in shell formation has been obtained by studying the effects of a manganese deficiency (Lyons, 1939; Longstaff and Hill, 1972; Leach and Gross, 1983). Egg production in manganese-depleted birds is reduced and

there is a decline in egg shell thickness and hatchability (Lyons, 1939; Leach and Gross, 1983). The shells produced by manganese-depleted birds are often marked by areas of translucence, the underlying mammillae of which are unusually large and appear to arise from the fusion of several mammillary cores (Leach and Gross, 1983). Longstaff and Hill (1972) reported a decrease in both the hexosamine and uronic acid content of shells produced by manganese-depleted birds. As Leach and Gross (1983) pointed out, this is consistent with the role of the element in mucopolysaccharide synthesis. Moreover, the translucence of the shells may arise from a reduced shell thickness and correspondingly high matrix : mineral ratio (Longstaff and Hill, 1972). Because the thin egg shells of manganese-depleted birds are not typical of prematurely expelled shells (Longstaff and Hill, 1972) it was suggested that the reduced synthesis of matrix mucopolysaccharide had inhibited the rate of shell deposition. Indirect evidence for such a relationship could be inferred from the relatively slow crystal growth of the mammillae in the absence of a well organised matrix and by the observation that naturally translucent shells, which do not possess the column region of low organic content, are thicker than normal shells (Talbot and Tyler, 1974a). As Longstaff and Hill (1972) pointed out, the relationship is unlikely to be as simple and in any case the deposition of the organic-poor layer was subsequently considered to have little effect on the rate of shell formation (Talbot and Tyler, 1974c).

Recent studies have tended to suggest that the organising ability of organic matrices in general has, in the past, been

over-emphasised (Mann, 1983; Silyn-Roberts and Sharp, 1986b). The incorporation of an organic phase into the developing shell certainly seems to confer strength to the whole structure and shells with a high organic content tend to be stronger (Petersen and Tyler, 1966). This could be due both to the mechanical properties of the organic material itself and to a reduction in shell crystal size (Terepka, 1963b; Petersen and Tyler, 1966; Quintana and Sandoz, 1978; Williams, 1984). In any case, the development of large, well oriented shell crystals appears to be hindered by the deposition of the organic phase (Silyn-Roberts and Sharp, 1986b). In general, the secretion of the organic component, along with changes in the inorganic composition of shell gland fluid, probably represent the mechanisms whereby biological influences act to determine the nature of inorganic shell development. A complete understanding of egg shell formation in the birds is, therefore, only likely to emerge from an approach which combines the physical chemistry of the calcifying system with the biological requirements of the finished shell.

PART II: THE ROLES OF MAGNESIUM, MANGANESE AND COPPER IN EGG SHELL FORMATION

PART II: CHAPTER 1

EXPERIMENTAL DETAILS

1.1 The birds

Pullets and eggs from two separate flocks (Table 1) were studied. All birds were individually caged in batteries and had continuous access to food and de-ionised water. Both flocks were kept under an artificial lighting regime consisting of fourteen hours of light (average intensity, 49.5 lux) and ten of darkness. Hut temperature was kept constant at 20 ± 1°C.

1.2 The diets

a Diet composition

The pullets were reared on a growers' ration until sexually mature, then switched to a layers' mash. The latter was routinely mixed at the PRC and resembled commercially available feedstuff. As the mash formed the staple diet of the majority of birds in both flocks it will henceforth be referred to as the standard diet.

Experimental diets were prepared in small-scale mixers (Bear Varimix, capacity 20 kg). These diets, which were used in studies with both flocks, were all based upon a single formulation (Table 2) derived from the reports of previous workers (Stafford and Edwards, 1973; Baumgartner et al.,1978; Leach and Gross, 1983). This formulation (the basal mixture) was intended to contain the lowest possible levels of the three elements, magnesium, manganese and copper.

During the study of Flock 1 the basal mixture was selectively

Table 1. The birds.

Flock	Number of birds*	Hatch date	Age at switch to layers' ration (weeks)	Age at which experimental diets administered (weeks)
1	192	14/10/83	18	33
2	102	17/09/85	18	21

* Warren ISA Brown strain (Robert Thompson Chicks, Thornhill, Dumfries.).

Table 2. Composition of the basal mixture.

Ingredient	Wt %
Cellulose	10.00
FP950	14.00
Casein	6.00
Corn oil	7.00
D-L methionine	0.50
Sucrose	52.13
Choline chloride	0.04
Vitamin mix*	0.39
Trace mineral mix**	0.06
NaCl	0.37
$CaHPO_4.2H_2O$	2.20
$CaCO_3$ (limestone flour)	6.80
KH_2PO_4	0.51
TOTAL	100.00

* supplies per 100 kg diet : Rovimix AD_3, 1.5 g; Rovimix E, 4.0 g; menapthone, 1.5 g; aneurine HCl, 10.0 g; nicotinic acid, 40.0 g; Ca pantothenate, 6.0 g; folic acid, 0.3 g; cyanocobalimin, 0.01 g; biotin, 1.5 g; riboflavin, 4.8 g; pyridoxine HCl, 1.8 g; ascorbic acid, 25.0 g; rice starch diluent. (after Dewar et al., 1974).

** supplies per 100 kg diet : Na_2SeO_3, 0.16 g; $Na_2MoO_4.2H_2O$, 0.83 g; KI, 0.26 g; $FeSO_4.7H_2O$, 33.3 g; ZnO, 6.26 g; $CoSO_4.7H_2O$, 0.195 g; rice starch diluent.

supplemented to produce four experimental diets. Three of the diets were deficient in either magnesium, manganese or copper and the fourth was supplemented with all three elements. The three experimental diets fed to selected birds of Flock 2 all contained different amounts of magnesium. The levels of added minerals used in the studies of both flocks are given in Table 3.

Table 4 gives the combinations of supplements used in the production of all experimental diets and provides a summary of the groups used in the present work.

b Diet analysis

The experimental diets fed to birds of Flock 1 were analysed by the PRC's Nutrition Department. The ingredients as well as the complete diets used in the study of Flock 2 were analysed for their magnesium content using atomic absorption spectroscopy (AAS) as follows.

Three 2.0 g samples of each material were taken and ashed at 500°C for 16 h. The cooled ash was dissolved in 10 ml of 6 M hydrochloric acid and the solution filtered (Whatman no. 1) into a 100 ml volumetric flask and filled to the mark with de-ionised water. To prevent interference by phosphate, 1 ml of the inhibitor lanthanum chloride (2.82% w/v) was added to 0.1 ml of the sample and the solution made up to 10 ml with de-ionised water.

All solutions were aspirated until the absorbance read-out of the spectrometer (Varian AA875, Varian, 28 Manor Road, Walton-on-Thames) became constant at which point an automatic average was made over 3 s and recorded onto a pen chart. All calibration curves

Table 3. Levels of mineral supplements used in the experimental diets.

Code	Element	Compound	Added amount (mg/kg diet)	Element content (mg/kg diet)
A	Magnesium	$MgSO_4.7H_2O$	2500	244
B	Manganese	$MnCO_3$	209	100
C	Copper	$CuSO_4.5H_2O$	13	4
D	Magnesium	MgO	2183	1300

Table 4. Combinations of mineral supplements used in the experimental diets.

Diet	Supplements used*	Flock	Group	Number of birds
Standard diet (STAND)	none	1	I	160
Basal mixture supplemented with Mg, Mn and Cu (BAS-SUP)	ABC	1	II	8
Magnesium-deficient (MAG-DEF)	BC	1	III	8
Manganese-deficient (MAN-DEF)	AC	1	IV	8
Copper-deficient (COP-DEF)	AB	1	V	8
Standard diet (STAND)	none	2	VI	66
Magnesium-supplemented (MAG-SUP)	BCD	2	VII	12
Magnesium-deficient (MAG-DEF)	BC	2	VIII	12
Magnesium-lowest (MAG-LOW)**	BC	2	IX	12

* relates to codes used in Table 3.

** see Section 2.5.

(calcium, 1-5 ppm; magnesium, 0.1-0.5 ppm) were subjected to linear regression analysis and the element concentrations of the sample solutions obtained.

In order to check instrument accuracy, solutions of known concentrations of calcium carbonate powder (Analar grade) and of pulverised limestone flour were also analysed by AAS.

1.3 Blood analysis

Blood was drawn from the wing vein into a heparinised syringe (2 ml) and then sealed in a heparinised plasma tube. After centrifugation at 1600 x g for 15 min the plasma was pipetted off and treated in one of two ways before being analysed by AAS:

(i) Plasma (0.1 ml) was added to 1 ml of the phosphate inhibitor strontium chloride (0.2% w/v) and the solution made up to 10 ml using de-ionised water.

(ii) Plasma was first added to an equal volume of 10% (w/v) trichloroacetic acid (TCA) in order to precipitate the plasma proteins and respun at 1600 x g for a further 15 min. The resulting clear liquid was drawn off and 0.2 ml added to 1 ml of a strontium chloride solution then made up to 10 ml with de-ionised water.

1.4 Monitoring egg production

When the flocks came into full lay, eggs were collected daily and a record kept for each bird. At regular intervals the mean egg weight (g) of each of the groups was measured, all eggs being weighed within 48 h of being laid.

Egg shell thickness was measured using an anvil-headed micrometer gauge. The eggs were broken, emptied and the membranes carefully peeled away. The inner surface of the shell was washed free of albumen and the average value of shell thickness (μm) at the shoulder of each egg calculated from three measurements made in close proximity to one another.

1.5 Shell element content

Egg shells were cut in half using a high speed cutting wheel (Cahill Dental Ltd., Henleaze, Bristol) and the membranes carefully peeled away. In a number of cases the half-shells were immersed for 15 min in 0.34 M ethylenediaminetetraacetic acid (EDTA) adjusted to pH 7.5. The cuticle and membrane remnants of these shells were then washed away. After drying overnight (Drierite, Koch Light Laboratories Ltd., Colnbrook, Bucks.) all shells were ground in a pestle and mortar and 1.0 g of material weighed into a Pyrex beaker and covered with a watch-glass. The samples were then ashed at either 600°C (Stafford and Edwards, 1973) or 700°C (Wright and Riner, 1972) for 16 h. The cooled ash was added to 5 ml of 6 M hydrochloric acid (HCl) which was then evaporated to dryness. Two ml of 36% (v/v) HCl was added dropwise and the solution boiled gently for 2 min after which 5 ml of de-ionised water was added. Each solution was made up to 50 ml and filtered (Whatman no. 541) into polythene bottles, the first few ml of filtrate being rejected.

All analyses of shell element content were carried out using a Pye Unicam SP9 atomic absorption spectrometer (Pye Unicam Ltd., York Street, Cambridge).

PART II: CHAPTER 2

RESULTS

Flock 1

2.1 Diet analysis

The results of the analysis of the basal mixture are given in Table 5. The relatively high level of available carbohydrate (41.4 wt %) was due to the diet being composed predominantly of sucrose. The gross reduction in dietary manganese in the sucrose-based diet of Leach and Gross (1983) was also achieved in the present work. The 'purified' diet of Baumgartner et al. (1978) was quoted to contain only 0.72 mg copper per kg diet compared to the present 3.0 mg/kg diet (Table 5). It therefore seems unlikely that the basal mixture was deficient in this element. The starch-based diet of Stafford and Edwards (1973) contained 118 mg compared with the 215 mg of magnesium per kg diet used in the present study. Both values are well below the recommended minimum requirement of 400 mg/kg diet.

After supplementation of the basal mixture to produce the four experimental diets the levels of the three elements were again measured. Due to an apparent reduction in instrument sensitivity (concentrations below 10 mg/kg diet were reported to be below the level of detection) the values for dietary copper content were not available. The levels of magnesium and manganese in the four experimental diets are given in Table 6 along with the magnesium content of the standard diet. It can be seen that the values for both elements approximate, in the experimental diets, to either the

Table 5. Analysis of basal mixture.

Constituent	Concentration	
	actual	recommended*
wt %		
Crude protein	16.80	16.00
Available carbohydrate	41.40	n.q.
Oil	7.20	n.q.
Ash	9.90	n.q.
Calcium	2.90	2.50 to 3.50
Phosphorus	0.47	0.40
mg/kg		
Magnesium	215.00	400.00
Manganese	4.80	100.00
Copper	3.00	3.50

* Anon. (1975).

n.q. not quoted.

Table 6. Analysis of diets used in study of Flock 1.

Diet	Element content (mg/kg)	
	magnesium	manganese
STAND	1522	n.a.
BAS-SUP	400	100
MAG-DEF	200	100
MAN-DEF	400	n.d.
COP-DEF	400	100

n.a. not available.

n.d. not detected.

basal levels given in Table 5 or else to the sum of the basal and supplemented values (the latter being given in Table 3).

2.2 Influence of diet on plasma magnesium content

Three birds from each of the four groups receiving the experimental diets (II, III, IV, V) were blood-sampled at regular intervals to assess the effects of the various diets on the relevant plasma elements. This proved to be impracticable in the case of manganese and copper due to the small amounts present even under normal conditions. As a result, information was obtained only on the plasma magnesium status of Group II (BAS-SUP) and Group III (MAG-DEF) birds. Figure 1 shows the mean plasma magnesium concentrations (mg %) of both groups plotted against the time (weeks) following administration of the experimental diets. In both cases a fall in plasma magnesium content occurred within the first week of feeding the diets. This effect was far more pronounced in the birds of Group III (MAG-DEF) than in those of Group II (BAS-SUP). Moreover, after a brief period of hypomagnesaemia the birds of Group II (BAS-SUP) were found to contain more normal levels of this element in the plasma. It is evident from Figure 1 that the marked reduction in dietary magnesium achieved in the experimental diet of Group III (MAG-DEF) was very quickly reflected in the plasma of these birds. Conversely, the provision of magnesium in the diet of Group II (BAS-SUP) maintained near-normal levels of this element in the blood.

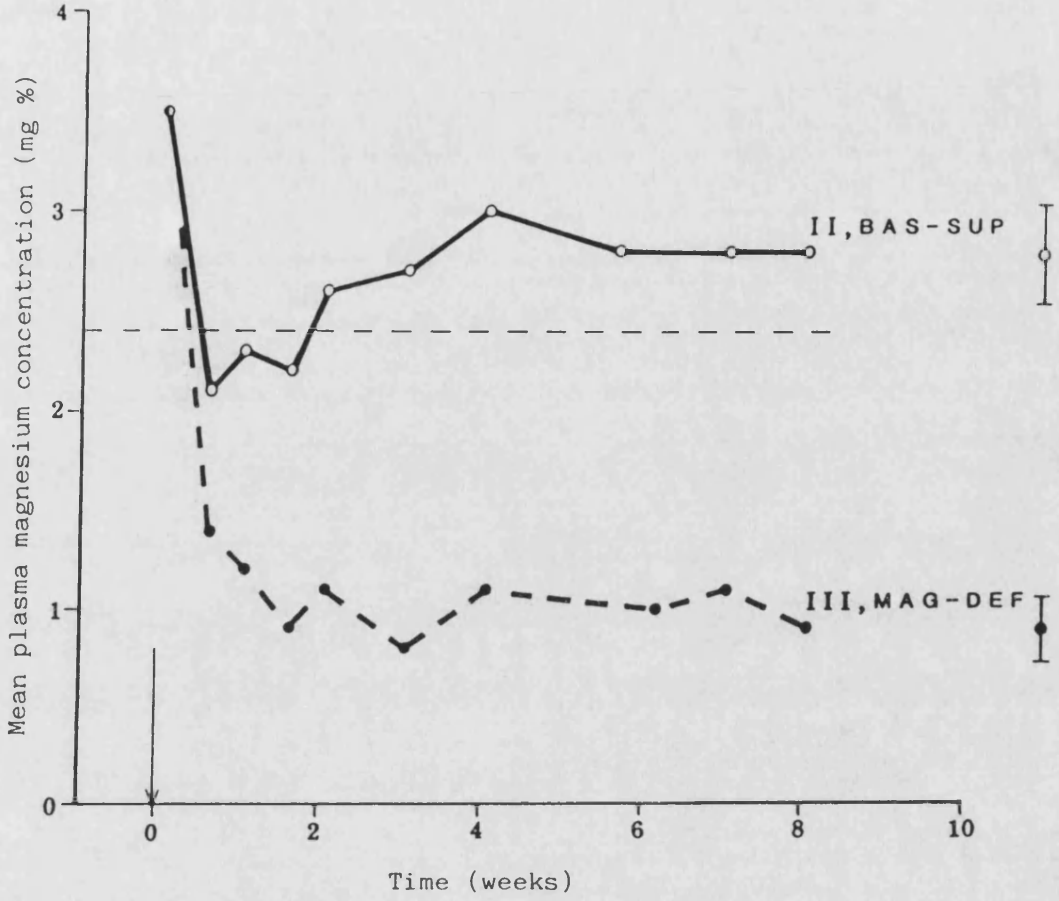


Figure 1. Mean plasma magnesium concentrations in Groups II and III following administration of experimental diets.

Vertical bars, mean standard errors (n = 3 birds).

Horizontal line, value below which birds are considered hypomagnesaemic (Stafford and Edwards, 1973).

Arrow, point at which experimental diets administered.

2.3 Egg production

a Egg number

The mean number of eggs per bird per week was plotted for each group in Flock 1 following onset of lay (Figures 2a, b). Figure 2a shows that egg production in Group I (STAND) reached a peak value of 90% in the sixth week of lay. Thereafter, production declined and after 27 weeks had dropped to 73% of maximum. Similar trends were evident in Groups II (BAS-SUP), IV (MAN-DEF) and V (COP-DEF). With Group III (MAG-DEF) birds, however, a decrease in egg production occurred within 2 to 3 weeks of administering the experimental diets. Indeed, after 12 weeks on the diet the group was out of lay. In summary therefore, a significant reduction in egg number was observed only in birds receiving the magnesium-deficient diet.

b Egg weight

The changes in group mean egg weight (g) during the study of Flock 1 are given in Figures 3a, b. In birds which received the standard diet throughout (Group I, STAND) the mean egg weight rose from 52.4 to 66.0 g during the 26 weeks of observation. The only noteworthy departure from this trend was seen in the birds of Group III (MAG-DEF) in which mean egg weight declined following administration of the experimental diets. In all other cases group mean egg weights were, on average, only slightly higher (Group V, COP-DEF) or lower (Group II, BAS-SUP; Group IV, MAN-DEF) than the corresponding Group I values, though these departures were not considered significant.

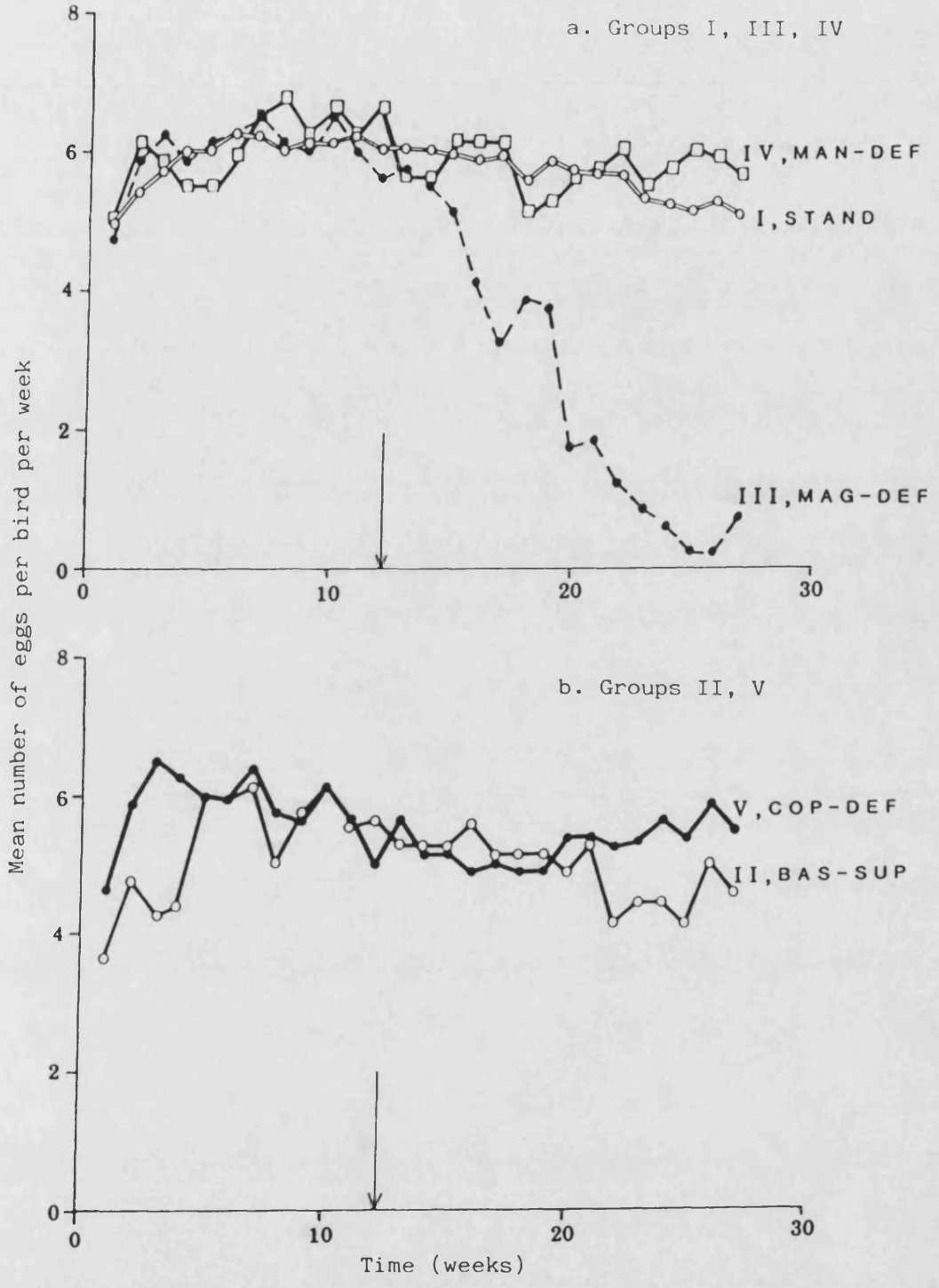
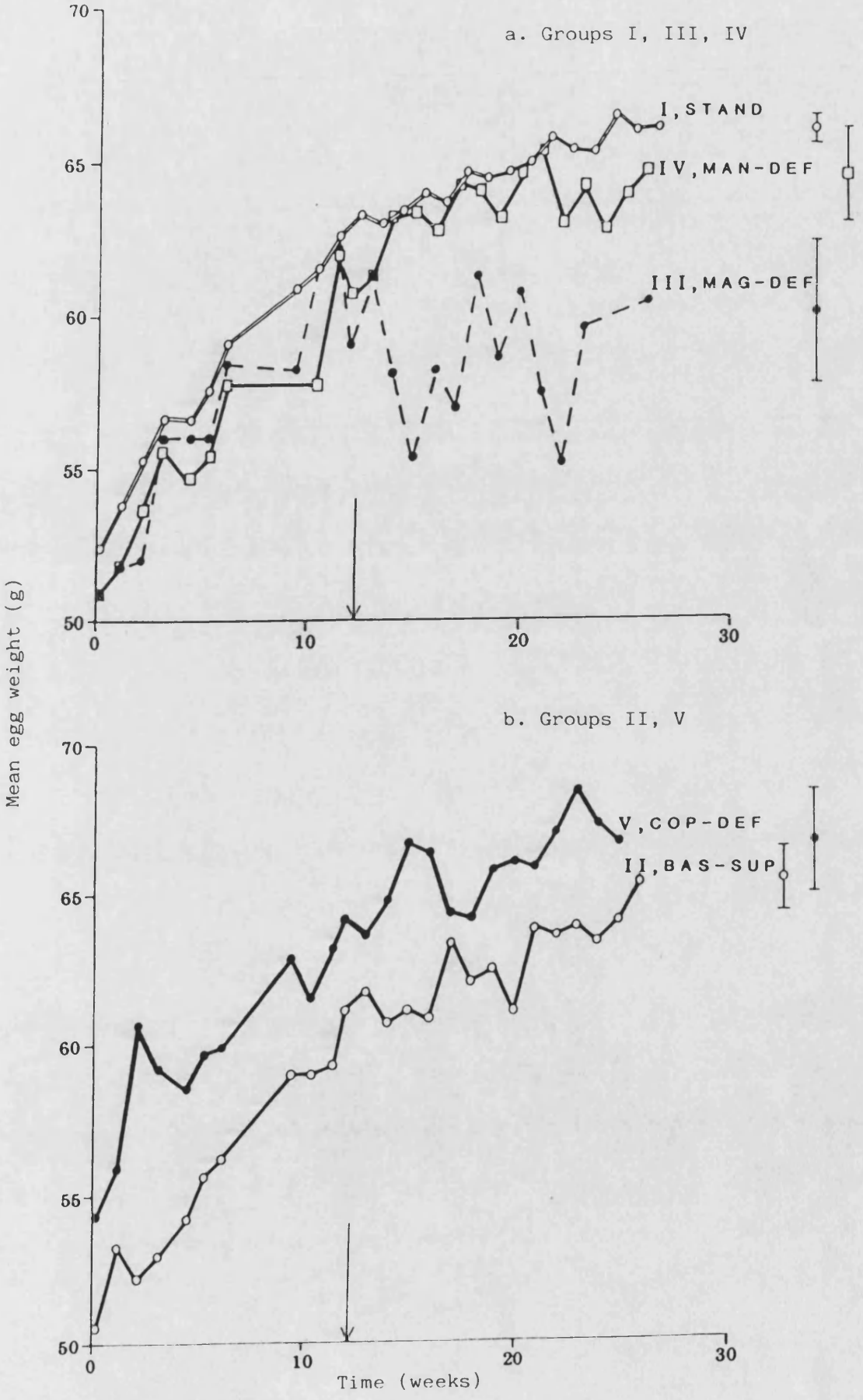


Figure 2. Mean number of eggs produced each week by Groups I to V following onset of lay.

Arrows, point at which experimental diets administered.

Figure 3. Mean egg weight in Groups I to V following onset of lay.
Vertical bars, mean standard errors.
Arrows, point at which experimental diets administered.


a. Groups I, III, IV
I, STAND
IV, MAN-DEF
III, MAG-DEF
b. Groups II, V
V, COP-DEF
II, BAS-SUP
Mean egg weight (g)
Time (weeks)
70
65
60
55
50
0
10
20
30

c Egg shell thickness

Mean egg shell thicknesses (μm) obtained from Groups II, III, IV and V following administration of experimental diets are shown in Figure 4. A decline in shell thickness was observed in all cases. By far the most severe reduction occurred in shells of Group III (MAG-DEF) birds.

2.4 Shell element content

Shell magnesium and calcium contents, as measured by AAS, are given in Figures 5a, b for the egg shells of three birds in Group II (BAS-SUP) and three birds in Group III (MAG-DEF) collected at weekly intervals following the administration of experimental diets. The ratios of the two elements in the shells are given in Figure 5c. It can be seen that despite the apparently high calcium content of Group III (MAG-DEF) shells at the end of 5 weeks (Figure 5b), the fall in the magnesium : calcium ratio (Figure 5c) was due entirely to a fall in absolute magnesium content (Figure 5a).

Flock 2

2.5 Diet analysis

The contributions of various dietary constituents to the magnesium content of the basal mixture, as measured by AAS, are given in Table 7. The relatively high magnesium content of the protein source, FP950, is evident and represents almost four times the amount found in casein. The vitamin and trace mineral mixes also contained magnesium though neither constituent was present in sufficient amounts, in the basal mixture, to be considered a major

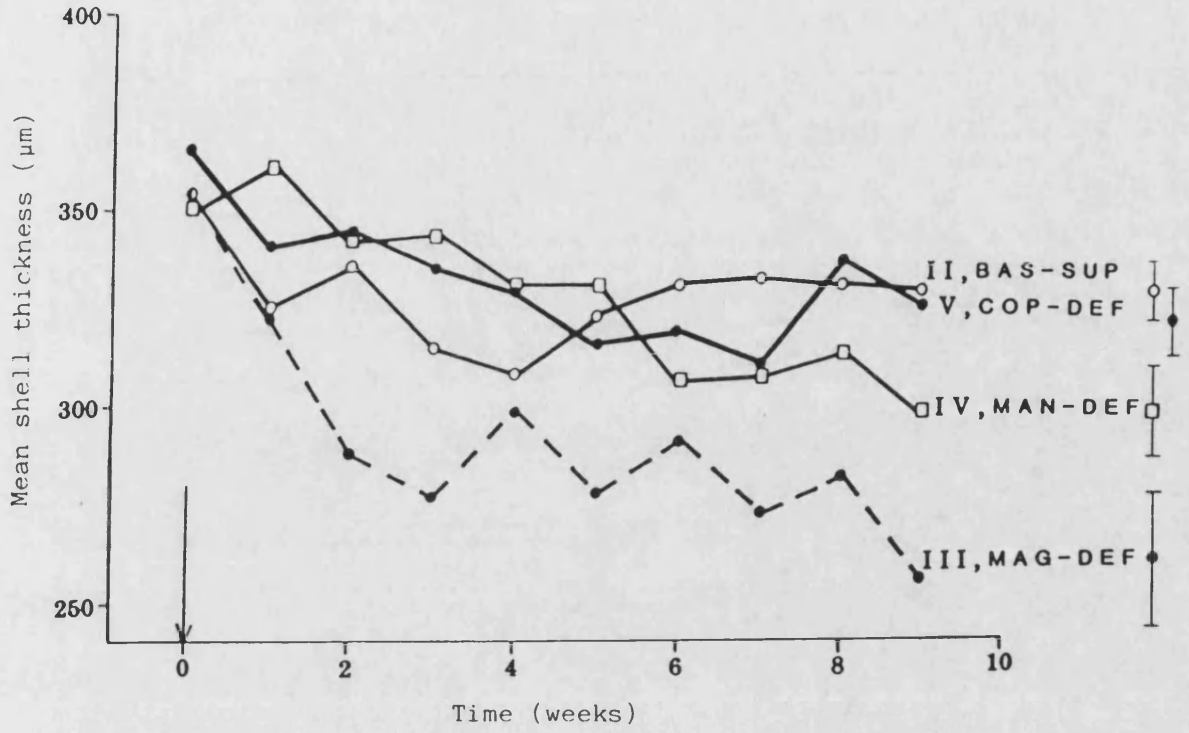


Figure 4. Mean egg shell thickness in Groups II to V following administration of experimental diets. Vertical bars, mean standard errors. Arrow, point at which experimental diets administered.

Figure 5. Mean egg shell element content in Groups II and III following administration of experimental diets. Vertical bars, mean standard errors (n = 3 shells). Arrows, point at which experimental diets administered.

a. Magnesium

b. Calcium

c. Magnesium : calcium ratio calculated from Figures 5 a, b.

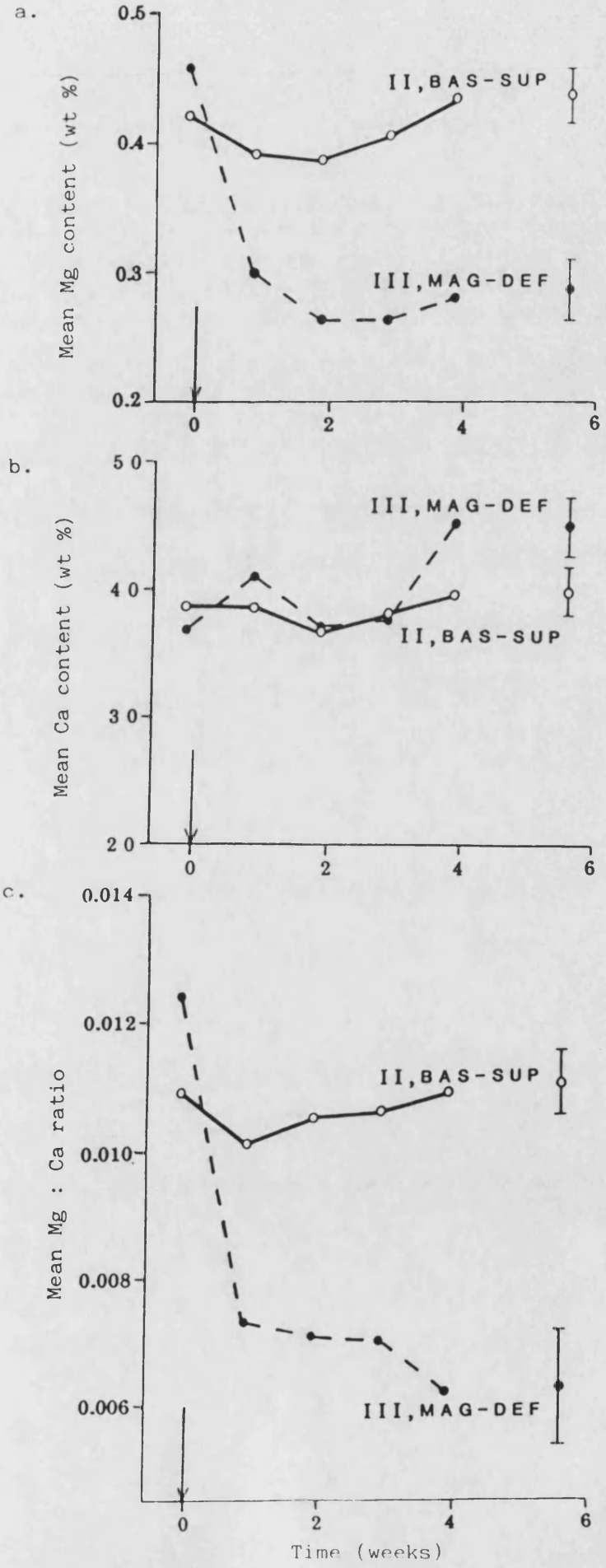

a.
Mean Mg content (wt %)
0.5
0.4
0.3
0.2
II, BAS-SUP
III, MAG-DEF
0
2
4
6
b.
Mean Ca content (wt %)
5 0
4 0
3 0
2 0
III, MAG-DEF
II, BAS-SUP
0
2
4
6
c.
Mean Mg : Ca ratio
0.014
0.012
0.010
0.008
0.006
II, BAS-SUP
III, MAG-DEF
0
2
4
6
Time (weeks)

Table 7. Magnesium content of four constituents of the basal mixture.

Constituent	Wt % constituent in diet	Magnesium content (mg/kg) ± S.E.	
		constituent	diet*
FP950	14.00	755 ± 40	106 ± 6
Casein	6.00	203 ± 1	12 ± 0.1
Vitamin mix	0.40	160 ± 4	0.6 ± 0.1
Trace mineral mix	<0.10	102 ± < 0.1	< 0.1

* overall level, 215 mg/kg (Table 5).

source of this element.

The replacement of FP950 by casein resulted in the production of a diet (MAG-LOW) containing less magnesium than previously obtained. Of the three remaining diets used in the study of Flock 2, one was the standard diet (STAND), one was the previously formulated magnesium-deficient diet (MAG-DEF) and the third was the basal mixture (Table 2) supplemented with sufficient magnesium so as to resemble the level found in the standard diet (MAG-SUP). Diets fed to Flock 2 birds contained normal levels of all other elements.

Table 8 gives the magnesium contents of the four diets. The relatively high level found in feedstuffs prepared on a large scale (STAND) is evident. Moreover, the attempt to match this level in an experimental diet (MAG-SUP) was successful. The value of 207 mg magnesium per kg diet found in the magnesium-deficient diet (MAG-DEF) was similar to the 215 mg/kg obtained during the study of Flock 1. The reduction in magnesium achieved in the MAG-LOW diet (207 to 132 mg/kg diet) resembles that reported by Stafford and Edwards (1973) when they also switched to a casein protein source (118 to 49 mg/kg diet).

Table 9 shows the calcium and magnesium contents (wt %) of the two reference materials used to confirm the reliability of the analytical method. The value of 39.3 wt % calcium for Analar grade calcium carbonate is acceptably close to the theoretical value of 40.0 wt % and the failure to detect magnesium in this material suggests there was little or no contamination by this element during sample preparation. Pulverised limestone flour (calcium

Table 8. Magnesium content of the four diets used in study of Flock 2.

Diet	Magnesium content (mg/kg) ± S.E.
Standard (STAND)	1522 ± 11
Magnesium-supplemented (MAG-SUP)	1323 ± 55
Magnesium-deficient (MAG-DEF)	207 ± 6
Magnesium-lowest (MAG-LOW)	132 ± 8

Table 9. Calcium and magnesium content of two reference materials.

Material	Element content (wt %) ± S.E.	
	calcium	magnesium
Calcium carbonate (Analar grade)	39.3 ± 1.7	n.d.
Pulverised limestone flour*	38.5 ± 1.4	0.10 ± < 0.01

* This material was a constituent of the basal mixture (Table 2).

n.d. not detected

content, 38.5 wt %) was also the principal source of calcium in the basal mixture. Despite its relatively low magnesium content (0.10 wt %) this material was almost certainly the only other major source of this element in the basal mixture.

2.6 Influence of diet on plasma magnesium content

Four birds from each of the groups of Flock 2 were blood-sampled (between 1400 and 1600 h) at regular intervals following the administration of experimental diets. Figure 6 shows the effects of feeding the various diets on the mean plasma magnesium concentration (mg %) of each group. The relationship between dietary magnesium content and the level maintained in the plasma is apparent. Furthermore, the hypomagnesaemia of birds in Groups VIII (MAG-DEF) and IX (MAG-LOW) was induced within 2 days of the switch to experimental diets.

2.7 Egg production

a Egg number

Figure 7 shows the mean number of eggs laid each week in Groups VI to IX following the onset of lay. In contrast to the study of Flock 1 there was only a 7 week period of observation. Nonetheless, the birds of Group VI (STAND) achieved near maximum production over the first 6 weeks of lay as did those of Group VII (MAG-SUP). Birds fed the magnesium-deficient diet (Group VIII, MAG-DEF) showed a decline in egg production, following the switch to experimental diet, similar to that seen in the corresponding birds of Flock 1. This, however, was not as severe as the disturbance in egg laying

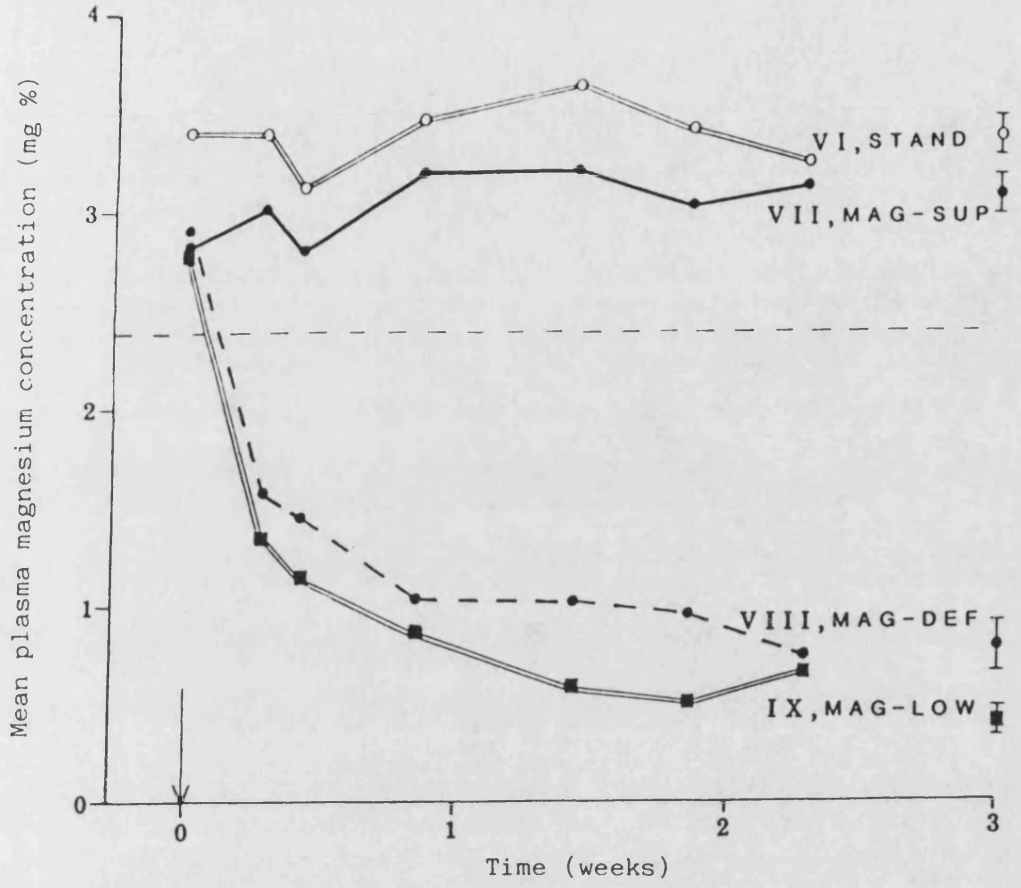


Figure 6. Mean plasma magnesium concentrations in Groups VI to IX following administration of experimental diets. Vertical bars, mean standard errors (n = 4 birds). Horizontal line, value below which birds are considered hypomagnesaemic (Stafford and Edwards, 1973).
Arrow, point at which experimental diets administered.

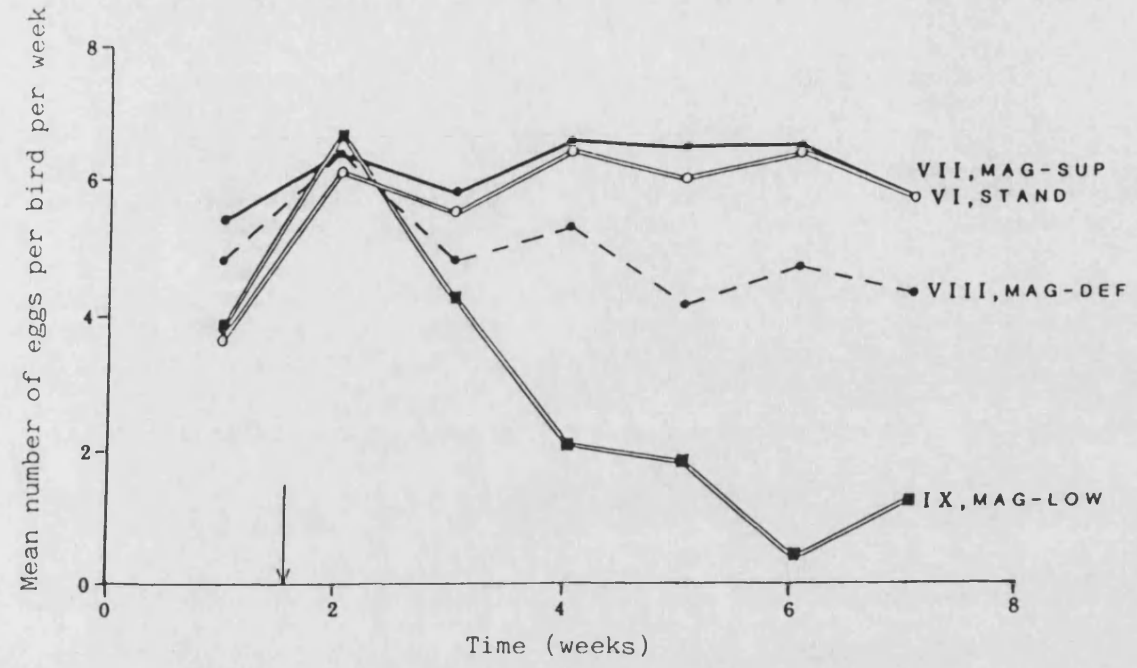


Figure 7. Mean number of eggs produced each week by Groups VI to IX following onset of lay.

Arrow, point at which experimental diets administered.

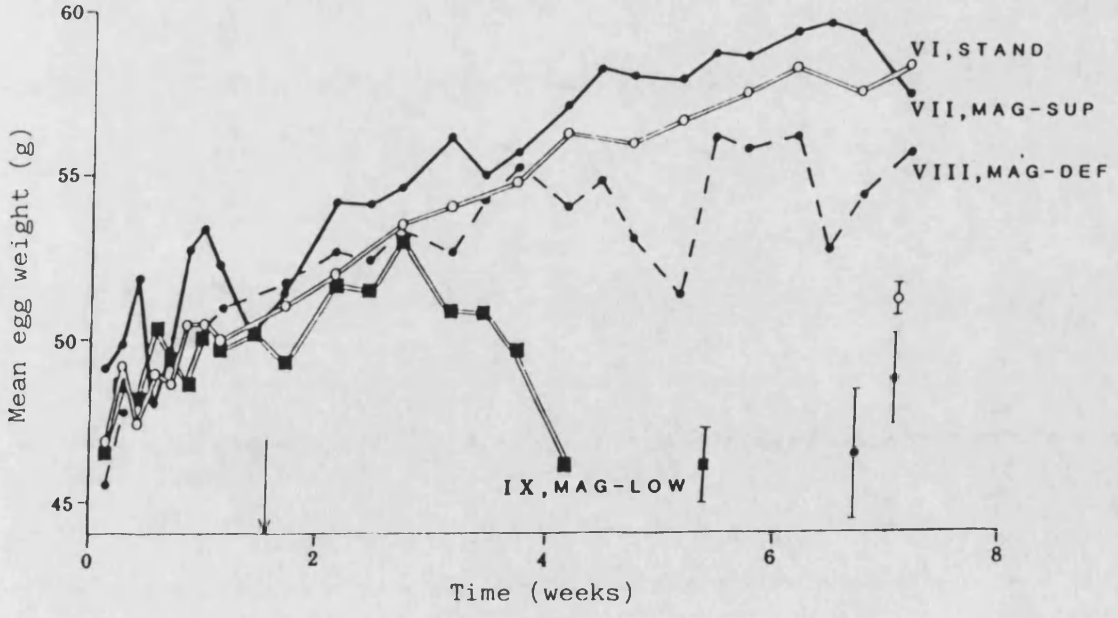


Figure 8. Mean egg weight in Groups VI to IX following onset of lay.

Vertical bars, mean standard errors.

Arrow, point at which experimental diets administered.

observed in birds fed the diet containing least magnesium (Group IX, MAG-LOW).

b Egg weight

The changes in mean egg weight (g) in groups VI to IX are given in Figure 8. As in the study of Flock 1 there was an increase in the mean egg weight of birds fed on the standard diet (Group VI, STAND). The initial mean egg weight of 46.9 g was lower than the 52.3 g seen in the corresponding Flock 1 birds but the absolute increase in mean egg weight over the first 7 weeks was very similar at 7.2 g and 7.3 g respectively. A similar increase in mean egg weight was also observed in Group VII (MAG-SUP) birds indicating that, fully supplemented, the basal mixture was capable of maintaining normal performance profiles. By contrast, the mean egg weight of Group VIII (MAG-DEF) birds stopped increasing after 2 weeks on the experimental diet. After only 1 week on the diet containing least magnesium the mean egg weight of Group IX (MAG-LOW) birds began to decrease and, after 3 weeks, had fallen below its initial value of 46.4 g.

c Duration of egg formation

On two occasions during the study of Flock 2 the mean duration of egg formation (h) was calculated for all birds. On each occasion, eggs were collected every hour following lights-on (0400 h) and this was repeated over four successive days. In this way a maximum of three intervals between successive ovipositions were obtained for each bird. Table 10a shows the group mean

Table 10. Mean intervals between successive ovipositions in Flock 2 birds.

a. At the time of administration of experimental diets.

Group	Mean interval (h) between eggs		
	1st-2nd egg	2nd-3rd egg	3rd-4th egg
VI (STAND)	24 (38)	24 (42)	24 (41)
VII (MAG-SUP)	23 (5)	24 (6)	23 (4)
VIII (MAG-DEF)	24 (8)	24 (8)	24 (5)
IX (MAG-LOW)	23 (9)	24 (9)	24 (8)

Figures in parentheses represent the number of observations used to calculate the mean.

b. Three weeks after administration of experimental diets.

Group	Mean interval (h) between eggs		
	1st-2nd egg	2nd-3rd egg	3rd-4th egg
VI (STAND)	24 (53)	24 (52)	24 (55)
VII (MAG-SUP)	24 (11)	24 (10)	24 (11)
VIII (MAG-DEF)	24 (6)	25 (5)	25 (6)
IX (MAG-LOW)	27 (5)	25 (2)	n.e.l

Figures in parentheses indicate the number of observations used to calculate the mean.

n.e.l. no eggs laid.

interval values (h) obtained over the 4 days prior to the administration of experimental diets. After 3 weeks the observations were repeated and the results are given in Table 10b. Although magnesium-deficiency caused a marked disturbance in egg number, egg weight and egg shell thickness, it appeared to have little effect on the residence time of the egg in the oviduct. There was an indication (Table 10b) that the mean interval between successive ovipositions was slightly increased in birds receiving least dietary magnesium (Group IX, MAG-LOW) but due to the severe reduction in egg production in this group it is difficult to attach significance to this observation.

2.8 Effect of egg formation on plasma calcium and magnesium content

Twelve birds from Group VI (STAND) were blood-sampled at regular intervals over a period of 24 h. Oviposition times were recorded the following day in order to confirm that at the time of sampling each bird was laying down egg material. The results from one non-laying bird were subsequently excluded from the analysis. The experiment was carried out when the birds were 24 weeks of age.

Figure 9a shows the variation in group mean plasma calcium content (mg %) over the 24 h period. The drop in circulating calcium which occurred at the end of the dark period is clearly shown though at all other times plasma calcium content tended to remain relatively constant. By contrast, the mean level of plasma magnesium (Figure 9b) rose steadily throughout the day reaching a peak value of 4 mg % shortly after lights-out (1800 h). Thereafter, the amount of this element in the blood declined throughout the

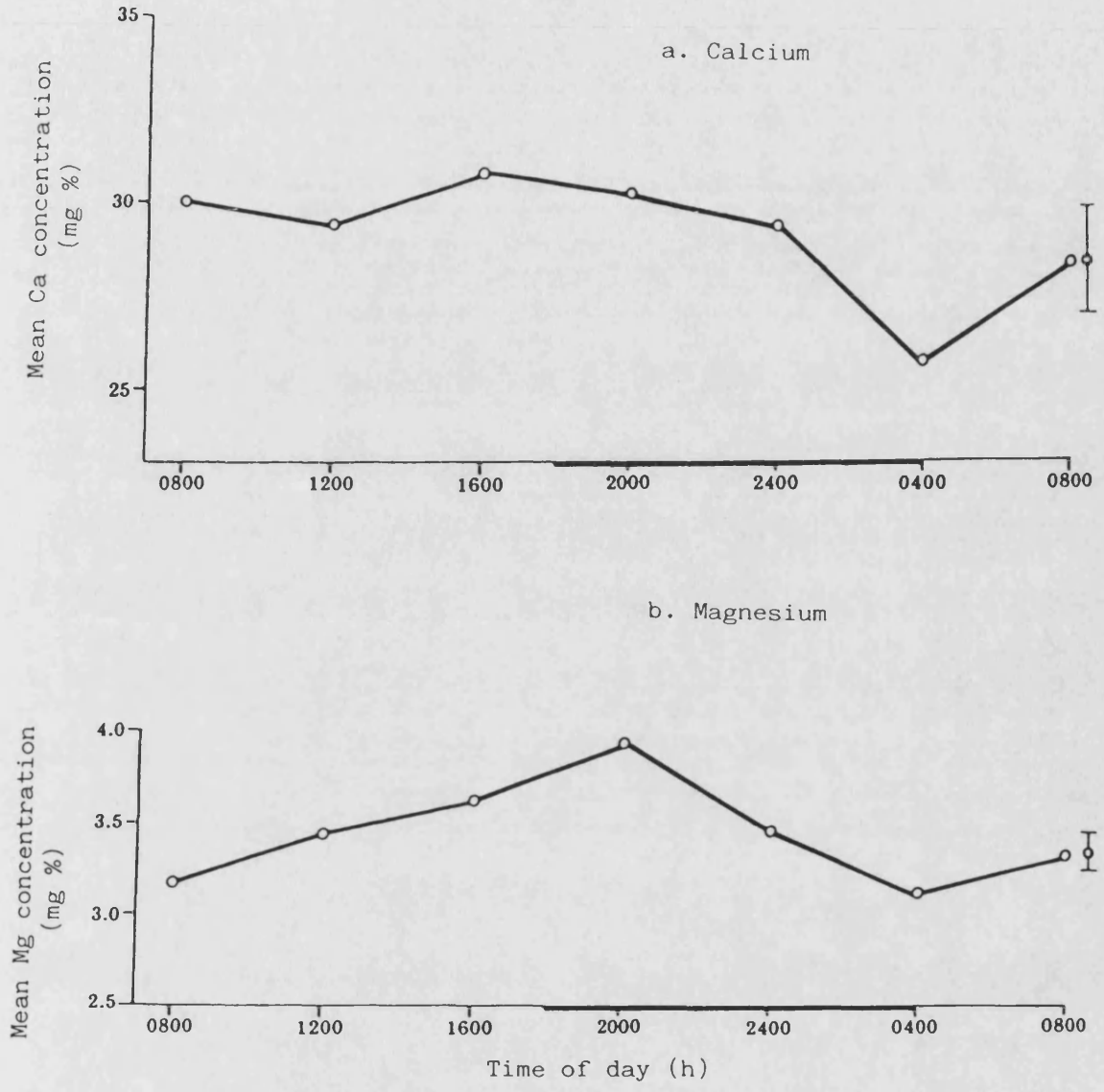


Figure 9. Diurnal variation in plasma calcium and magnesium levels in selected birds of Group VI (STAND). Vertical bars, mean standard errors (n = 11 birds).

dark period, reaching a minimum value of around 3.2 mg % immediately prior to lights-on (0400 h). It seems that despite the apparent differences in the daily variations of these two elements in the plasma, a common feature of both was the marked fall which occurred towards the end of the dark period.

In a second experiment, selected birds of Groups VI (STAND), VII (MAG-SUP) and VIII (MAG-DEF) were blood-sampled at the beginning and end of the dark period. The birds were 26 weeks old and had received the experimental diets for 5 weeks. Group IX (MAG-LOW) birds were no longer in lay at this time and were excluded from the analysis. Table 11a shows the changes in mean plasma calcium content (mg %) which occurred overnight in Groups VII (MAG-SUP) and VIII (MAG-DEF). In both cases plasma calcium content was lower at the end of the dark period than at the beginning but these differences were not considered to be significant. It should be noted however that the mean plasma calcium level of group VIII (MAG-DEF) birds was consistently lower than that found in the other groups; the value of 23 mg %, seen at the end of the dark period, was the lowest group mean value observed in the present work (Table 11a).

Table 11b shows the corresponding group mean plasma magnesium concentrations (mg %). The marked fall in plasma magnesium level seen in Figure 9b was observed in a further four birds of Group VI (STAND). A similar result was obtained from eight birds of Group VII (MAG-SUP), the mean plasma magnesium level in both cases falling by approximately 25% during the dark period. The hypo-magnesaemia of birds fed on low-magnesium diets (Figure 6) was once again evident in birds of Group VIII (MAG-DEF).

Table 11. Plasma element concentrations in selected birds of Groups VI to VIII at the beginning and end of the dark period.

a. Calcium

Group	Mean plasma calcium concentration (mg %) ± S.E.	
	beginning	end
VI (STAND)*	30.2 ± 1.1	25.7 ± 0.4
VII (MAG-SUP)**	28.4 ± 0.9	27.1 ± 1.8
VIII (MAG-DEF)***	25.4 ± 1.6	23.0 ± 1.4

* values taken from Figure 9a, not independently determined, n = 11.

** n = 4.

*** n = 8.

b. Magnesium

Group	Mean plasma magnesium concentration (mg %) ± S.E.	
	beginning	end
VI (STAND)*	3.8 ± 0.3	2.9 ± 0.1
VII (MAG-SUP)**	4.0 ± 0.2	2.9 ± 0.1
VIII (MAG-DEF)**	0.8 ± 0.1	0.5 ± 0.02

* n = 4.

** n = 8.

2.9 Shell element content

The eggs of six birds from each of Groups VII to IX were collected on two occasions: once on the day of the switch to experimental diet and again 2 weeks later. Table 12a gives the group mean shell calcium contents (wt %), as measured by AAS, obtained for these times. There was little change in the level of shell calcium and no effect due to diet. Table 12b shows the marked correlation between dietary magnesium level and shell magnesium content (wt %). As in the earlier findings (Figure 5a) there was a reduction in the absolute amount of this element in the egg shells of birds receiving the magnesium-deficient diets. It appears that as well as being thinner such shells also contain less magnesium per unit mass.

The values of shell calcium and magnesium content were adjusted according to the results obtained, by AAS, for solutions of both Analar grade calcium carbonate and magnesium sulphate. The derivation of each correction factor (calcium, x 0.903; magnesium, x 1.08) is shown below the relevant table.

Table 12. Egg shell element content in selected birds of Flock 2 before and after administration of experimental diets.

a. Calcium*

Group	Mean shell calcium content** (wt % ± S.E.)	
	before administration	after 2 weeks
VII (MAG-SUP)	38.8 ± 0.7	39.7 ± 0.7
VIII (MAG-DEF)	37.3 ± 1.6	35.1 ± 1.7
IX (MAG-LOW)	37.9 ± 1.1	38.2 ± 0.6

* reference calcium carbonate, theoretical content 40.0 wt %, actual 44.3 wt %, correction x 0.903.

** n = 6.

b. Magnesium*

Group	Mean shell magnesium content** (wt %) ± S.E.	
	before administration	after 2 weeks
VII(MAG-SUP)	0.37 ± 0.02	0.35 ± 0.01
VIII (MAG-DEF)	0.32 ± 0.02	0.19 ± 0.01
IX (MAG-LOW)	0.32 ± 0.02	0.15 ± 0.01

* reference magnesium sulphate, theoretical content 9.74 wt %, actual 8.98 wt %, correction x 1.08.

** n = 6.

PART II: CHAPTER 3

DISCUSSION

3.1 Flock 1

The inability in the present study to induce a copper deficiency in Group V (COP-DEF) birds underlines the difficulty in excluding trace elements from a diet. From Table 5 it can be seen that the unsupplemented basal mixture probably contained sufficient copper (3.0 ppm) to provide for the requirements of laying hens (recommended, 3.5 ppm; Anon., 1975). The exclusion of copper proved to be impracticable in an experimental diet which had to be produced in relatively large quantities (> 1000 kg) and which had to match as closely as possible a normal formulation. The absence of any adverse effects on egg number (Figure 2b), egg weight (Figure 3b) and egg shell thickness (Figure 4) in Group V (COP-DEF) birds indicates that an experimental diet containing sufficient amounts of all elements was capable of maintaining normal performance profiles. This is confirmed in the birds receiving the basal mixture purposely supplemented with magnesium, manganese and copper (Group II, BAS-SUP) in which egg production (Figures 2b, 3b) was in all respects similar to that seen in Group I birds receiving the standard PRC diet throughout (Figures 2a, 3a). It can be concluded, therefore, that there were no adverse effects on egg laying caused by administering the supplemented basal mixture. This is supported by the observations that food consumption, although not accurately measured, appeared normal and, with the exception of a single bird in Group II (BAS-SUP), there were no early mortalities in experimental birds.

Although the experimental diet fed to Group IV (MAN-DEF) birds contained only about 5% of the recommended level of manganese (Table 5), there were no adverse effects on egg number (Figure 2a), egg weight (Figure 3a) or egg shell thickness (Figure 4). This confirms that birds which are already in lay can tolerate low levels of this element in the diet (Hill and Mathers, 1968). If pre-laying birds are given a manganese-deficient diet then the eggs which are subsequently produced will exhibit the rough, translucent shells characteristic of this deficiency (Hill and Mathers, 1968; Longstaff and Hill, 1970; Leach and Gross, 1983). It seems that the success of the early work on manganese-deficient laying birds (Lyons, 1939) was due to high levels of dietary phosphorus which act to reduce manganese availability (Longstaff and Hill, 1971). It has also been reported that a manganese deficiency in laying birds does not significantly affect cholesterol and lipid metabolism and that magnesium, calcium and zinc can all substitute for this element in the activation of enzymes involved in the synthesis of yolk cholesterol (Klimis-Tavantzis et al., 1983).

The attempt to restrict the level of magnesium in the basal mixture and hence in the experimental diet (Group III, MAG-DEF) was successful (Tables 5, 6). The formulation given in Table 2 contains only about one half (215 ppm) of the AFRC's recommended minimum requirement for this element in laying birds (400 ppm; Anon., 1975). Moreover, there was a fall in plasma magnesium content in Group III (MAG-DEF) birds within the first week of feeding the experimental diets (Figure 1). This observation is in accord with that of Stafford and Edwards (1973) who noted a similar decrease

within 4 d. The supplemented basal mixture fed to Group II (BAS-SUP) birds also caused a sudden reduction in plasma magnesium level though there was some evidence of a recovery in these birds (Figure 1). Nevertheless, the level of plasma magnesium in this group was initially below the level at which birds are considered hypomagnesaemic (Stafford and Edwards, 1973). This confirms that 400 ppm dietary magnesium should be considered as a minimum requirement for laying birds especially in commercial feedstuffs where complete mixing of minor constituents may not be achieved (Gilbert et al., 1981).

Within 3 weeks of the switch to the experimental diets, there was a marked reduction in egg number (Figure 2a), egg weight (Figure 3a) and egg shell thickness (Figure 4) in Group III (MAG-DEF) birds. These observations are in agreement with previous reports on magnesium-depleted birds (Cox and Sell, 1967; Sell et al., 1967; Stafford and Edwards, 1973). As suggested by Stafford and Edwards (1973) the production of fewer, smaller eggs may result from atrophy of the liver leading to a reduced production of yolk constituents in magnesium-depleted birds. It is not clear, however, why an induced magnesium deficiency results in the production of thin egg shells. The suggestion that magnesium is required for the PTH-induced resorption of medullary bone (Stafford and Edwards, 1973) may not explain fully the often severe reduction in shell thickness. Normally, the provision of shell calcium from the skeletal reserves only becomes important towards the end of shell formation. The majority of shell calcium (60-75%) is derived from the diet (Comar and Driggers, 1949; Van de Velde and Vermeiden,

1984). Moreover, the reduction in mean shell thickness in Group III (MAG-DEF) birds shown in Figure 4 was probably an underestimate of the effects of the deficiency on shell deposition. The large variations in egg weight (Figure 3a) and shell thickness (Figure 4) in Group III (MAG-DEF) suggest that individual birds differed in their response to the low-magnesium diet. Mean shell thickness in this group would therefore be kept disproportionately high by the gradual cessation of lay in the most susceptible birds with only a small number of more resistant birds contributing to the mean estimate. Furthermore, although not quantified in the present work, the proportion of soft-shelled and shell-less eggs increased in Group III (MAG-DEF) birds compared to other groups. If soft-shelled and shell-less eggs had been included in the calculations of all group mean shell thickness values then a much more acute response to magnesium deficiency would have been apparent. In view of the severe reduction in both shell deposition (Figure 4) and shell magnesium content (Figures 5 a-c) in magnesium-depleted birds, it was decided to investigate further the role of this element in the provision of calcium for egg shell formation.

3.2 Flock 2

At the beginning of the studies with Flock 2 birds there was a successful attempt to reduce the magnesium content of the basal mixture. This was achieved by replacing all the FP950 in the diet with casein, generally considered to be a purer source of protein. This led to the production of an experimental diet (MAG-LOW) containing only 132 ppm magnesium (Table 8). The contribution of

FP950 to the overall level of dietary magnesium (Table 7) was of the same order in the experimental diet of Stafford and Edwards (1973) who reported a similar reduction upon switching to a casein protein source.

The recognition that 400 ppm dietary magnesium should be considered as a minimum level led to the production of an experimental diet, the magnesium content of which (1323 ppm) resembled that observed in the standard diet (1522 ppm). Of the four diets used during this phase of the study, two were deficient in magnesium (MAG-LOW, 132 ppm; MAG-DEF, 207 ppm) and two were within the normal range (MAG-SUP, 1323 ppm; STAND, 1522 ppm).

Due to a large increase in the frequency of blood sampling necessary to monitor the plasma magnesium status of the four groups, all plasma had to be stored longer prior to analysis. In order to prevent interference by the blood proteins in subsequent element determinations, all samples were treated with TCA (10% w/v) immediately after collection and centrifugation and respun at 1600 g. From Figure 6 it is clear that the level of magnesium in the plasma is sensitive even to small differences in dietary magnesium intake and that a response occurred in depleted birds within 3 d. The magnitude of this response in birds of Group VIII (MAG-DEF) was very similar to that seen in the comparable birds of Flock 1 (Group III, MAG-DEF) indicating that TCA-treatment is not necessary for the accurate determination of plasma element content, at least in fresh samples (cf Willis, 1960 a, b).

Adverse effects on egg number (Figure 7) and egg weight (Figure 8) were also observed in the magnesium-depleted birds of Flock 2.

Moreover, the severity of the response was determined by the degree of deficiency and in this respect there was a clear distinction between birds receiving 132 ppm (Group IX, MAG-LOW) and those receiving 207 ppm dietary magnesium (Group VIII, MAG-DEF). The same disparity did not exist between birds receiving sufficient, though different, amounts of magnesium in the diet (Groups VI, STAND; VII, MAG-SUP) even though the difference was, in absolute terms, far greater. This seems to suggest that within the normal range of dietary magnesium content birds are able to maintain constant performance profiles. Indeed, it has recently been reported that even excess levels of dietary magnesium (up to 7700 ppm) caused no severe disruption either to egg number or egg weight although there was a tendency for the eggs to be heavier (Atteh and Leeson, 1983a).

It is evident from Tables 10 a, b that the mean interval between successive ovipositions remained largely unchanged in all groups of Flock 2 following the administration of experimental diets. Magnesium deficiency therefore had no effect on the duration of egg formation and, by inference, on that of shell formation, assuming the latter occupied the same proportion of the total interval in all groups (Talbot and Tyler, 1974c; Nys, 1986). This finding is important because it shows that the reduced egg shell thickness in magnesium-depleted birds is due to a decreased rate of shell deposition rather than to a reduction in the residence time of the egg in the shell gland. Indirect support for this comes from the observation that 75% of the variation in shell thickness between good- and poor-quality egg shells in birds receiving a

normal diet was due to a reduced rate of shell deposition. Only 25% of the variation could therefore be attributed to changes in the duration of shell formation (Nys, 1986). Moreover, the thin shells produced by ducks administered DDE result from a reduced rate of calcium secretion by the shell gland mucosa and not from premature expulsion of the egg (Lundholm, 1980, 1982, 1984 a-c). Although the normal duration of shell formation in magnesium-depleted birds is consistent with the mechanism of shell thinning proposed by Stafford and Edwards (1973), their contention that a reduced resorption of medullary bone leads to the production of thin shells may not be wholly correct. Examination of the changes in plasma element content during shell formation in normal and magnesium-depleted birds in the present study has indicated that a more direct relationship exists between magnesium and shell formation in the hen.

Figure 9a shows that there was a decline in total plasma calcium during shell formation in birds receiving the standard diet (Group VI, STAND). In agreement with previous findings (Hertelendy and Taylor, 1961; Hodges, 1969) the level of calcium had declined to around 85% of its normal value at the end of shell formation. This would suggest that the reduction in ionised plasma calcium associated with shell calcification (Luck and Scanes, 1979a; Singh et al., 1986) is normally sufficient to affect the total calcium content of the blood despite the buffering ability of the bound calcium fraction. A reduction in total plasma calcium following shell formation was also observed in birds receiving the magnesium-supplemented (Group VII) and magnesium-deficient (Group VIII) diets

(Table 11a) though the effects were not as pronounced as those shown in Figure 9a. Nevertheless, the mean plasma calcium level of 23 mg % observed in magnesium-depleted birds at the end of the dark period was the lowest mean value recorded in the present work. Although this indicates a slight hypocalcaemia in shell-forming, magnesium-depleted birds, the reduction seems insufficiently severe to account for the effects on egg production occurring at this time (Figures 7, 8) and, more specifically, for the reduction in shell thickness previously shown to be typical of birds receiving this diet (Figure 4). According to Stafford and Edwards (1973) birds receiving diets containing only 118 ppm magnesium had a total plasma calcium level approaching 94% of the normal value. The corresponding value from Table 11a, both before and during shell formation, is 87%. Only when the diet contained 49 ppm magnesium did Stafford and Edwards (1973) record a severe reduction in plasma calcium content. It is interesting to note that DDE-induced egg shell thinning in ducks has been shown to result from a reduced secretion of calcium by shell gland epithelial cells even though the uptake of calcium from the blood is largely unaffected (Lundholm, 1984a). Although the presence of DDE tended to reduce total plasma calcium content to about 85% of normal, the effect was not considered significant (Lundholm, 1984a). In fact the similarity between DDE poisoning in ducks and magnesium deficiency in the domestic hen may be the key to understanding the role of this element in shell formation.

It is proposed that in Galiforme birds, including the domestic hen, the majority of ATP-dependent calcium transfer across the shell gland can only occur in the presence of magnesium which,

acting as a co-factor, also passes into the shell gland lumen. The proportion of calcium transport systems not dependent upon magnesium will be small though during a magnesium deficiency these will continue to transport calcium into the shell gland. In most non-Galliforme birds the relative contributions of each system are reversed with most calcium transfer occurring independently of magnesium. Further evidence for this is given in Figure 9b and Table 11b in which the decline in plasma magnesium content during shell formation in the hen is evident. In birds receiving a normal diet (Group VI, STAND) the level of circulating plasma magnesium invariably fell by around 25% during the dark period (Figure 9b, Table 11b). This was also the case in birds receiving the supplemented basal mixture (Group VII, MAG-SUP). The consistency of these values, in view of the often variable results obtained for total plasma calcium, may reflect the fact that the ionised magnesium fraction of the blood accounts for almost 50% of the total amount (Stafford and Edwards, 1973). Any changes in the physiological demand for this fraction are therefore likely to be reflected in the level of total plasma magnesium. The severe hypomagnesaemia of Group VIII (MAG-DEF) birds after 5 weeks on the experimental diet is clearly shown in Table 11b. In this group magnesium would be largely unavailable during shell formation which consequently proceeds at a rate determined by the ratio of magnesium-dependent and magnesium-independent calcium transfer across the shell gland. As this ratio could conceivably vary between individual birds, this might be the basis of the observed variability in response to magnesium deficiency. The increased dependence on shell calcium

transported in the absence of magnesium will reduce the magnesium : calcium ratio in the shell. That this is indeed the case (Figure 5 a-c; Tables 12a, b) is suggestive though other, more direct, explanations for this, such as the severely depleted plasma magnesium level itself, cannot be ruled out at this stage.

A considerable amount of evidence now exists to support the contention that shell gland physiology in the Galliformes differs in some way from that of other orders of birds. Galliformes are considered to be relatively resistant to the DDE-induced shell thinning now considered partly responsible for the decline in certain populations of raptors (Ratcliffe, 1967; Cecil et al., 1972; Cooke, 1973; Lundholm, 1980). An extensive investigation into the mechanism of shell thinning in DDE-sensitive species has been carried out (Miller et al., 1976; Greenburg et al., 1979; Lundholm, 1980, 1982, 1984 a-c, 1985). It has been demonstrated that DDE can block ATP-dependent calcium binding at any one of several stages in the transcellular movement of this element (Lundholm, 1984a). Miller et al. (1976) have shown that DDE is a potent inhibitor of calcium-activated ATPase in duck shell gland homogenate and that the corresponding enzyme in the hen is far less sensitive. In addition, magnesium-activated ATPase was also relatively unaffected by DDE in homogenates of this kind (Miller et al., 1976). This suggests that biochemical reactions dependent upon magnesium are far more specific for the activating element than those involving calcium. The finding that magnesium-dependent ATPase will continue to operate in the presence of DDE (Miller et al., 1976) is consistent with the idea that a change in the proportions of the various

ATPases in the shell glands of birds from different orders is the basis of the observed differences in DDE sensitivity. It has been observed that the magnesium content of mallard egg shells increases significantly in DDE-poisoned birds (Cooke, 1973). This results, it is suggested, from an increased reliance on magnesium-dependent calcium transport which in most non-Galliformes, like the mallard, normally plays only a minor role in the provision of shell calcium. Lundholm (1984a) studied the effects of DDE on several subcellular fractions in both sensitive and non-sensitive species. There was a reduction in calcium-binding by the plasma membrane, by 'secretory granules', and by the mitochondrial fraction in cell homogenates from susceptible birds. Similar effects were not observed in the subcellular fractions of shell gland homogenates obtained from domestic fowl. Coty and McConkey (1982) observed a magnesium-independent enhancement of basal ATPase activity in shell gland epithelial tissue from the domestic fowl when a 0.75 mM calcium chloride solution was added to the reaction medium. This highly specific calcium-stimulated ATPase was also extremely localized and was not found in tissue taken from the isthmus or magnum regions of the oviduct. The high specificity for calcium and the localization of the ATPase in the shell gland was taken as evidence of the importance of the enzyme in calcium transport during shell formation (Coty and McConkey, 1982). These authors seem unaware, however, of the observation that the magnum also can transport calcium into the oviduct against a concentration gradient and that the activity of this region almost certainly contributes to the composition of the shelling fluid (Moynihan and Edwards, 1975). The

absence of magnesium-independent activity in the magnum is, if anything, evidence of the element's involvement in the uptake of calcium from the blood and its subsequent release into the oviduct lumen, at least in the domestic hen. When Coty and McConkey (1982) removed magnesium from the reaction medium containing shell gland epithelium, there was a reduction of >95% in the 'basal' level of ATPase activity though these authors considered that this multi-enzymatic complex played no part in calcium transport. Pike and Alvarado (1975) in a study of another Galliforme, the Japanese quail, noted the presence of a less specific calcium- or magnesium-stimulated ATPase complex in the shell gland and suggested that this was responsible for the transport of shell calcium. In Galliformes, the contribution of calcium-specific ATPase activity would be small and, it seems, confined to the shell gland. A reduction in active calcium transport observed in the excised shell glands of DDE-poisoned ring doves was not observed in magnum tissue from the same source (see Cooke, 1973). This suggests that, if it occurs at all, the transport of calcium by the magnum in non-Galliformes is also mediated by a magnesium-dependent ATPase. Nevertheless, it is proposed that in non-Galliformes this type of calcium transport plays only a minor role in the provision of shell calcium. One testable prediction from this is that DDE-sensitive, non-Galliforme birds should be resistant to the effects of a magnesium deficiency at least in terms of shell thickness.

A second, major line of evidence for the proposed difference in Galliforme and non-Galliforme shell gland physiology is provided by the electron probe microanalysis studies of Board and Love (1980, 1983).

The presence of appreciable amounts of magnesium in the column layer of the completed shell is a feature almost entirely confined to the Galliformes. This strongly suggests that it is only in these birds that magnesium is present in relatively high concentrations in the calcifying fluid. Although this would support the contention of the present work there are several other possible explanations of the observed differences in shell magnesium content and it is now necessary to investigate in detail the nature of the incorporation of this element into the developing shell.

PART III: THE INFLUENCE OF MAGNESIUM DEFICIENCY ON EGG SHELL STRUCTURE AND COMPOSITION

PART III: CHAPTER 1

EXPERIMENTAL DETAILS

1.1 Scanning electron microscopy (SEM)

a Principle of technique

A schematic diagram of the main components of a scanning electron microscope is shown in Figure 10. The technique involves focusing a beam of electrons, of energy in the range 5-30 kV, into a spot approximately 5 nm in diameter and scanning the specimen surface in a rectangular raster. A fraction of the incident electrons are backscattered from the surface and secondary electrons are also produced. Both types of emission are collected on a scintillator and the light produced transmitted via a perspex light guide to a photomultiplier. The ratio of backscattered to secondary electrons used for imaging may be varied by adjusting a biasing electrode placed in front of the scintillator. The signal which is generated is then used to modulate the brightness of the electron beam in a cathode ray tube (CRT) which is scanning in synchronism with the electron beam in the microscope column. The image formed on the CRT screen thus relates to the distribution of electrons obtained from the specimen surface and the spatial resolution in the image to, essentially, the diameter of the focused incident electron beam. Image magnification is given by the size of the scanned area on the CRT divided by the size of the scanned area on the specimen and can readily be altered by increasing or decreasing the electric current flowing through the scanning coils in the electron column. Typical magnifications range

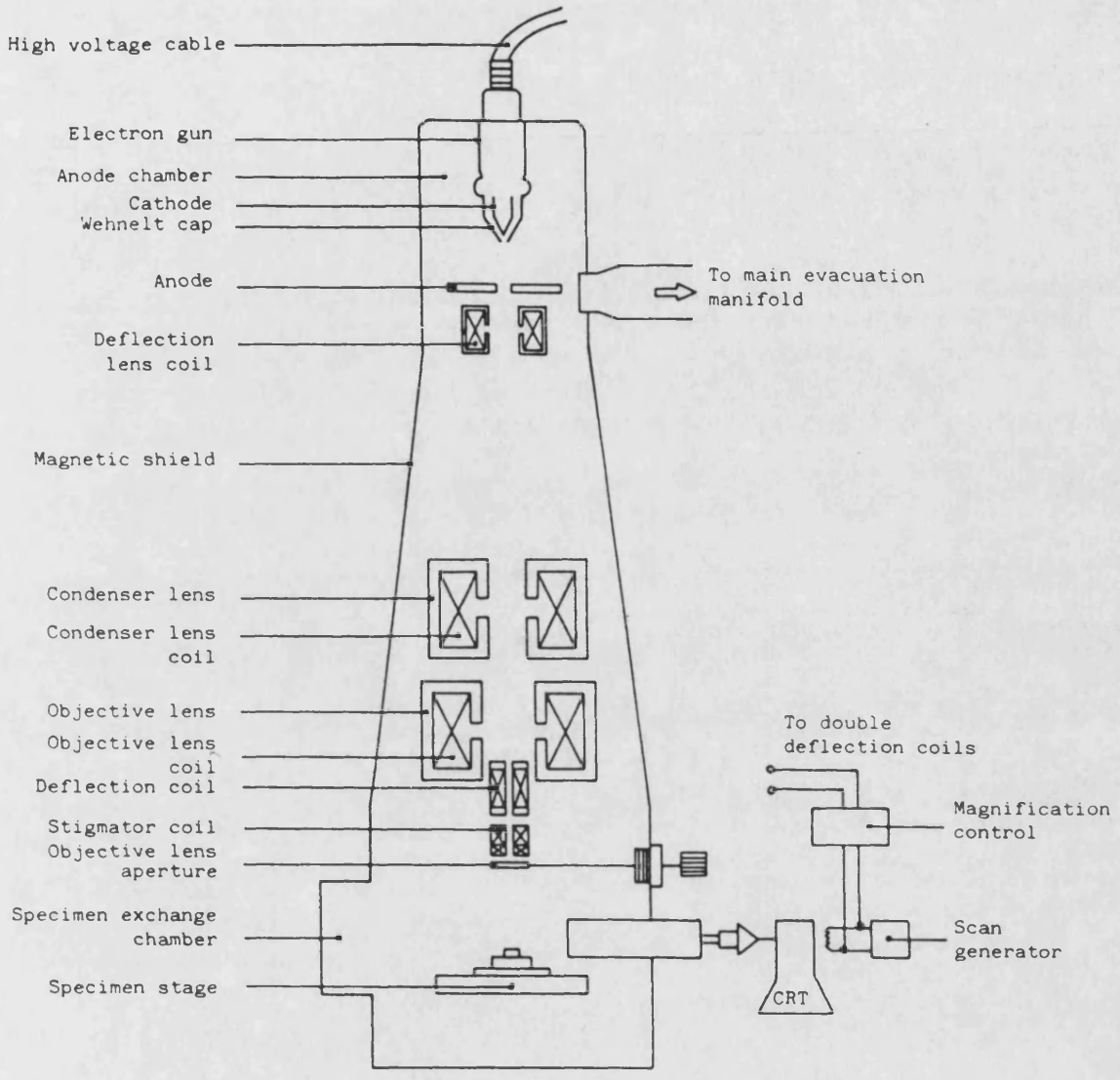


Figure 10. Schematic diagram of a scanning electron microscope.

Adapted from Goldstein et al. (1975) and Murr (1982).

from x20 to x50,000 in most modern scanning electron microscopes. In the present work a Joel 35C instrument (Joel (UK) Ltd., Colindale, London) operating at 15 kV was used.

b Specimen preparation

Shell samples (approximately 20 x 10 mm) were removed from the shoulder of each egg using a high speed cutting wheel and then prepared for SEM studies in one of two ways:

(i) After washing and drying, the cut samples of shell were immersed in liquid nitrogen for 10 s. They were fractured to produce a radial section and mounted, fractured face uppermost, onto aluminium planchettes using graphite paste (Polaron Equipment Ltd., Watford, Herts). A thin layer of gold was then deposited on the radial face of the shell using a sputter coater (Edwards S150B, Edwards High Vacuum, Crawley, Sussex) and electrical contact was made between the gold and the planchette with a conducting paint (DAG 915, Acheson Colloids Co., Prince Rock, Plymouth). The coating was necessary to remove charging of the specimen during irradiation with the electron beam in the SEM.

(ii) The inner surface of the shell was examined in order to determine the number of cones per unit area. This necessitated the removal of the shell membranes which was achieved in one of two ways. Egg shell pieces from birds of Flock 1 were immersed in 0.34 M ethylenediaminetetraacetic acid (EDTA) for 30 min after which the membranes and cuticle could be readily detached. Following Stevenson (1980) egg shell material from Flock 2 birds was incubated at 37°C for 72 h with a non-specific protease (Pronase E,

Sigma Chemical Co., Poole, Dorset) in 0.01 M tris buffer solution. After washing and drying, all shells were mounted, inner surface uppermost, onto aluminium planchettes and coated with a thin conducting film, as outlined above, prior to SEM examination.

1.2 Pore counts

The number of pores per cm^2 of shell surface was calculated using a modified version of Tyler's (1953) method. Two pieces of shell (each approximately 20 x 10 mm) were removed from the shoulder of each egg and boiled for 5 min in 5% (w/v) sodium hydroxide solution. The shell pieces were washed and then immersed for 5 s in concentrated nitric acid. After careful rinsing and drying, the shells were mounted over an aperture of 10 mm^2 at the centre of an otherwise opaque projector slide. Upon projection, the light spots corresponding to the pores were copied onto a plain sheet of paper acting as a screen.

1.3 Electron probe microanalysis (EPMA)

a Principle of technique

When, in an electron microscope, energetic electrons are incident on the specimen, X-rays are given off as well as secondary electrons etc. The X-rays consist of a continuous background of energies, sometimes called 'Bremsstrahlung' or 'white' radiation, superimposed upon which are discrete X-ray lines of energies characteristic of the elements contained within the irradiated volume of material. Each element (above He, Z = 2) in the periodic table will produce characteristic X-ray lines of different energy.

Hence measurement of the energy (or wavelength) of the X-ray lines enables the elements in the sample to be identified. Moreover, the intensity of a line gives an indication of the amount of the respective element present. An important advantage of the EPMA technique is that with the focused beam of electrons chemical compositions may be obtained on very small volumes (approximately 1 μm^3) of material. In order to obtain quantitative data, a reference sample of known composition is used for comparison purposes and the ratio of the relevant X-ray line intensities multiplied by correction factors. For a detailed discussion of quantitative analysis and correction procedures the reader is referred to the publication by Scott and Love (1983).

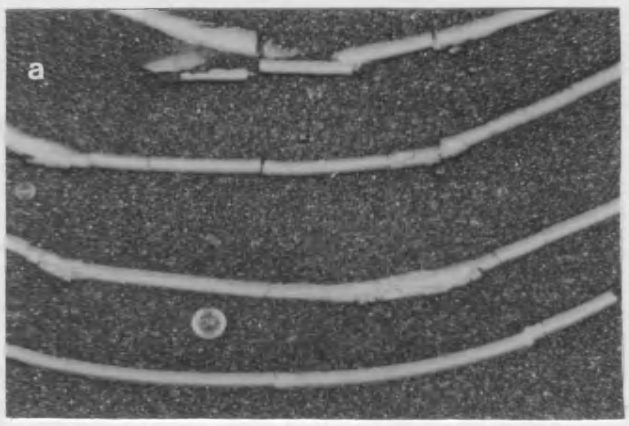
EPMA was carried out, at an electron accelerating voltage of 15 kV, using a Joel JXA733 microprobe fitted with three Bragg-type crystal spectrometers to analyse the X-ray emission. Each spectrometer contained a choice of two analysing crystals to disperse the X-ray spectrum and a proportional counter for detecting the beam of X-rays diffracted from the crystal. Rotation of the crystal through a range of Bragg angles enables the X-ray emission spectrum to be plotted on a chart recorder and the position of the peaks in the recorder trace can be measured to identify the elements present. Once an element is detected the spectrometer can be set so that X-rays emitted from that element only are recorded. In this way it is possible to study the distribution of selected elements by translating the specimen beneath the focused electron beam. For the quantitative determination of calcium, reference samples of calcite were used which meant that no correction factor was needed.

Magnesium contents were obtained using a pure metal magnesium reference standard and applying the absorption correction factor proposed by Philibert (1963).

b Specimen preparation

Following the procedure of Board and Love (1980) pieces of egg shell obtained from Flock 1 birds were mounted in plastic resin (Metaserv Laboratories, Betchworth, Surrey). A radial face of each piece was exposed using a coarse grade emery cloth (particle size, 70 μm) mounted on a polishing wheel (Knuth Rotor, Struers, Copenhagen, Denmark). After polishing on a series of successively finer grade papers (particle sizes, 57, 46 and 33 μm) the shell faces were given a two-stage polish using alumina pastes (particle sizes, 7 and 2 μm). A final finish was obtained using a velvet cloth impregnated with diamond compound (particle size, 1 μm) and supported by plate glass. Shell material from birds of Flock 2 was mounted in a copper-based resin (Struers, Copenhagen, Denmark) as was a series of shell pieces taken from successfully incubated eggs. Each mount was then subjected to the polishing procedure outlined above. The polished radial surfaces of all shells were first examined under an optical microscope (Plates 1a to d) and then coated with a thin layer of gold prior to EPMA.

It was subsequently found that variations in the thickness of the gold coat could have a profound effect on the intensity of X-ray emission from the specimen due to X-ray absorption by the coating. The gold applied to all Flock 2 shell mounts was therefore removed using diamond paste (particle size, 1 μm) and the shells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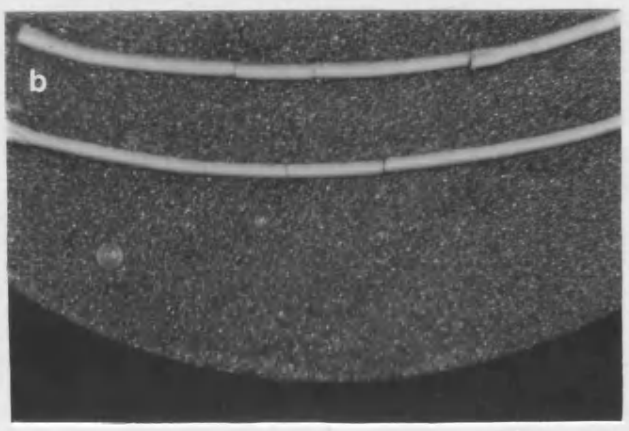
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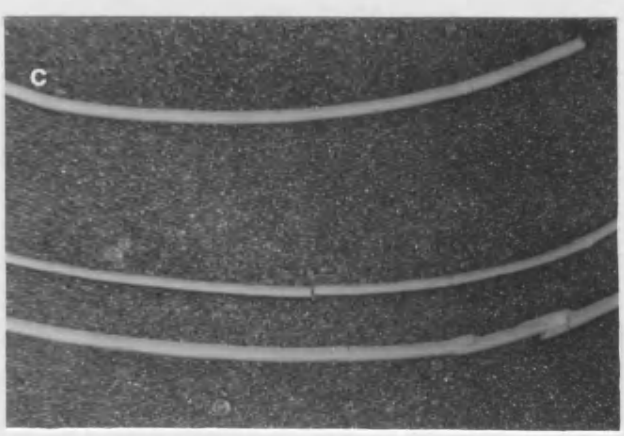
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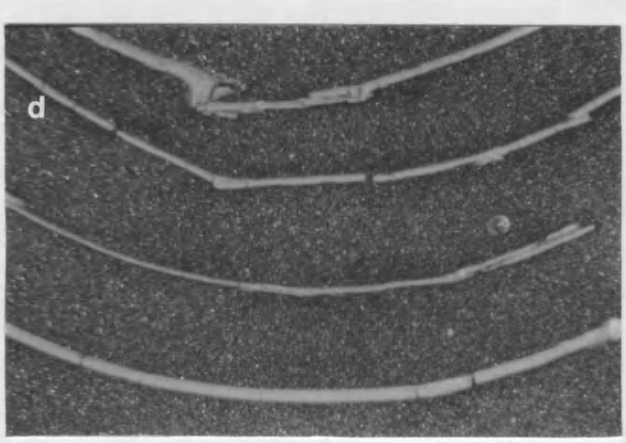

d

re-coated, under vacuum, with carbon, a material causing much less absorption of X-rays. Although the relative distribution of each element across individual shells was found not to differ appreciably between gold-and carbon-coated specimens, all estimates of shell element content were based upon results obtained from carbon-coated materials.

PART III: CHAPTER 2

RESULTS

2.1 Scanning electron micrographs of radial shell surface

Plate 2 shows the radial shell surface of a commercially produced hen's egg. The close association between the cones (or mammillae) and shell membrane fibres is evident and the cone layer as a whole accounts for only about one quarter of total shell thickness. Both features are typical of good quality shells. Plate 3 shows the radial shell surface typical of eggs produced by birds receiving the basal mixture supplemented with all minerals (Group II, BAS-SUP). In general, shell thickness did not differ from that of eggs produced commercially (Plate 2). There was, however, some evidence of pitting in the column layer suggesting a reduction in shell quality. This feature was more pronounced in the column layer of the thin shells (Plate 4) laid by birds receiving the magnesium-deficient diet (Group III, MAG-DEF). In both Groups II and III the characteristic division of the shell into cone and column layers was apparent.

2.2 Inner surface studies

a Cone counts

Table 13 summarizes the values for mean cone number (per 0.126 mm^2) in selected shells from Groups II (BAS-SUP), III (MAG-DEF), VI (STAND), VII (MAG-SUP), VIII (MAG-DEF) and IX (MAG-LOW). There appeared to be little effect due to diet on the number of cones per unit area of shell. The largest difference was

Plate 2. Electron micrograph of the radial face of the shell of a commercially produced hen's egg.
ce l, cone layer.
cm l, column layer.
Note close association of cones (c) and membrane fibres (mf)

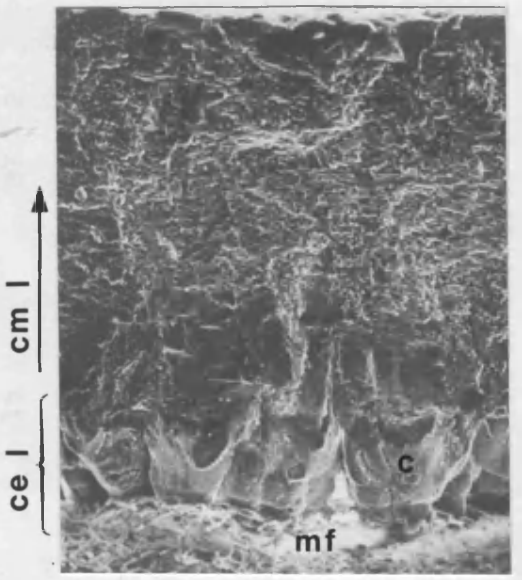


Plate 3. Electron micrograph of the radial face of the shell of an egg produced by a Group II (BAS-SUP) bird.
ce l, cone layer.
cm l, column layer.
Note pitting (p) of column layer.

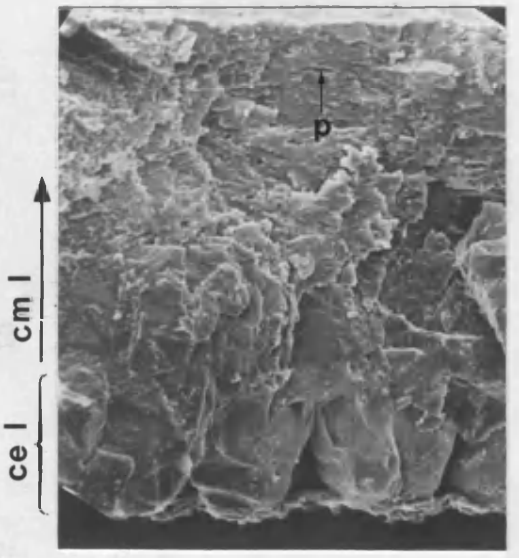


Plate 4. Electron micrograph of the radial face of the shell of an egg produced by a Group III (MAG-DEF) bird.
ce l, cone layer.
cm l, column layer.
Note pitting (p) of column layer and reduced shell thickness

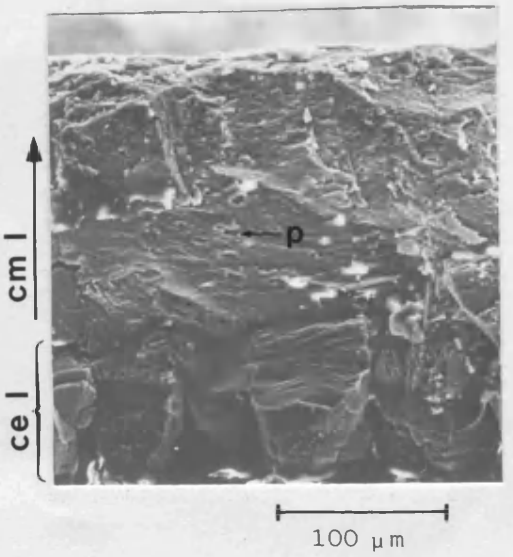


Table 13. Mean egg shell cone number in selected groups from both flocks.

Group	Flock	Sample size			Mean cone number per 0.126 mm^2 ± S.E.
		number of birds	number of shells	successful counts*	
II (BAS-SUP)	1	4	6	12	34 ± 2
III (MAG-DEF)	1	5	6	10	31 ± 2
VI (STAND)	2	5	5	8	32 ± 2
VII (MAG-SUP)	2	6	7	12	33 ± 3
VIII (MAG-DEF)	2	10	11	15	37 ± 2
IX (MAG-LOW)	2	8	13	19	32 ± 2

* two pieces taken from each shell hence maximum number of counts = number of shells x 2.

observed between shells of Groups III and VIII both of which had received the magnesium-deficient diet (MAG-DEF). The difference, however, is small and is unlikely to be significant given the large variation in cone density between individual shells within each group. In conclusion, there was no evidence of a major change in cone density occurring as a consequence of feeding the various diets. Plates 5 to 7 confirm the similarity between groups in Flock 2 regarding cone numbers.

b General appearance

The normal appearance of the inner surface of the hen's egg shell is typified by Plates 5 to 7 which show the numerous knob-like cones or mammillae formed during early shell development. The innermost surface of these cones is in close contact with the outer shell membrane, the removal of which leaves characteristic grooves or tracks in the cone tips. Plate 8, obtained from the same shell surface as Plate 6, shows, at higher magnification, the appearance of the cone tips following removal of shell membranes by boiling in sodium hydroxide solution. In some cases the removal of shell membrane in this way led to the removal of shell material, whilst other cones remained intact. The origin of the shell pores, at the junctions between cones, is also clearly shown in Plate 8.

During the measurement of cone numbers it became apparent that uncharacteristic, sphere-like deposits were present in the cone layers of egg shells produced by certain magnesium-depleted birds (Groups VIII, MAG-DEF and IX, MAG-LOW). Plates 9 a-d show, at progressively higher magnifications, the inner egg shell surface of

Plate 5. Electron micrograph showing inner shell surface of an egg produced by a Group VII (MAG-SUP) bird.

c, cone.

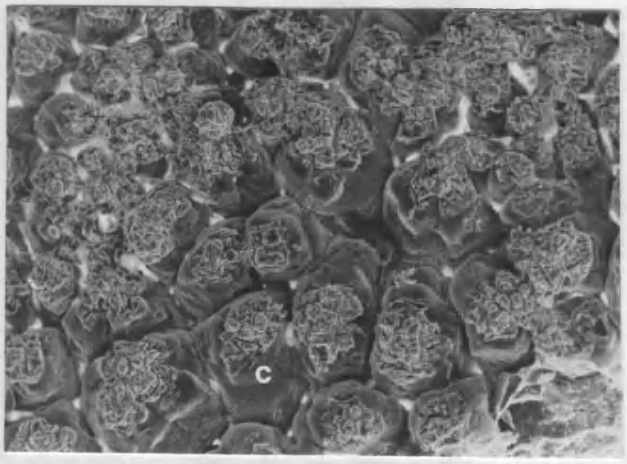


Plate 6. Electron micrograph showing inner shell surface of an egg produced by a Group VIII (MAG-DEF) bird.

c, cone.

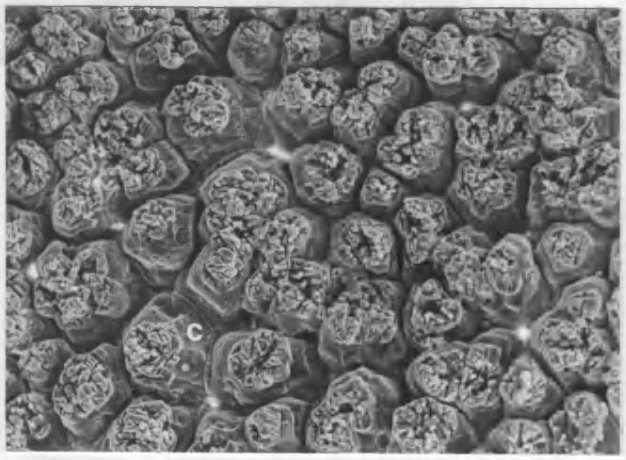
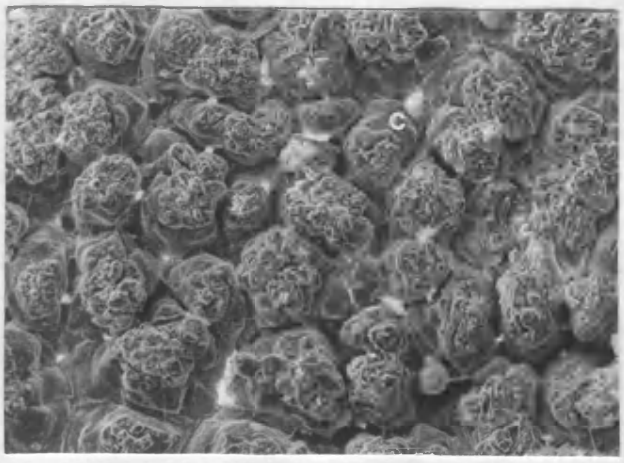
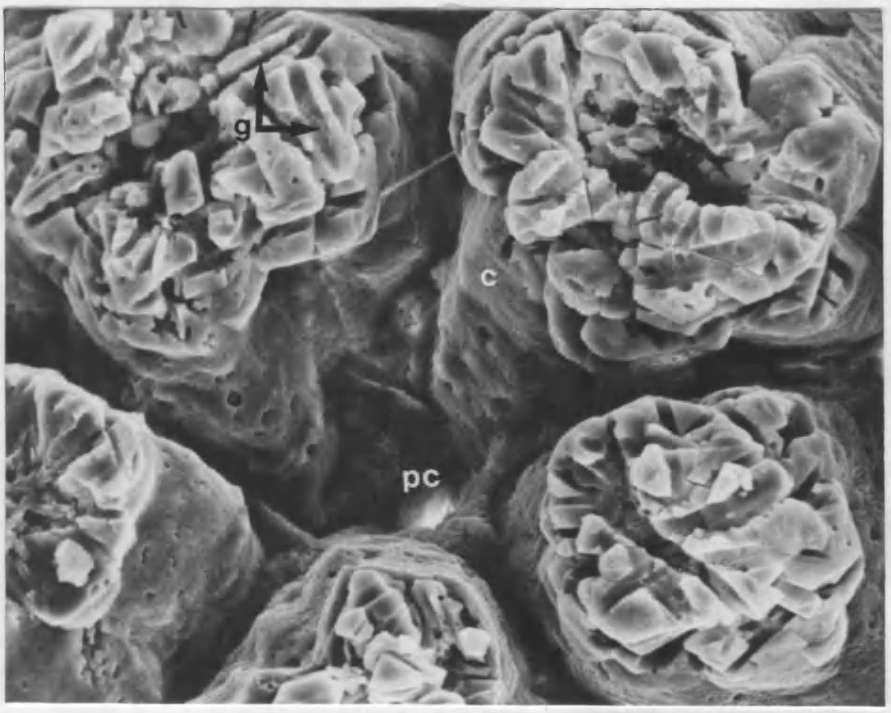


Plate 7. Electron micrograph showing inner shell surface of an egg produced by a Group IX (MAG-LOW) bird.

c, cone.





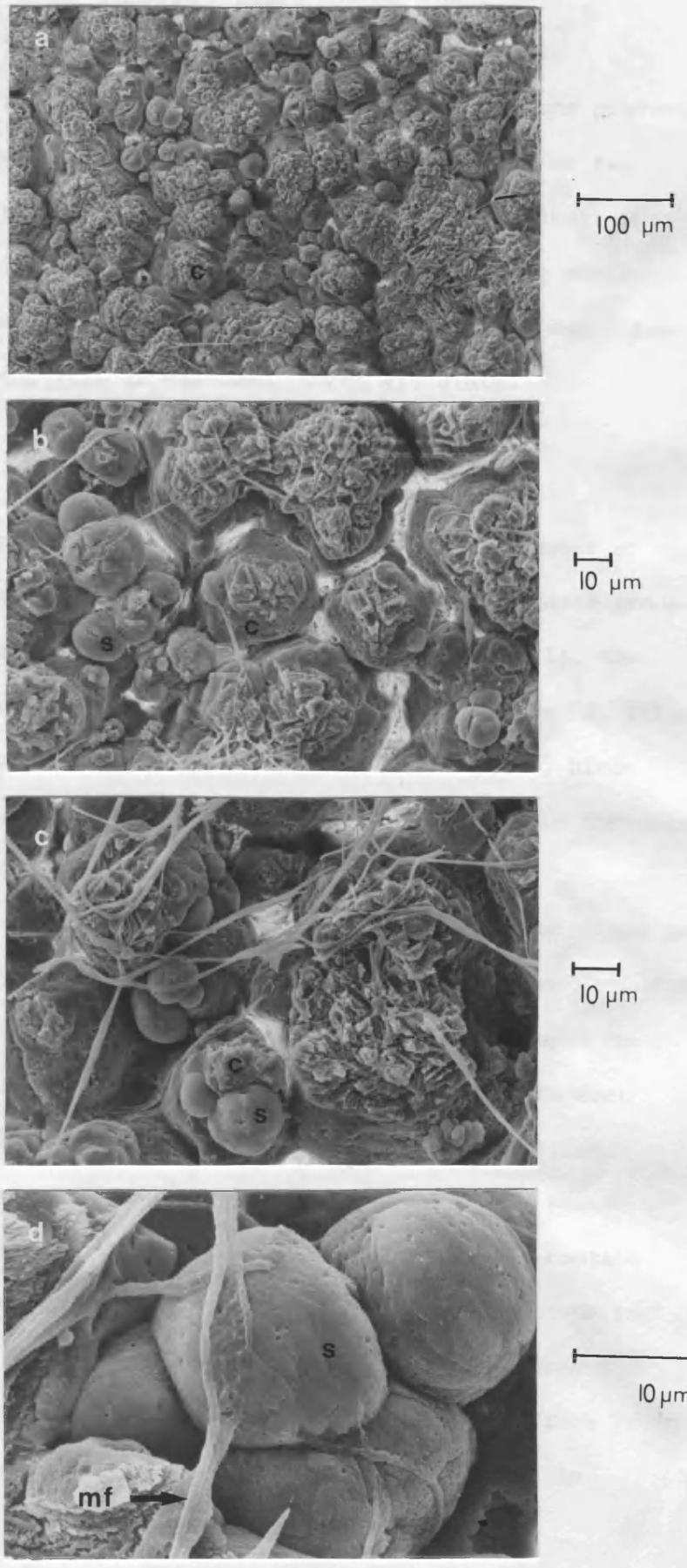
10 µm

Plate 8. Electron micrograph of inner shell surface shown in Plate 6, at higher magnification.

g, grooves remaining after removal of shell membranes.

Note origin of pore canal (pc) at the junction between cones (c).

Plate 9. Electron micrographs of inner shell surface of an egg produced by a Group IX (MAG-LOW) bird, showing sphere-like mineral deposits (s) on cones (c) and membrane fibres (mf).


a
c
100 µm
b
s
c
10 µm
c
c
s
10 µm
d
s
mf
10 µm

an egg produced by a bird of Group IX (MAG-LOW). There is widespread mineralisation of both the cone tips and, where present, the shell membrane fibres. These features were observed in two shells produced by different Group IX birds and in one shell from a Group VIII (MAG-DEF) bird. They were not observed in egg shells produced by Flock 1 birds or by Flock 2 birds receiving magnesium-supplemented (Group VII) or standard (Group VI) diets.

2.3 Pore counts

After 4 weeks on the experimental diets, the mean number of egg shell pores per cm^2 surface area was calculated for selected groups in Flocks 1 and 2. In the case of Flock 1 (Groups II, III), the eggs were laid at 37 weeks of age and for Flock 2 (Groups VI, VII, VIII, IX) at 25 weeks. Egg shells from Group IX (MAG-LOW) birds were very thin and were etched in concentrated nitric acid for only 2 s as opposed to 5 s for all other groups.

Table 14 gives the group mean pore number (per cm^2) obtained in each case. There appeared to be a reduction in pore number in the shells of those birds receiving low-magnesium diets compared to those receiving the supplemented or standard diets. Within each flock a correlation between pore number and dietary magnesium content was evident. There was also a difference between flocks in that the eggs of the older birds (Flock 1) were found to contain fewer pores per unit area than those laid by comparable birds in Flock 2. This may have been due to a reduction in pore density attendant upon increased egg size in the older birds of Flock 1. In the case of Groups III (MAG-DEF) and VIII (MAG-DEF) there is

Table 14. Mean egg shell pore number in selected groups from both flocks.

Group	Flock	Sample size			Mean pore number per cm^2 surface area ± S.E.
		number of birds	number of shells	successful counts*	
II (BAS-SUP)	1	5	11	22	142 ± 5
III (MAG-DEF)	1	7	13	24	103 ± 5
VI (STAND)	2	6	6	12	150 ± 4
VII (MAG-SUP)	2	9	14	24	155 ± 5
VIII (MAG-DEF)	2	10	20	39	131 ± 4
IX (MAG-LOW)	2	10	20	21	99 ± 6

* two pieces from each shell hence maximum number of counts = number of shells x 2.

indirect evidence for this surmise in that the group mean egg weights at this time were 57.8 g and 55.7 g respectively. The difference between birds in Flocks 1 and 2 receiving the standard diet (Groups I, VI) was even greater at 63.7 g and 57.2 g respectively.

2.4 Electron probe microanalysis (EPMA)

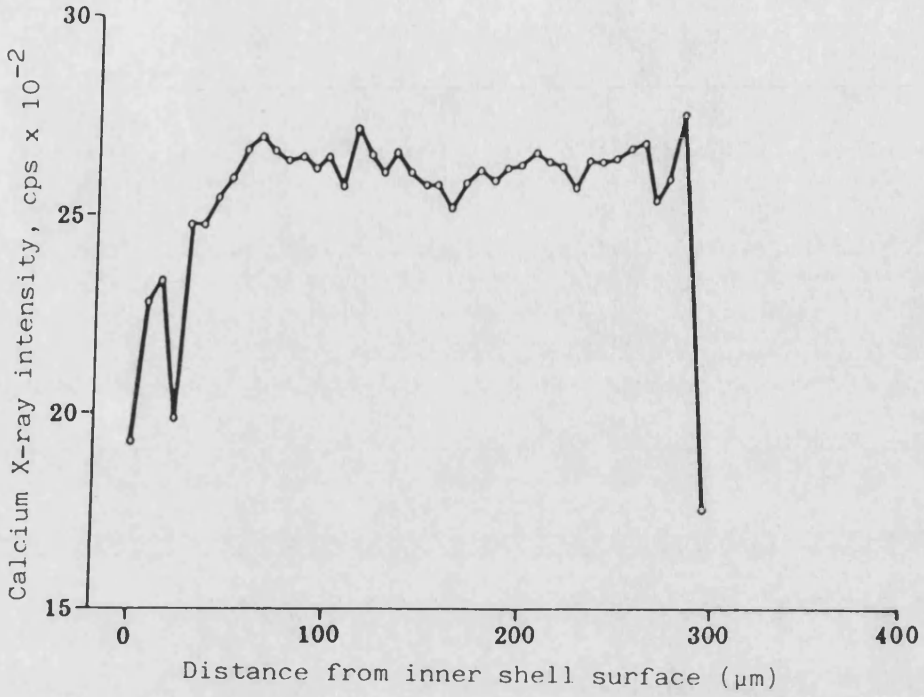
Plate 14a is an electron micrograph of the polished radial surface of an egg shell following EPMA. In general, the largest cones were selected for analysis since it could be assumed that in cross-section they represented the more central regions of each mammilla. The specimen was positioned such that the focused electron beam (< 1 µm diameter) was located just off the inner surface of the shell (point A in Plate 14 a). The X-ray counts were then recorded at regular intervals across the shell as the specimen was moved in a direction normal to the outer shell surface. In the case of Plate 14 a the step interval was 10 µm, each position being sampled for 20 s. The dark spots are contamination marks produced during each analysis.

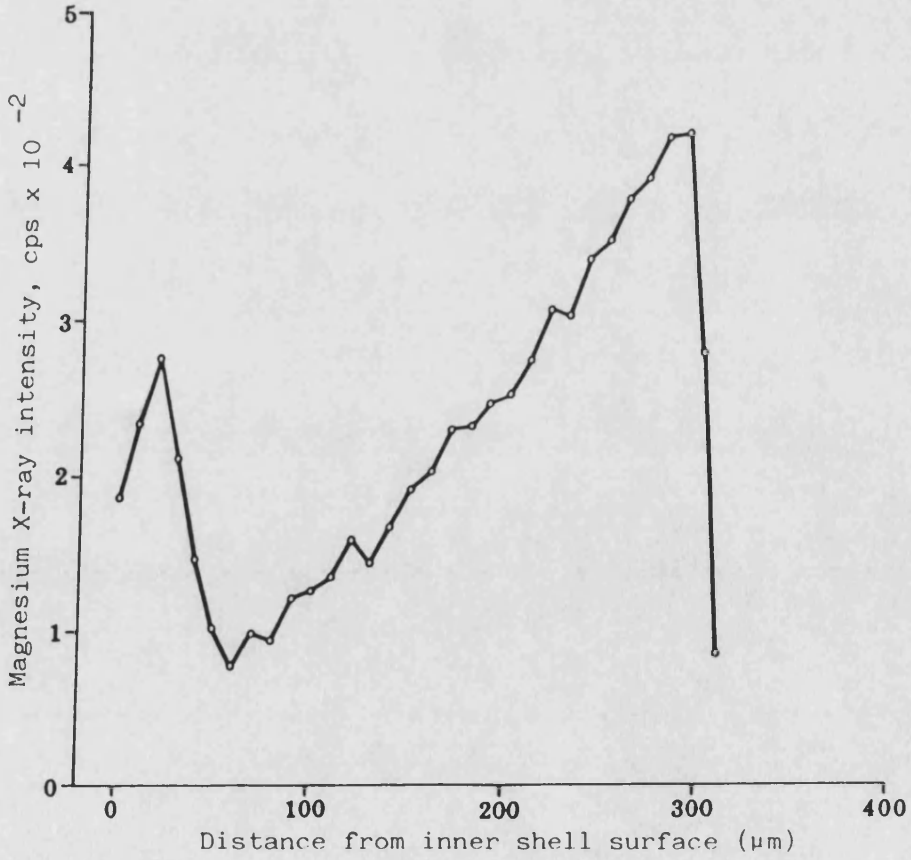
a. <u>Distribution of calcium, magnesium, phosphorus and sulphur across the shell</u>.

Figure 11 shows the distribution of calcium across the egg shell of a bird fed on the standard diet (STAND). The apparent variation in X-ray counts in the first 30 µm or so is due mainly to cavities associated with the removal of organic material, principally outer shell membrane fibres. The remainder of the distance-

Figure 11. Variation in calcium X-ray intensity across the thickness of the egg shell.
0 - 100 μm, cone layer; 100 - 300 μm, column layer.

Figure 12. Variation in magnesium X-ray intensity across the thickness of the egg shell.
0 - 100 μm, cone layer; 100 - 300 μm, column layer.


30
25
20
15
Calcium X-ray intensity, cps x 10^{-2}
0
100
200
300
400
Distance from inner shell surface (μm)


5
4
3
2
1
0
Magnesium X-ray intensity, cps x 10^{-2}
0
100
200
300
400
Distance from inner shell surface (μm)

concentration profile remained substantially constant apart from small-scale changes in count rate probably due to effects of surface topography. The corresponding magnesium profile is given in Figure 12. It shows two important features. Firstly, a relatively high level of magnesium $K\alpha$ counts was recorded within the cone layer, this high count rate decreasing markedly with distance into the shell. Thereafter, the magnesium count increased progressively to reach a maximum value in the region of the shell exterior. Figure 13 indicates a fairly constant level of phosphorus across most of the shell with a sharp rise in count rate occurring towards the outer surface. Measurement of sulphur (Figure 14) shows relatively high levels within the shell membranes but little in the shell proper. At this stage no attempt was made at a quantitative determination of shell mineral content, the main purpose being to demonstrate the relative distribution of each element across the shell.

b. Influence of diet on shell magnesium

(i) Flock 1 shells

The typical distribution of magnesium across the egg shells of birds receiving the basal mixture supplemented with all minerals (Group II, BAS-SUP) is shown in Figure 15a. The profile is similar to that observed in Figure 12 and represents the normal distribution of this element across the hen's egg shell. By contrast, the egg shell magnesium profile of birds fed on the magnesium-deficient diet (Group III, MAG-DEF) was found to be substantially different (Figure 15b). Although there was still a relatively high level of

Figure 13. Variation in phosphorus X-ray intensity across the thickness of the egg shell.

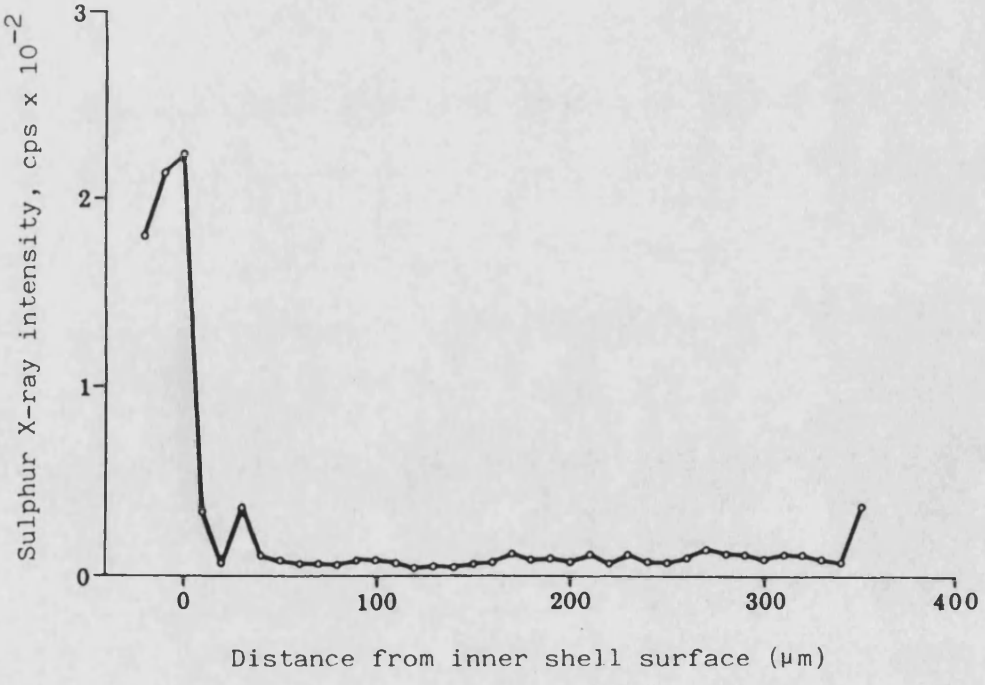
0 - 100 µm, cone layer; 100 - 300 µm, column layer.

Figure 14. Variation in sulphur X-ray intensity across the thickness of the egg shell.

0 - 100 µm, cone layer; 100 - 350 µm, column layer.

Note that peak in sulphur content occurs outside the true shell.


Phosphorus X-ray intensity, cps x 10^{-2}
30
20
10
0
0
100
200
300
400
Distance from inner shell surface (μm)


Sulphur X-ray intensity, cps x 10^{-2}
3
2
1
0
0
100
200
300
400
Distance from inner shell surface (μm)

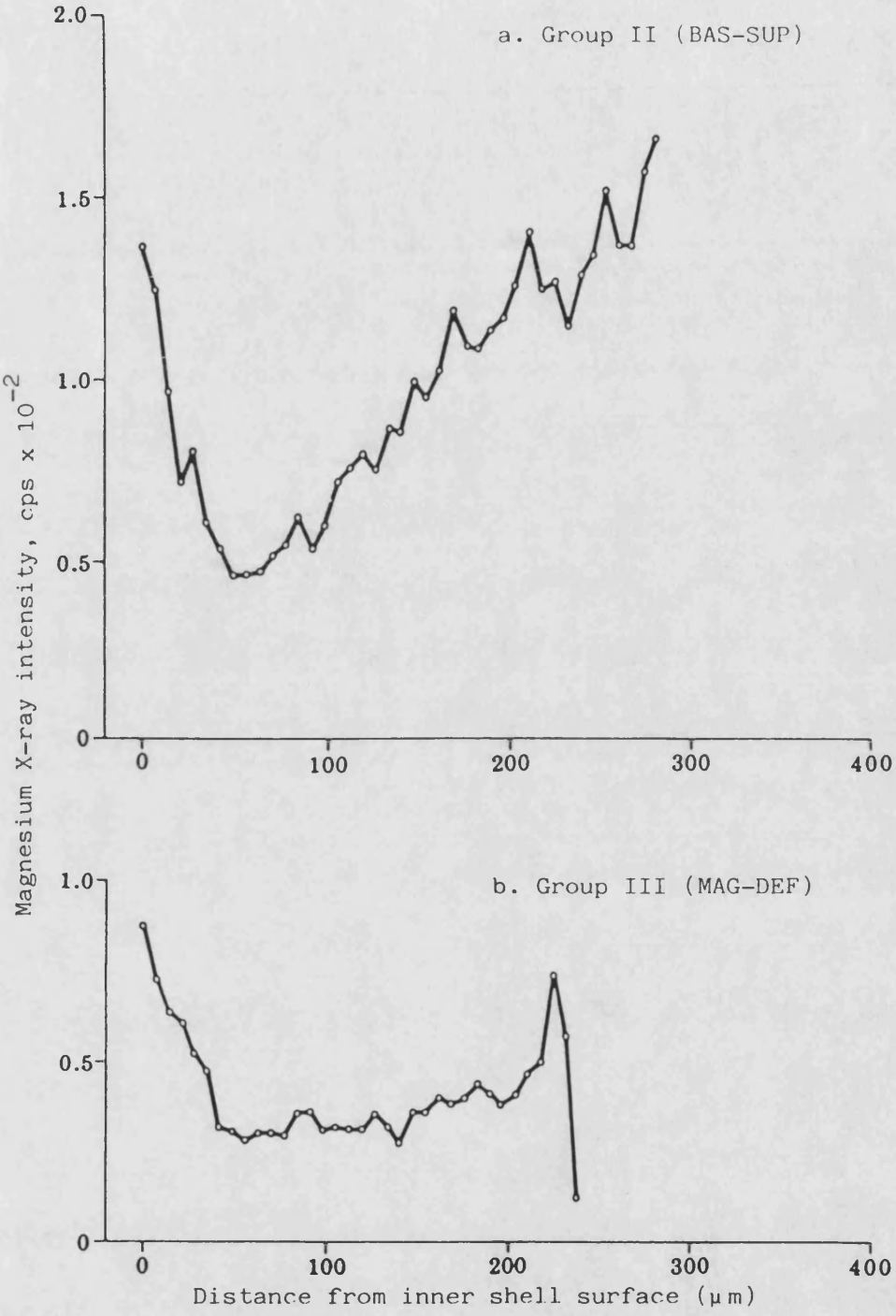


Figure 15. Variation in magnesium X-ray intensity across the thickness of the egg shell in Groups II and III. Results typical of 42 and 37 observations respectively. 0 - 100 µm, cone layer; 100 µm onwards, column layer.

magnesium Kα counts at the cone tips, there was no progressive increase across the shell, apart from a small peak in the outer layer. These results, typical of many observations made in the two groups (n = 42 and 37 respectively), clearly indicate that the distribution of magnesium across the shell is determined by the amount of this element in the diet. In the following section emphasis was placed on obtaining quantitative data on the microdistribution of calcium and magnesium across the egg shells of birds fed on diets containing various amounts of magnesium.

(ii) Flock 2 shells

Figure 16a shows the typical calcium and magnesium distributions (n = 7) across the egg shells of Group VI (STAND) birds. Both elements show the characteristic profiles seen in Figures 11 and 12. This was also the case in three egg shells from birds fed on the magnesium-supplemented diet (Group VII, MAG-SUP), as is shown in Figure 16b. As previously noted, the egg shells of birds receiving the magnesium-deficient diet (Group VIII, MAG-DEF) are thinner and this is confirmed in Figure 16c. Using the calcium line as a guide it can be seen that, in the example shown, shell thickness fell by around 15% compared to the above groups. Although there was a peak in magnesium content at the cone tips of these shells (n = 4), the level of this element across the rest of the shell remained low. This was also the case in eight shells taken from Group IX (MAG-LOW) birds (Figure 16d) though shell thickness in this group was reduced to only around 50% of the normal values obtained from Figures 16a, b. The distribution of calcium across

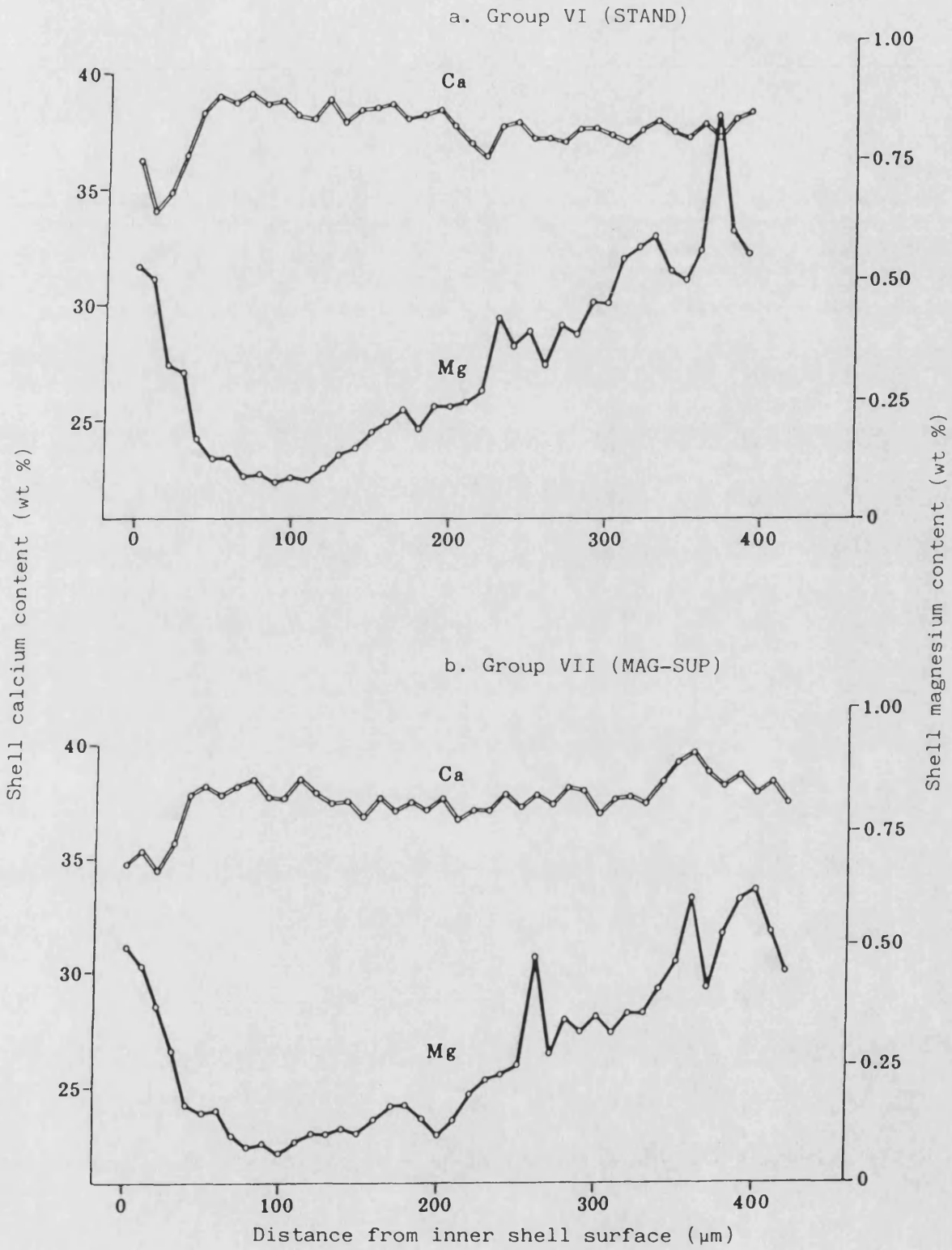


Figure 16. Distribution of calcium and magnesium across the thickness of the egg shell in Groups VI to IX.

0 to 100 μm, cone layer; 100 μm onwards, column layer.

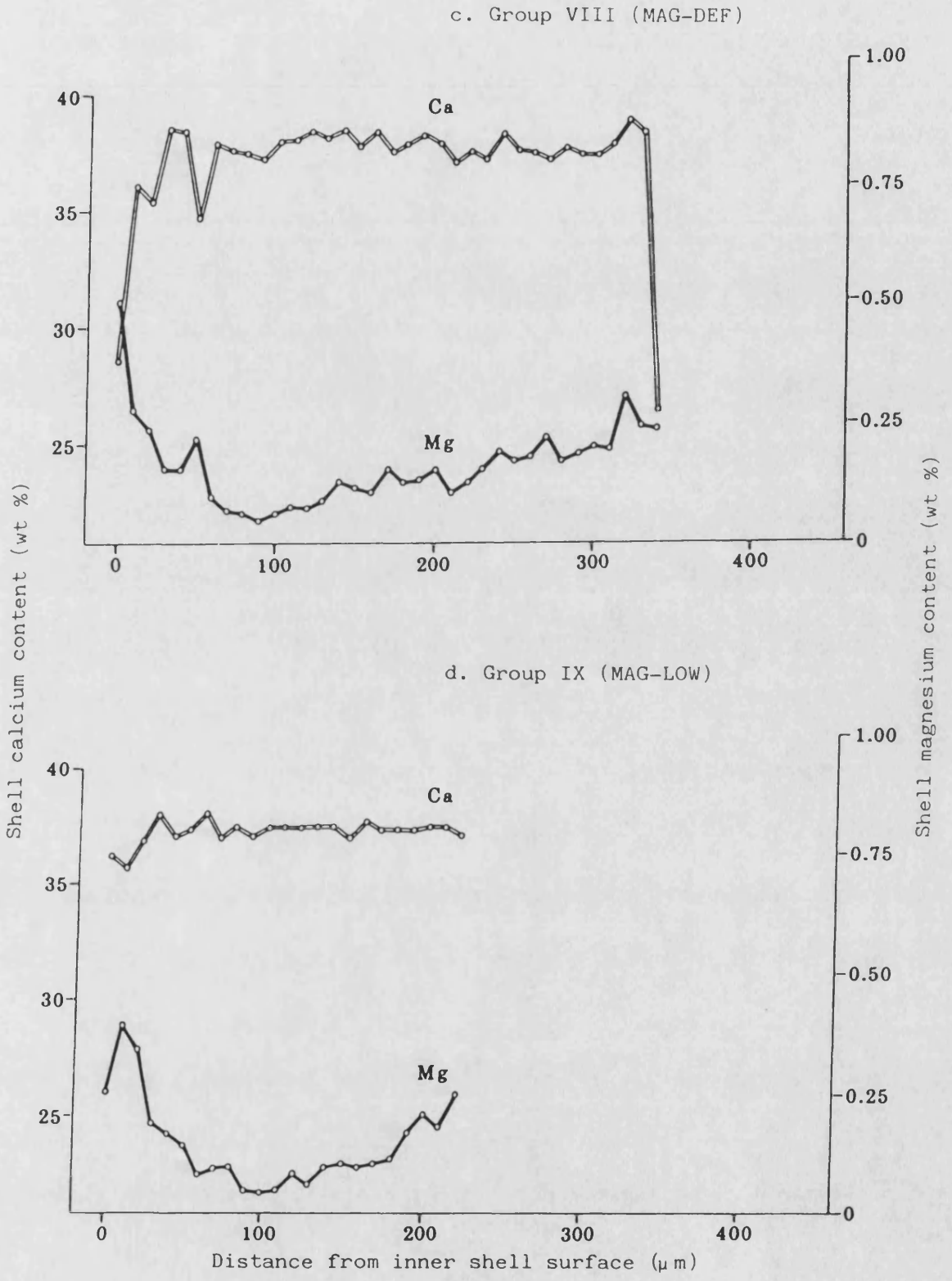


Figure 16. (contd.).

the shell remained relatively constant in all four groups and in all cases represented around 37.5 wt %. Although magnesium was distributed unevenly across all the shells, the amount present was readily obtained by measuring the area under the curve. Table 15 compares the values of shell calcium and magnesium content (wt %) determined by both AAS (Table 12) and EPMA. There is substantial agreement between the results obtained from the two techniques. The values for both elements as measured by AAS are slightly higher than those from EPMA and there was more variation between group mean shell calcium content when AAS was used. Nevertheless, the overall mean calcium content of all groups was similar at 37.7 and 37.4 wt % for AAS and EPMA respectively. Moreover, the correlation between dietary magnesium level and shell magnesium content was evident using both techniques (Table 15).

In order to eliminate any local variations in element concentration due to surface topography, porosity etc., during EPMA, the magnesium : calcium ratios across each of the four shells of Figures 16 a-d were calculated. Figures 17 a-d show ratios of the two elements, calculated from their wt % values, and confirm the previous findings regarding the relative distribution of each element across the shell.

c The three-dimensional distribution of shell magnesium

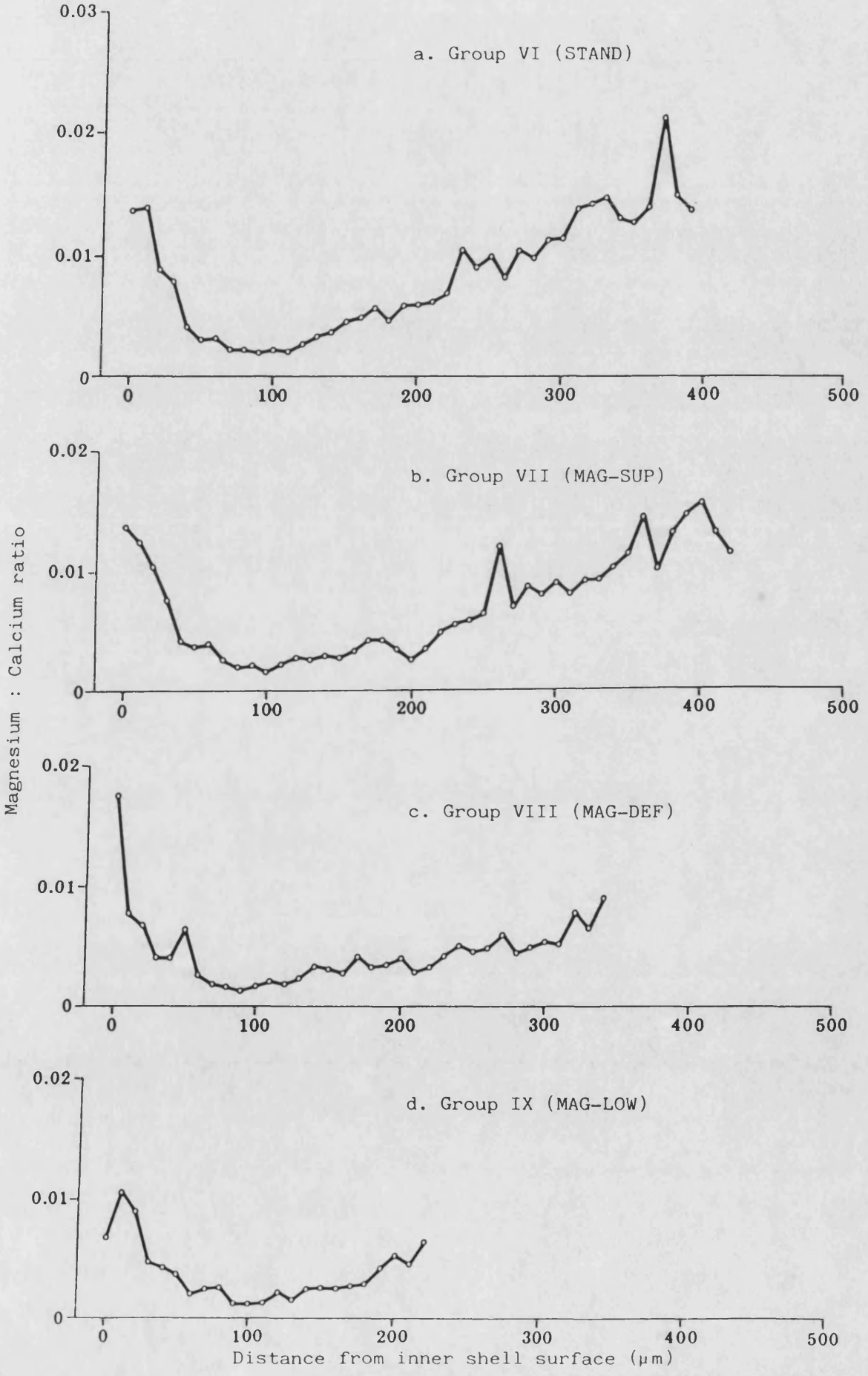
During the present study it became apparent that the distribution of magnesium within the column layer was relatively consistent within any one shell regardless of whether the analysis began at the centre or to either side of an individual mammilla.

Table 15. Comparison of egg shell element content as determined by atomic absorption spectroscopy (AAS) and electron probe microanalysis (EPMA).

Group	Shell element content (wt %)			
	calcium		magnesium	
	AAS ± S.E.	EPMA	AAS ± S.E.	EPMA
VI (STAND)	n.a.	37.7	n.a.	0.31
VII (MAG-SUP)	39.7 ± 0.7	37.5	0.35 ± 0.01	0.27
VIII (MAG-DEF)	35.1 ± 1.7	37.1	0.19 ± 0.01	0.16
IX (MAG-LOW)	38.2 ± 0.6	37.3	0.15 ± 0.01	0.14

n.a. not available.

Figure 17. Magnesium : calcium ratio across the egg shells of Groups VI to IX calculated from the wt % values of each element obtained from Figures 16 a-d.
0 - 100 μm, cone layer; 100 μm onwards, column layer.


a. Group VI (STAND)
b. Group VII (MAG-SUP)
c. Group VIII (MAG-DEF)
d. Group IX (MAG-LOW)
Magnesium : Calcium ratio
Distance from inner shell surface (µm)
0.03
0.02
0.01
0
0
100
200
300
400
500

The same was not true of the inner magnesium peak, the magnitude of which was variable and seemed to depend upon the position of the electron beam in relation to the mammillary structure. For this reason all EPMA results were obtained after positioning the electron beam as close as possible to the central line of each mammilla.

From Figures 16 a-d it can be seen that, although a reduction in dietary magnesium intake markedly reduced the magnesium content of the column layer, the peak at the cone tips still persisted. In order to compare the distribution of magnesium in the cone layers of normal and reduced-magnesium shells it was necessary to eliminate any variations arising from the heterogeneous distribution of this element. Plate 10 shows a Group VI (STAND) shell in which a regularly arranged series of single line scans was carried out across the width of a single mammilla. In contrast to the earlier work each line scan was produced by moving the specimen in a direction parallel to the outer shell surface. After completion of each line the shell was displaced in a direction normal to the outer surface and the analysis repeated. In this way a map of the distribution of calcium and magnesium was slowly built up across the shell. The technique was applied to a single shell from each of Groups VII (MAG-SUP) and IX (MAG-LOW), the same shells having been used to produce Figures 16 b, d.

The porosity of the cone tips, due to the removal of shell membrane fibres, is also shown in Plate 10 and this effect was probably the cause of the variation in shell calcium content seen in Figure 11.

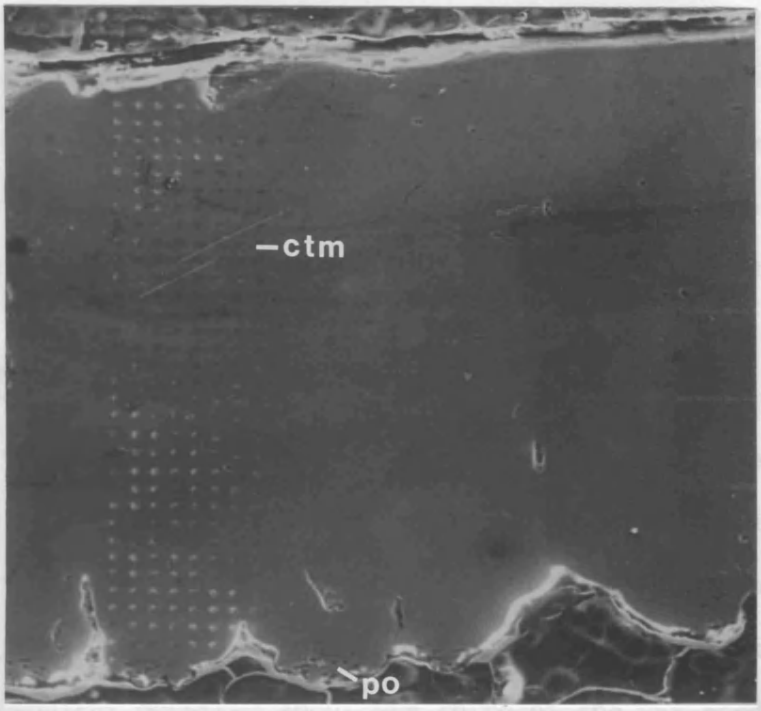


Plate 10. Electron micrograph showing appearance of shell following repeated EPMA.
Magnification, x 215
ctm, contamination marks left by the electron beam during element analysis.
Note porosity (po) of shell material at the cone tips due to removal of shell membrane fibres.

Plate 11a shows the magnesium distribution across the egg shell of a Group VII (MAG-SUP) bird. The step interval across the shell was reduced to 5 μm for more detailed analysis, the X-rays at each position being measured for 20 s. Most of the magnesium was present in the column layer and the relatively consistent distribution of magnesium in this region gives rise to the correlation between bulk estimates of its content (AAS) and estimates derived from single line scans (Table 15). Magnesium in the cone layer was concentrated within a central zone at the tip of the mammilla. The enhanced magnesium signal persisted for a short distance, especially along the central line but very quickly fell away as the beam passed into the shell. From Plate IIa it is also evident that an increase in magnesium content occurred about one third of the way across the shell, at the point of fusion of individual mammillae.

The relatively high magnesium content of the cone layer of reduced-magnesium shells (Group IX, MAG-LOW) is confirmed in Plate 11b which also shows the marked reduction in column layer magnesium content.

The scales used for each trace in Plates 11 a, b are proportional to those given in Figure 16. For clarity, each trace was separated from the next in Plates 11 a, b , though in reality the width of the analysed area was much smaller in relation to its length (Plate 10). In both cases the results for calcium were used to delimit the shell boundaries and the curvature of the mammillae can be seen, especially in Plate 11a.

One further feature of the normal distribution of magnesium

Plate 11. Distribution of magnesium across the egg shells of birds in Groups VII and IX as revealed by EPMA.

a. Group VII (MAG-SUP)

b. Group IX (MAG-LOW)

Step interval, 5 µm; sampling time, 20 s.

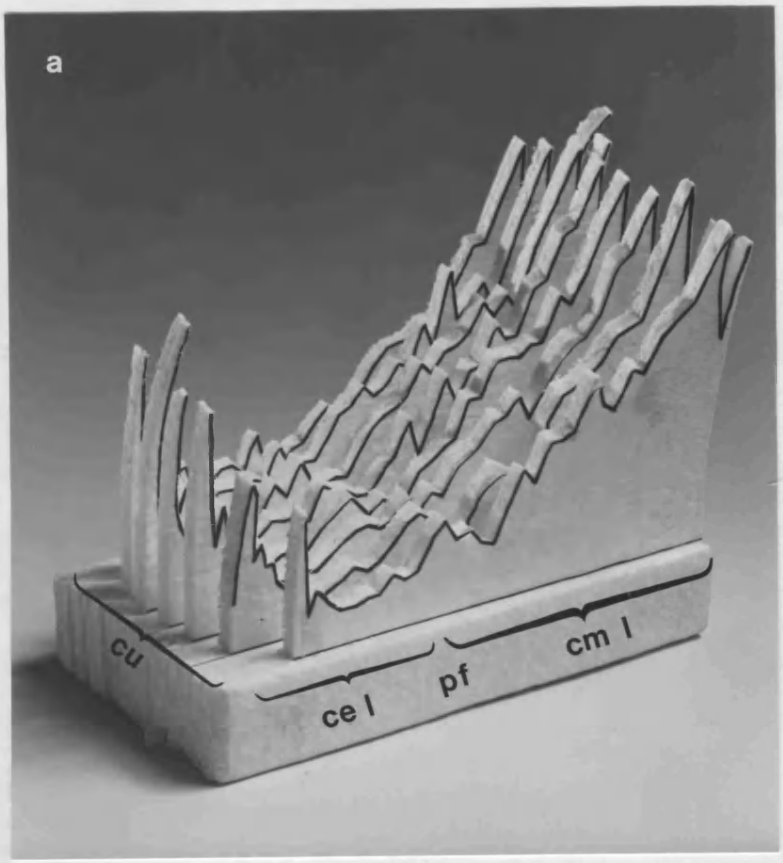
ce 1, cone layer.

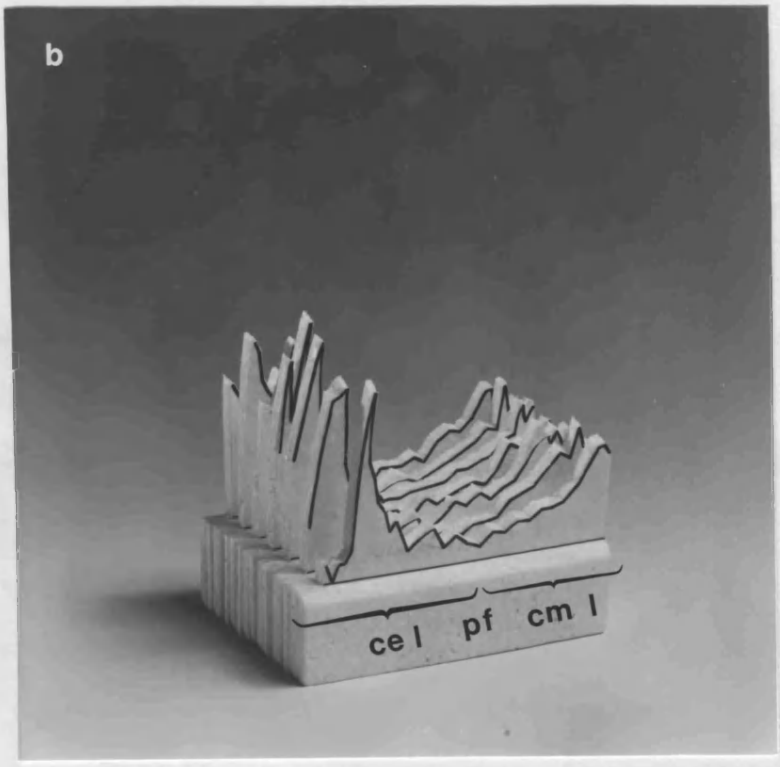
pf, point of fusion of cone layer.

cm 1, column layer.

Note curvature (cu) resulting from the rounded outline of a single cone.

Scales proportional to those used in Figures 16 a - d.


a
cu
ce l
pf
cm l


b
ce l
pf
cm l

across the hen's egg shell is its precise correlation with the known distribution of organic material. Plate 12 shows the polished radial shell surface of an unincubated egg obtained from a commercial hatchery. The lighter regions in this micrograph correspond to the relatively high concentrations of shell organic material found at the cone tips, at the point of fusion of the cone layer and in the outer half of the shell (Plate 12). This is precisely the distribution of magnesium as revealed by Plate 13 (see legend for details) which represents the results of an earlier unpublished study (courtesy of R.G. Board). The enhancement of the magnesium signal at the point of fusion of the cone layer (Plates 11a, 13) was not always obvious in single line scans but is quite evident when presented in this way.

d Effect of incubation on shell magnesium

Figures 18a, b show the calcium and magnesium distributions across the shell of a hatched egg. Figure 18a, obtained from the region of the blunt pole, shows the characteristic distribution of these elements previously seen in Figures 11 and 12. Figure 18b, obtained from a piece of shell taken from the equator of the egg, shows two main features. Firstly, the distribution of magnesium across the column layer is similar to that in Figure 18a supporting the contention that the profile is consistent within single shells. Secondly, the peak in cone layer magnesium content, shown in Figure 18a, is very much reduced, the same difference being observed in a further two, successfully incubated, eggs. It seems that the erosion of the cone tips during late incubation (Plates 14 b, 15 b)

Plate 12. Electron micrograph of the radial shell face of an incubated egg.

Regions of high organic content appear lighter.

Magnification, x 215.

o ce, organic material in the cone layer.

o pf, organic material at the point of fusion of cone layer.

o cm, organic material in the column layer.

Plate 13. Distribution of magnesium across the hen's egg shell as revealed by EPMA.

Step interval, 2 μm; sampling time 20 s.

Count rate plotted as $\log_{10}$ transformations.

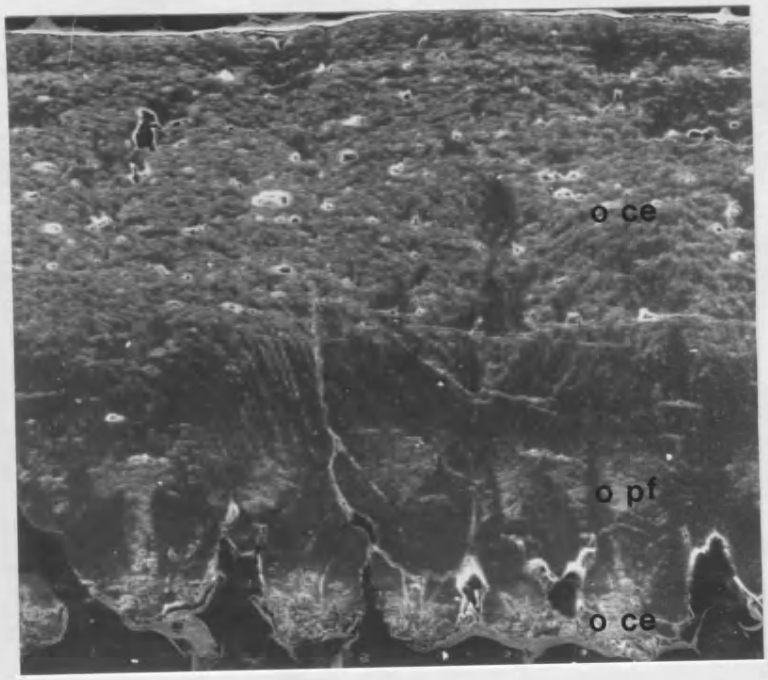
ce 1, cone layer.

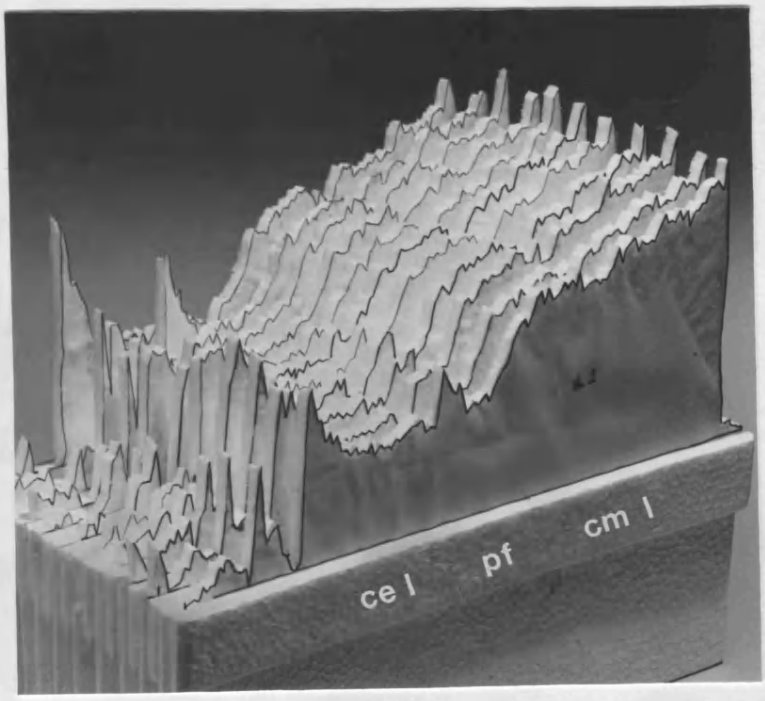
pf, point of fusion of cone layer.

cm 1, column layer.

Note similarity between the distribution of magnesium and that of organic material (Plate 12).

Also note step-wise distribution of magnesium in the column layer.


o ce
o pf
o ce


ce l
pf
cm l

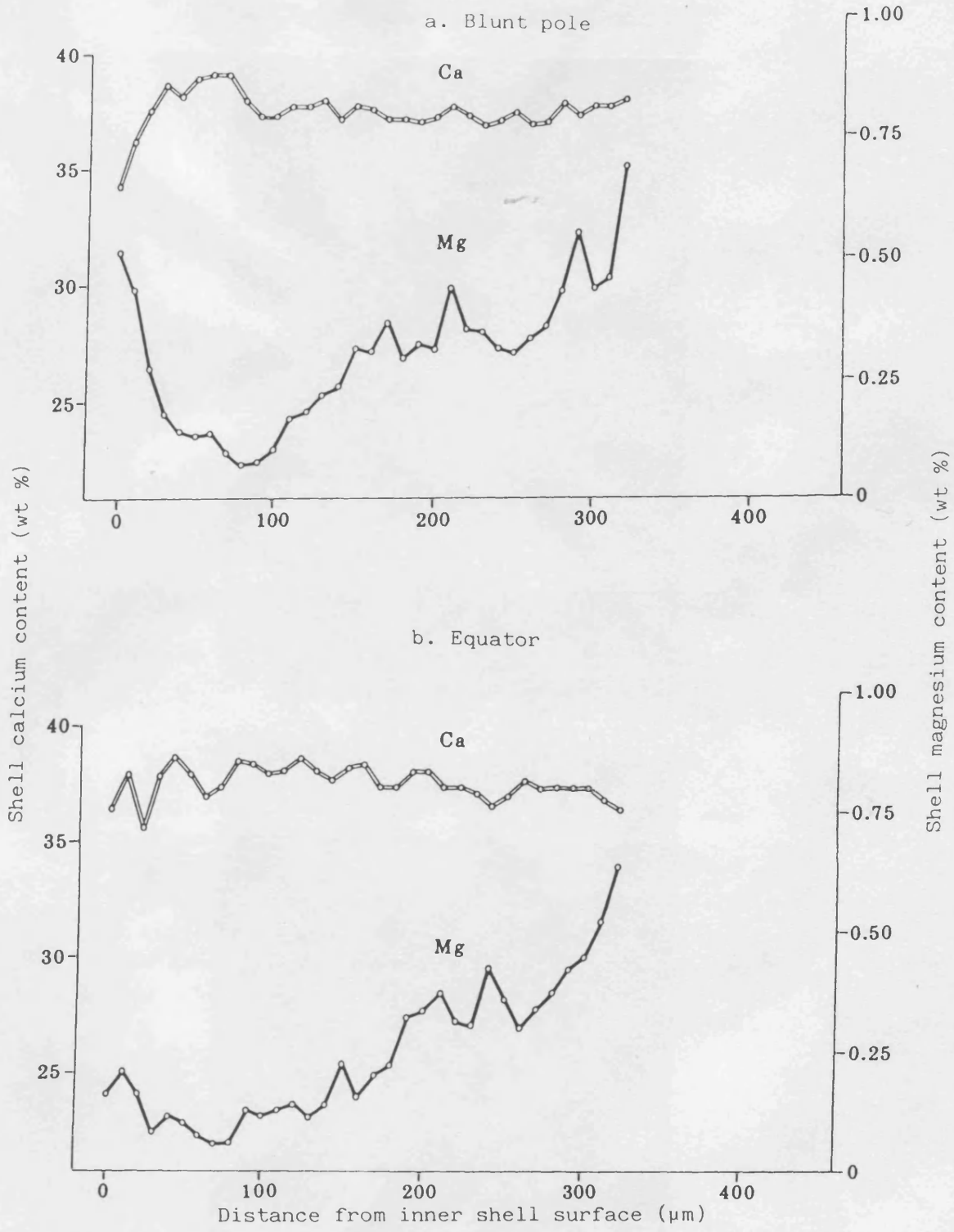


Figure 18. Distribution of egg shell calcium and magnesium at different regions of a hatched egg.

0 - 100 μm, cone layer; 100 μm onwards, column layer.

Plate 14. Electron micrographs of the radial shell face at different regions of a hatched egg.

a. At the blunt pole.

b. At the equator.

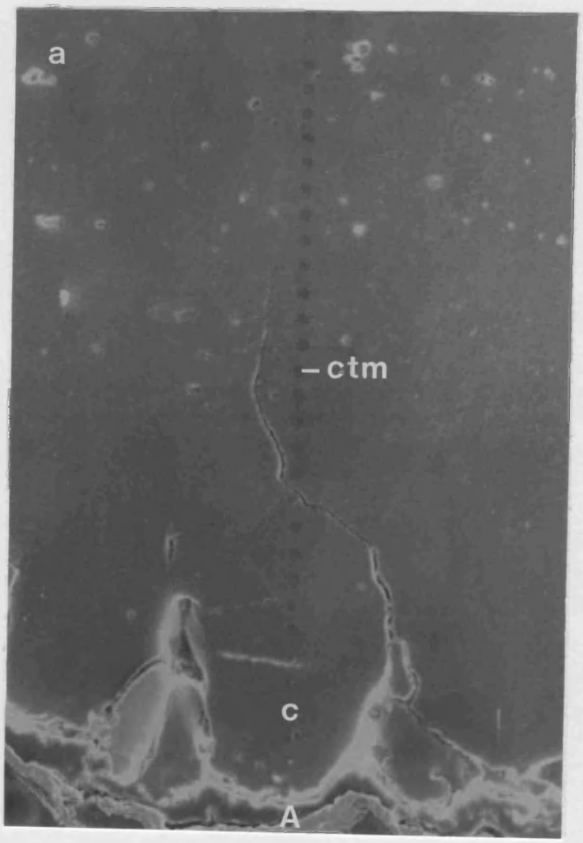
Magnification x 280.

A, position of the electron beam relative to the shell prior to EPMA.

c, cone.

ctm, contamination marks left by the electron beam during element analysis.

Note erosion (er) of the cones in shell obtained from the region of the equator.


a
–ctm
c
A

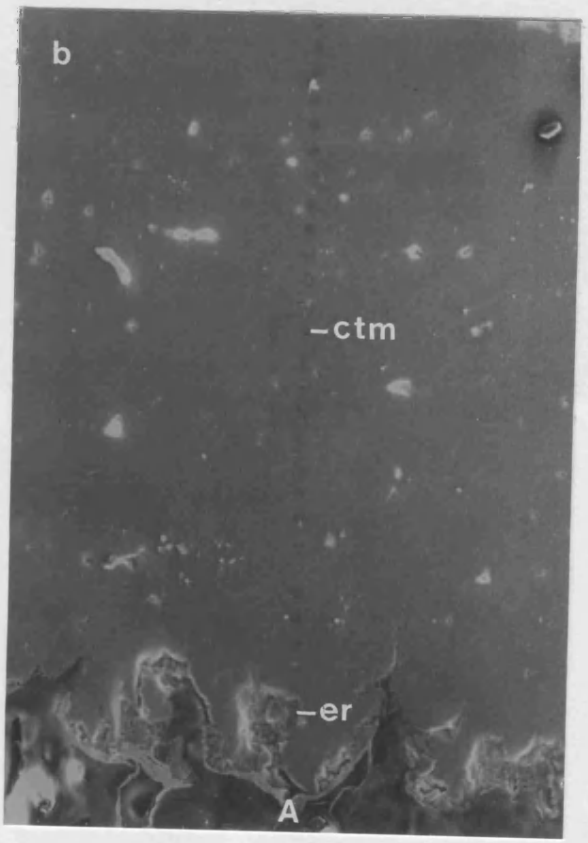

b
–ctm
–er
A

Plate 15. Electron micrographs of the radial surface of individual cones obtained from the equatorial regions of unincubated and incubated eggs.

a. Unincubated egg.

b. Incubated egg.

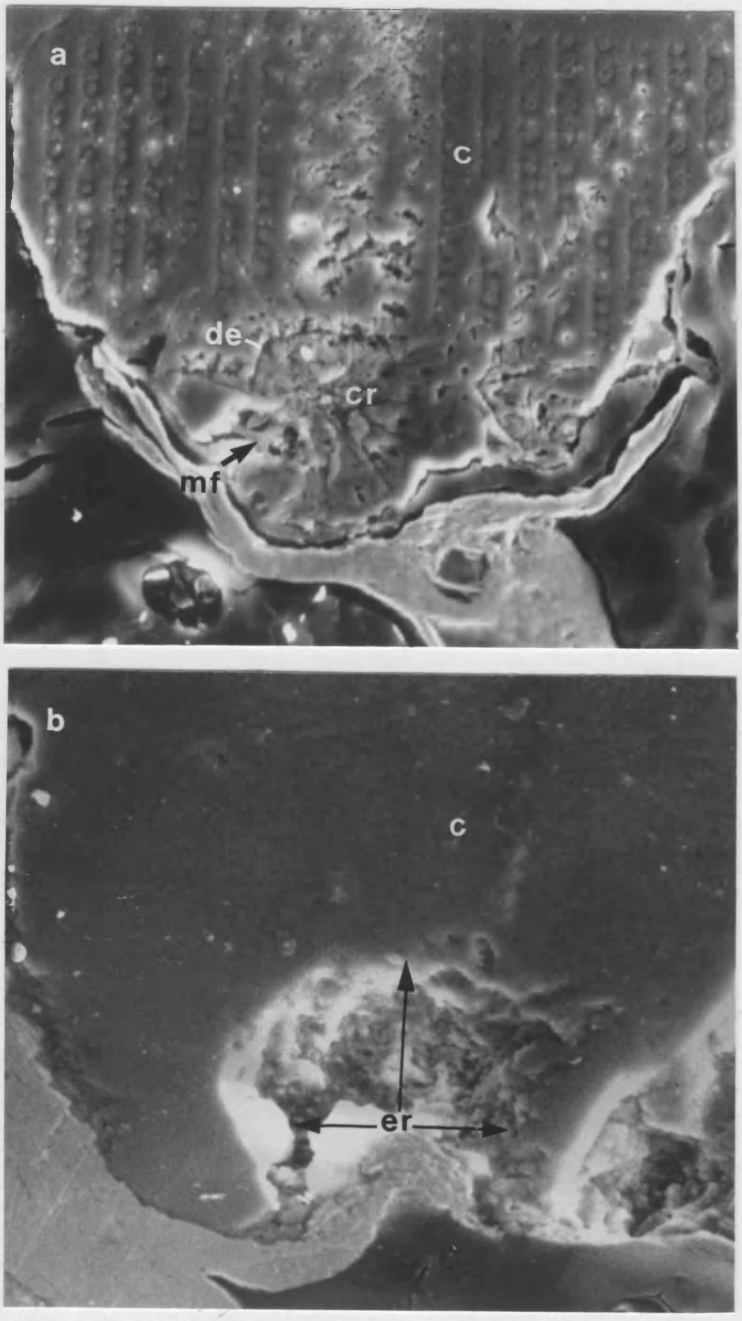
c, cone

cr, core region of cone.

de, demarcation between core and remainder of cone.

mf, membrane fibres.

er, erosion of cone, characteristic of successfully incubated eggs.


a
c
de
cr
mf
b
c
er
10 µm

is associated with a loss of magnesium from this region. This loss does not occur in cones at the blunt pole (Figure 18a, Plate 14 a) which, during early incubation, become separated from the egg contents by the formation of the air space. Note also that in the cone layer of unincubated shells (Plate 15a) there is a clear demarcation between the core of each mammilla and the remainder of the structure.

PART III: CHAPTER 3

DISCUSSION

In Part II it was shown that magnesium played a vital role in the maintenance of egg production in the domestic hen. In birds receiving low-magnesium diets there was a decline in egg number (Figure 2), egg weight (Figure 3) and egg shell thickness (Figure 4). It was also demonstrated, using atomic absorption spectroscopy (AAS), that there was a reduction in the magnesium content of the thin egg shells produced by magnesium-depleted birds (Figures 5 a-c, Table 12b). It is clear, therefore, that the requirement for magnesium during egg formation results in part from the involvement of this element in egg shell deposition. In order to resolve the exact role of magnesium in shell formation a detailed examination was made of the structure and composition of egg shells produced in Part II. The use of electron probe microanalysis (EPMA) enabled the precise determination not only of shell element content but also of shell element distribution. Consequently, it became apparent that the involvement of magnesium in egg shell formation and function should be considered in the following contexts:

3.1 The mechanism of shell thinning in magnesium-depleted birds

3.2 The incorporation of magnesium into the developing shell

3.3 The role of magnesium in shell function.

3.1 The mechanism of shell thinning in magnesium-depleted birds

The marked reduction in egg shell thickness in birds receiving the low-magnesium diets (Figure 4) was also apparent in optical (Plates 1 a-d) and electron (Plates 3, 4) micrographs obtained in the present section. It can further be concluded that it was the rate of shell deposition which was reduced in magnesium-depleted birds since the duration of egg formation was largely unaffected (Tables 10 a, b). A reduction in the amount of magnesium available to the actively calcifying shell gland could impair the rate of shell deposition in one of two ways:

a By reducing the amount of calcium transported into the shell gland lumen during calcification. This would be consistent with the observed similarities between the effects of magnesium deficiency and those of DDE administration, as discussed in Part II.

b By interfering with the normal development of the shell matrix, either by reducing the amount deposited or by altering its composition. The same mechanism has been proposed to explain the shell-thinning effects of a manganese deficiency (Longstaff and Hill, 1972; Leach and Gross, 1983).

The degree to which the organic material organises shell development will be the key to determining which of these two possibilities is the most likely. It has often been assumed that organic matrices are capable of acting as very specific determinants of crystal development in a wide range of biominerals (Simkiss and Tyler, 1958; Watabe and Wilbur, 1960; Terepka, 1963b; Longstaff and Hill, 1972; Abatangelo et al., 1978; Krampitz and

Witt, 1979). It has also been reported that the production of thin egg shells is correlated with disruptions in the normal distribution of organic material across the shell (Longstaff and Hill, 1972; Krampitz and Witt, 1979). The correspondence between the distributions of magnesium and organic material observed in Plates 12 and 13 is consistent with the idea of a causal relationship between the deposition of the two shell components. If magnesium was largely unavailable during shell formation then some self-organising property of the organic matrix could conceivably be lost leading to a reduction in the rate of shell calcification.

The concept of an organising shell matrix has, however, been undermined by several recent studies. These studies have tended to suggest that the organising ability of organic macromolecules has been overemphasised, both in the case of the bird's egg shell (Silyn-Roberts and Sharp, 1986) and in biominerals generally (Mann, 1983). The major role of the organic material would appear to be to provide mechanical strength to the shell rather than organising its development (Petersen and Tyler, 1966; Bond et al., 1986; Silyn-Roberts and Sharp, 1986b). In view of this, the correlation between the distributions of magnesium and organic matter is likely to be more relevant when considering the incorporation of magnesium into the shell and will therefore be discussed in the following section.

A reduction in the availability of calcium during egg shell formation therefore remains the most likely explanation of the reduced shell thickness in magnesium-depleted birds. The results obtained from SEM studies in the present work are consistent with this. Given that the maximum demand for calcium occurs during formation of the column layer (Talbot and Tyler, 1974c) it might be

expected that this will be the region most affected by a reduced provision of this element, as indeed appears to be the case (Plates 3 to 7). The essentially normal development of the cone layer in shells produced by magnesium-depleted birds (Table 13; Plates 5 to 7) may be explained by the slower rate at which this region is normally formed (Bradfield, 1951; Talbot and Tyler, 1974c). There is some evidence that the rate of shell formation only becomes maximal following fusion of the individual cones (Eastin and Spaziani, 1978a, b) due partly perhaps to the cessation of plumping (Talbot and Tyler, 1974c). As suggested in Part II, the active transport of calcium across the shell gland in the domestic hen may result from magnesium-dependent ATPase activity. Under the conditions of magnesium deficiency, the shell gland may be unable to respond to the sustained demand for shell calcium, especially towards the end of the dark period. One consequence of the dependence upon magnesium for the provision of calcium is that, in Galliforme birds at least, relatively large quantities of magnesium will pass into the shell gland lumen during shell calcification. The implications of this, in terms of shell composition, will now be considered.

3.2 The incorporation of magnesium into the developing shell

The results of the present study indicate that it is only in regions of high organic content that magnesium is incorporated into the developing shell (Plates 12, 13). This can be explained in terms of the relationship between the shell mineral and organic phases. The major role of column layer organic material is probably

to provide mechanical strength to the finished structure (Bond et al., 1986; Silyn-Roberts and Sharp, 1986b). The organic matrix can increase shell strength either by acting as a fibrous network or by restricting the grain size of the calcite crystals during shell formation. Indeed, Quintana and Sandoz (1978) reported that crystal size is inversely related to organic content in different regions of the quail shell and proposed that this is true of biominerals generally. Moreover, both a high organic content and small crystal grain size are features of the unusually thick and strong egg shells of the guinea fowl (Petersen and Tyler, 1966). Further evidence that the organic matrix disrupts crystal growth was provided by Silyn-Roberts and Sharp (1986b) who demonstrated that the incorporation of shell organic material hindered the development of preferred orientation in the calcite crystals of the hen's egg.

Further work needs to be done to determine the precise nature of the hindrance to calcite formation caused by the deposition of the shell organic phase. Organic macromolecules can, in general, either inhibit or promote the rate of biomineral deposition (Williams, 1984). Assuming that the binding of organic material to the developing crystal face inhibits the rate of shell calcite growth then the deposition of a more thermodynamically stable mineral form might become possible, if not favoured (S. Mann, pers. comm.). Under the conditions of at least one study (Stumm and Morgan, 1981) it was shown that calcite containing a small amount of magnesium was indeed more thermodynamically stable than pure calcite. From Figure 16a (Group VI, STAND) it can be seen that the maximum level

of magnesium in the column layer was about 0.8 wt %.

The characteristic distribution of magnesium across the column layer of the hen's egg shell (Figures 12, 16a; Plates 11 a, 13) could therefore result from a progressive increase in the amount of this element passing into the shell gland during calcification (Itoh and Hatano, 1964; El-Jack and Lake, 1967). Alternatively, the same distribution could result from a corresponding change in either the amount or composition (Cooke and Balch, 1970b) of shell organic material across the column layer. A step-wise increase in the density of organic matter would be consistent with the step-wise disruption of shell crystal orientation reported by Silyn-Roberts and Sharp (1986b). In Plate 13 there is a suggestion of a step-wise distribution of magnesium across the column layer similar to that reported for the quail shell (Quintana et al., 1980). If the incorporation of magnesium into the shell was indeed linked to changes in the deposition of organic material then the increase in the amount of this element across the column layer could occur independently of changes in the magnesium content of the shelling fluid. In fact, Solomon (1971) reported that in the hen both the magnesium content and the magnesium : calcium ratio of the shell gland fluid remained constant during shell formation.

Further evidence of the link between the incorporation of shell magnesium and the deposition of the organic phase is provided by the increase in magnesium content at the point of fusion of the cone layer (Plates 11a, 13). This increase is unlikely to reflect a similar change in the magnesium concentration of the shelling fluid and more probably results from the presence of organic material in

this region (Terepka, 1963b; Plate 12) - possibly organic material extruded from the egg surface by the developing cones (Fujii, 1974; Sparks, 1985). The paucity of magnesium in the shell region just above this could result from a lag period between fusion of the cone layer and the onset of shell matrix secretion by the shell gland. When this region of low organic content does not occur, the resulting shells are translucent (Talbot and Tyler, 1974a). It would be interesting to establish the distribution of shell magnesium in such cases.

The extremely localized peak in the magnesium content of the cone layer (Figure 12, Plate 11a) is a feature apparently common to the egg shells of all birds (Board and Love, 1980, 1983). This peak in magnesium content appears to occur at the cone tips and therefore corresponds to the site of shell crystal nucleation. The organic cores on the surface of the egg in the tubular shell gland (red region) are mineralized extremely rapidly (Tyler, 1969b), suggesting the presence of active element-binding sites. This will tend to favour, at least initially, the deposition of kinetically preferred mineral forms such as high-magnesium calcites, aragonite and even amorphous calcium carbonate. The presence of aragonite in this region has in fact been reported (Erben, 1970) though this finding has never been confirmed. In any case, the prompt mineralization of the surface of the organic cores will block the active binding sites thereby preventing further rapid calcification. The subsequently reduced rate of mineralization could then favour calcite formation (S. Mann, pers. comm.) giving rise to the radially oriented crystals characteristic of the cone

layer (Schmidt, 1957; Silyn-Roberts and Sharp, 1986b).

The occurrence of high-magnesium calcite is likely to be confined to the surface of the cores and as a result will be extremely localized even within individual cones. For this reason, the peak in magnesium content at the cone tips will have tended to be underestimated by averaging the amount of magnesium even in a volume of 1 μm^3 . Nevertheless, the observation that the peaks in cone and column layer magnesium content are similar (Figure 12, Plate 11a) points to the 'avidity' of the former for this element (Board and Love, 1980). Although the magnesium content of the fluid secreted by the red region is low compared to that found in shell gland fluid, there is a corresponding difference in calcium content (El-Jack and Lake, 1967). The ratio of the two elements could therefore be such as to allow the deposition of high-magnesium calcite to occur during the earliest stages of shell formation occurring in the red region.

In summary, it is suggested that the distribution of magnesium across the hen's egg shell reflects the changes occurring in the amount or composition of organic material incorporated into the developing shell. If this is correct then it provides further evidence for the contention in Part II that it is only in the Galliformes that magnesium enters the shell gland in relatively large quantities, as the presence of organic material is common to the egg shells of all birds.

3.3 The role of magnesium in shell function

An important function of the porous avian egg shell during incubation is to allow gaseous exchange to occur between the developing embryo and the external environment. Any disruption in the normal course of pore formation might therefore be expected to have a profound influence on the role of the shell during embryogenesis.

The abnormal mineral deposits on the cones and outer membrane fibres of shells produced by birds receiving low-magnesium diets (Plates 9 a-d) might be related to the reduction in egg shell pore number observed in the same groups (Table 14). It is tempting to speculate that a reduced level of magnesium in the calcifying fluid of magnesium-depleted birds was directly responsible for both these effects.

It has been suggested that during the early stages of shell formation it is magnesium in the shell gland fluid which maintains the geometry of the cones (Board and Love, 1980). As the characteristic development of the cone layer is responsible for the initiation of the pore canals (von Nathusius - see Tyler, 1964; Tullett, 1976; Tyler and Fowler, 1978) then the concentration of magnesium in the calcifying fluid may be one determinant of shell porosity. The reductions in egg shell pore number in magnesium-depleted birds as shown in Table 14 are consistent with this as are similar results obtained from turkey egg shells (Christensen and Edens, 1985). A distinction must be made, however, between the mechanisms responsible for the development of potential pore canals during cone layer formation and the mechanisms which prevent pore

closure during the growth of the column layer.

Despite the relatively high levels of magnesium likely to be present during shell formation in the shell gland lumen of Galliforme birds, the element is unlikely to be important in preventing the closure of the pore canals indicated by Table 14. Although the presence of magnesium has been observed to inhibit the nucleation of calcite (Pytkowicz, 1965; Bischoff, 1968), calcification is not prevented because the growth of the aragonite polymorph becomes favoured (Kitano, 1964; Simkiss, 1964a; Bischoff, 1968; Watabe, 1974; Berner, 1975). Moreover, any direct effect of magnesium on shell formation, and hence function, especially during development of the column layer is likely to be overwhelmed by the antagonistic effects of organic macromolecules present in the shelling fluid (Kitano, 1962, 1964). As suggested in the previous section, the inhibition of shell crystal growth rate caused by the incorporation of an organic phase will tend to favour the deposition of more thermodynamically stable mineral forms such as calcite and low-magnesium calcite (see Kitano and Furutsu, 1959), even though the calcifying fluid may contain relatively high levels of magnesium.

An alternative explanation of the observed effects of magnesium deficiency on shell ultrastructure is that the decreased provision of calcium during shell formation leads to a general reduction both in the rate of shell formation and in the volume of bulk shell gland fluid released into the lumen. The continual passage of fluid along the pores and into the egg during shell formation may normally prevent the mineralization of the pore canals (Tyler and

Simkiss, 1959). It is noteworthy that the types of abnormal shell mineralization shown in Plates 9 a-d are also a feature of some poor-quality egg shells (Bunk and Balloun, 1978). A reduction in the rate of shell formation is likely to be responsible for the production both of the thin egg shells of magnesium-depleted birds and of thin, poor-quality shells generally.

The importance of the avian egg shell as an external source of calcium during embryo development has long been recognised (Johnston and Comar, 1955). The absence of an external mineral supply in shell-less cultures severely restricts embryo development during the second half of the incubation period (Ono and Wakasugi, 1984).

The mechanism of egg shell calcium resorption by the avian embryo has received considerable attention (Terepka, 1963b; Schmidt, 1964, 1965; Moriarty and Terepka, 1969; Garrison and Terepka, 1972; Crooks and Simkiss, 1974; Kyriakides and Simkiss, 1975; Saleuddin *et al.*, 1976; Tuan, 1983). Terepka (1963b) suggested that the presence of a relatively soluble mineral form at the tips of the cones was responsible for the characteristic erosion of these regions during incubation, though Schmidt (1964) disputed this. Kyriakides and Simkiss (1975) suggested that it was hydrochloric acid and not carbonic acid that was responsible for the decalcification of the shell.

The importance of shell-derived magnesium in chick viability was pointed out by Christensen *et al.* (1964). Although Taylor *et al.* (1975) considered that the yolk was the only source of this element during embryo development, Ono and Wakasugi (1984) demonstrated

that approximately 24% of the magnesium content of newly hatched chicks came from the shell. Moreover, hatchability in turkey eggs has been found to be positively correlated with shell magnesium content (Christensen and Edens, 1985). This suggests that magnesium, like calcium, is sequestered from the shell by the developing embryo during late incubation. Evidence for this can be seen in Figures 18a, b which show a reduction in cone layer magnesium content in the equatorial regions of hatched egg shells.

Plate 15a shows the interface which exists between the core region of the individual cones and the rest of the structure. The shell minerals taken up by the developing embryo are removed from this interface and when the shell membranes of hatched eggs are removed, a characteristic cavity is left in each cone (Plate 15b). One consequence of the rapid deposition of high-magnesium calcite onto the surface of the core during early shell formation is an increase in the solubility of shell mineral in this region. This may be due either to the high magnesium content itself or to a reduction in crystal size, both of which could result from the initial interference to calcite development caused by the rapid mineralization of the cores.

In Plate 15a, it can be seen that remnants of the membrane fibres are still firmly attached to the inner region of the cone. In the incubated egg, these fibres are part of the continuous link between the shell minerals, the shell membranes and the chorioallantoic circulation of the developing embryo.

It is worth noting that previous correlations between hatchability and egg shell magnesium content (Christensen, 1964; Christensen and Edens, 1985) have been based upon relatively crude

determinations of whole shell magnesium content. In the hen, the amount of this element in the cone layer was found to be small compared to that found in the remainder of the shell (Figures 12, 16 a, b). Moreover, the peak in cone layer magnesium content was observed in shells produced by magnesium-depleted birds even though the amount of this element in the column layer was severely reduced (Figures 16 c, d; Plate 11b). This would seem to indicate that the amount of magnesium in the column layer can vary without any corresponding change in the level of cone layer magnesium. Consequently, in the case of the domestic hen, it cannot be assumed that the amount of magnesium available to the developing embryo is accurately reflected in estimates of total shell magnesium content.

The successful cultivation of the avian embryo in the absence of the shell and shell membranes is likely to become increasingly important in future research. Any attempt to artificially provide the minerals required during late embryogenesis will have to allow for the important role of egg shell magnesium in avian reproduction.

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