RELATIONSHIPS BETWEEN EGGSHELL QUALITY AND BIOCHEMICAL PARAMETERS OF CALCIUM METABOLISM

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Summary

To determine relationships of biochemical parameters involved in Ca metabolism with eggshell quality, serum Ca level, duodenal and uterine calcium binding protein (CaBP) and uterine carbonic anhydrase (CA) activities were measured using 102-week old hens. Three groups of chickens were selected, those showing high quality (HQ) and low quality (LQ) eggshells and non-laying activity (NE). NE hens exhibited significantly ($p < 0.05$) lower serum Ca levels than laying hens. HQ and LQ hens were not different in the Ca level, indicating that serum Ca level was not good indicator of hen's ability to produce different quality eggshells. Duodenal CaBPs was highest in HQ and lowest in NE ($p < 0.10$). Uterine CaBPs of LQ and NE were not different and lower significantly ($p < 0.10$) than that of HQ, suggesting that CaBP played an important role in determining eggshell quality. Uterine CA activities of the three groups were significantly different ($p < 0.01$); highest in HQ and lowest in NE, suggesting intimate relationship between CA and eggshell quality.

(**Key Words** : Eggshell Quality, Blood Ca Level, CaBP, Carbonic Anhydrase)

Introduction

The eggshell of a hen weighs about 5 g and averages about 320 μm in thickness (Voisey and Hamilton, 1976). It consists of about 98% calcium carbonate (as calcite) and 2% protein (Simkiss and Taylor, 1971).

Although there are some evidences indicating that the isthmus initiates shell formation, the main process of shell calcification occurs in the shell gland (Robinson and King, 1963; Creger et al., 1976; Stemberger et al., 1977). The raw materials for the calcite crystals in eggshell are Ca^{2+} and CO_3^{2-}. Calcium in the diet can ionize in the digestive tract and is absorbed in the small intestine. The greatest absorption of calcium occurs between the duodenum and the lower jejunum (Hurwitz and Bar, 1970). Blood calcium plays an important role in the calcium pool (Hurwitz and Bar, 1967). Calcium enters the pool through calcium absorption and bone resorption.

The movement of calcium across the shell gland occurs through the involvement of a vitamin D-dependent calcium-binding protein (CaBP) (Hurwitz and Bar, 1973; Nys et al., 1986). Since vitamin D_3-induced CaBP in the intestinal mucosa of chicks was identified by Wasserman and Taylor (1966), a large number of workers have demonstrated a vitamin D requirement for its synthesis (DeLuca, 1974) and the importance of its role in calcium metabolism (Wasserman and Taylor, 1968; Taylor and Wasserman, 1969; Emtage et al., 1974). Nys and De Laage (1984) observed that uterine CaBP was reduced in hens laying shelless eggs. Bar et al. (1984) reported that uterine CaBP was reduced in hen laying poorly calcified eggs and that a significant correlation existed between uterine CaBP and shell calcium.

Mongin and Carter (1977) constructed a shell gland model. The shell gland mucosa consisted of two types of cells, glandular cells and columnar cells. The main function of the glandular cells was formation of HCO_3^- from CO_2 and water in the presence of carbonic anhydrase (CA). They postulated that the transfer of Ca^{2+} from

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blood into the lumen of the shell gland was brought about by the columnar cells, and that translocation of the ions might require CaBP. The calcification of egg shell occurred in the lumen of the shell gland from the precipitation of Ca^{2+} by HCO_3^- as $CaCO_3$.

The CA activity of the shell gland epithelium was reported by Common (1941) to be higher than that of the other tissues of the oviduct. Some workers failed to observe significant correlation between shell quality and CA activity of the shell gland of hen (Heald et al., 1968; Gutowska and Mitchell, 1945). However, Pearson et al. (1977) observed that the uterine CA activity of laying quail was twice that of molting birds and five times that of nonlaying bird. Thus they concluded that this enzyme activity might play a role in the active calcium transport in the avian uterus. However, none of the literature reported so far has clearly shown the mechanism for the shell thining as hens age.

The present study was undertaken to determine whether certain biochemical parameters involved in calcium metabolism are related to shell quality.

Materials and Methods

Two strains laying hens(Babcock B-300 and Hyline W-36), 102 weeks of age, were used. A total of 80 birds were placed in individual cages and were fed experimental diet for 24 days (table 1).

Egg production and the number of soft shelled eggs laid were recorded daily. One egg per hen was collected every two days, and was subjected to measurement of shell thickness by use of dial pipe gauge(FHK, Ozaki CO.) and shell strength as determined by the water loading pressure device (Kang et al., 1996). At the end of the 24-day period, 30 birds were selected, based on the eggshell breaking strength and their laying state, as follows; 10 birds laying eggs with high quality shells, 10 birds laying eggs with poor quality shells, and 10 birds out of production during the 24-day period.

From 1 to 2 p.m. on a given day, the birds were killed by slashing the jugular vein. A blood sample was collected in a centrifuge tube at the time. The duodenum and the shell gland of each bird were removed and kept in an ice bath for later determination of CaBP and CA activity.

Serum Calcium Level

Each blood sample collected was kept at room temperature for about 24 hours and allowed to clot. Serum was separated from the clotted sample by means of centrifugation at 12,000 × g for 10 minutes. The total calcium level in the blood serum was assayed using a Perkin-Elmer 303 atomic absorption spectrophotometer.

TABLE 1. COMPOSITION OF EXPERIMENTAL DIET

Ingredients	16% protein layer diet (%)
Corn (ground yellow)	66.0
Soybean meal, dehulled	20.0
Alfalfa meal (17% protein)	2.0
Dicalcium phosphate	2.0
Limestone	5.0
Yellow grease	4.0
Mineral mix[a]	0.5
Vitamin mix[b]	0.5
Total	100.0
Calculated analysis	
Crude protein	15.9
ME_n (kcal/kg)	3,061.2
Calcium[c]	2.64
Phosphorus	0.65
Methionine + cystine	0.52
Lysine	0.80

[a] Supplied per kg of diet: sodium chloride, 4.8 g; zinc, 18 mg; iron, 10 mg; manganese, 10 mg; magnesium, 7.5 mg; copper, 1.5 mg; cobalt, 0.25 mg and iodine, 0.35 mg.

[b] Supplied per kg of diet: vitamin A, 5280 U.S.P.,; vitamin D_3, 1375 U.S.P.,; vitamin E, 22 I.U.; vitamin B_{12}, 0.0088 mg; niacin, 44 mg; choline chloride, 440 mg; riboflavin, 6.6 mg; d-calcium pantothenate, 8.8 mg; vitamin K, 1.1 mg; folic acid, 1.1 mg and biotin, 0.11 mg.

[c] For maximum utilization of dietary calcium, calcium content is lower than the NRC requirement.

Duodenal CaBP Activity.

Immediately after a bird was sacrificed, the duodenum was removed and rinsed with ice-cold 0.7% NaCl solution, it was excised and blotted dry with disposable wipes. The mucosa was scraped off from the muscle layers and homogenized in tris-buffer, pH 7.4, as described by Wasserman and Tayler (1966). The homogenate was centrifuged at 37,000 × g for 20 minutes. The supernatant liquid was heated at 60℃ for 10 minutes and centrifuged again at 12,000 × g for 10 minutes. The supernatant liquid was used for the determination of CaBP activity by the Ca^{45} and chelex-100 method described by Wasserman and Tayler (1966). The Ca^{45} activity was counted using a radiation counter (RCL Scaler Mark 13 Model-1).

The protein concentration of the sample was measured by the Lowry method (1951).

Uterine CaBP activity

The shell gland was immediately rinsed with ice-cold glass distilled water and kept in an ice bath. After being blotted dry, the shell gland was cut into two portions longitudinally. One of these was cut into small pieces with a scissors and homogenized in tris-buffer, pH 7.4. The homogenate was centrifuged, heated, and assayed for CaBP activity using the same method as that for the duodenum. The protein concentration was measured by the Lowry method (1951).

Uterine Carbonic Anhydrase Activity

The other portion of the shell gland from the CaBP activity assay was minced finely with scissors into a cold weighing dish kept on ice, and homogenized in ice-cold glass distilled water. The homogenate was centrifuged at 1,500 × g at 4℃ for 10 minutes.

The enzyme activity was determined on the supernatant liquid using modifications (Worthington Enzymes Mannal, 1977) of the electrometric method of Wilbur and Anderson (1948), in which the time required for a saturated CO_2 solution to lower the pH of 0.02 M tris-buffer from 8.3 to 6.3 in the presence of the enzyme was measured at 2℃.

As before, the protein content of the supernatant liquid was measured by the Lowry method. (1951)

The data were analyzed statistically by analysis of variance. The group means were compared by using the least significant difference (Steel and Torrie, 1960).

Results

The relationships between breaking strength, shell thickness and level of serum Ca are presented in table 2. Hens laying eggs with an average breaking strength of 3.00 kg or more were taken as high quality shell producers and hens laying eggs with an average below 2.00 kg were taken as low quality shell producers. Hens classified as non-layers had been out of production for a 24-day period.

Serum Calcium Level. As shown in table 2, the serum calcium level of the low quality shell producers (27.8 mg %) was not significantly different from that of the high quality shell producers (26.9 mg%). On the other hand, the non-layer group had a significantly ($p < 0.05$) lower serum calcium value of 17.7 mg% than the two layer groups.

Duodenal and Uterine CaBP Activity. The results of CaBP activity determined in the duodenum and shell gland of hens from the three groups are presented in table 3.

TABLE 2. SERUM CA LEVELS OF HENS SELECTED ON THE BASIS OF SHELL QUALITY

	Non-layer	Low quality shell	High quality shell
Eggs produced/hen during 24-day period	–	10	14
Breaking strength (kg)	–	1.64 ±0.07	3.60 ±0.16
Shell thickness (mm × 10^{-2})	–	27.2 ±0.7	37.1 ±0.7
Ca in blood serum (mg/100 ml)	17.7 ±5.7[a]	27.8 ±8.8[b]	26.9 ±5.5[b]

[ab] Means within a row with different superscripts are significantly different ($p < 0.05$).

TABLE 3. DUODENAL AND SHELL GLAND CABP ACTIVITIES OF HENS SELECTED ON THE BASIS OF SHELL QUALITY

	Non-layer	Low quality shell	High quality shell
Duodenal CaBP activity			
Units/g tissue	3.87 ±0.10[a]	6.83 ± 2.49[b]	13.75 ± 5.79[c]
Units/mg protein (× 10^{-2})	19.2 ±4.8[a]	33.2 ±14.6[b]	67.4 ±26.6[c]
Shell gland CaBP activity			
Units/g tissue	3.10 ±1.00[a]	3.11 ± 1.00[a]	6.54 ± 2.23[c]
Units/mg protein (× 10^{-2})	14.2 ±3.3[a]	20.3 ± 9.8[a]	46.8 ±20.3[c]

[ab,c] Means within a row with different superscripts are significantly different ($p < 0.01$).

There were significant differences ($p < 0.10$) in the CaBP activity among the three groups as concerns the duodenal mucosa in terms of either units per gram of tissue or units per mg of protein (table 3). The duodenal calcium binding activity of hens from the low quality shell group was 6.83 units per gram tissue or 0.332 unit per mg protein which was about one half that for hens from the high quality shell group with 13.75 units per gram of tissue or 0.674 unit per mg of protein. However, it is twice as high as that of hens from the non-layer group with 3.87 units per gram of tissue or 0.192 unit per mg of protein.

Uterine CaBP activity, on either the per gram tissue basis or per mg protein basis, was almost the same in both the non-layer group and the low quality shell group, i.e., 3.10 units per gram of tissue or 0.142 unit per mg of protein vs. 3.11 units per gram of tissue or 0.203 unit per mg of protein. On the other hand, the activity of the high quality group of 6.54 units per gram of tissue or 0.468 unit per mg of protein was twice that of the other groups.

Uterine Carbonic Anhydrase Enzyme Activity. As shown in table 4, CA activity of the shell gland from the high quality shell group was 464.4 units per gram of tissue or 11.15 units per mg of protein. This is about twice that of the shell gland from the low quality shell group of 269.6 units per gram of tissue or 6.85 units per mg of protein. The value for the non-layer group was 142.3 units per gram of tissue or 3.34 units per mg of protein which were about half of that for the low quality shell group.

TABLE 4. SHELL GLAND CARBONIC ANHYDRASE ACTIVITY OF HENS SELECTED ON THE BASIS OF SHELL QUALITY

	Non-layer	Low quality shell	High quality shell
Shell gland carbonic anhydrass activity			
Units/g tissue	142.3 ±68.7[c]	269.6 ±82.2[b]	464.4 ±52.2[a]
Units/mg protein	3.34 ± 1.47[c]	6.85 ± 2.22[b]	11.15 ± 1.11[a]

[a,b,c] Means within a row with different superscripts are significantly different ($p < 0.01$).

Discussion

The nonsignificant difference in serum calcium concentrations between the two different shell quality groups (table 2) is in good accord with the data presented by Hurwitz and Bar (1967). They found plasma calcium levels of 26.1 mg% in a thick shell group and 26.0 mg% in a thin shell group. Paul and Snetsinger (1969) observed a negative correlation for plasma calcium levels and breaking strength for 8-month old layers, but this was not statistically significant. On the other hand, a similar positive correlation was obtained with 20-month old laying hens, but this again was not significant. In the light of the results of this experiment together with the data reviewed, shell quality appears to be related not to serum calcium concentration, but to other factors involved in calcium transport across mucosa of the avian shell gland.

The low serum calcium concentration of the non-layer group shown in table 2 is similar to that reported by Miller et al. (1978) for non-layers which exhibited regressed reproductive organs.

Calcium homeostasis is achieved by balancing the efficiency of intestinal Ca absorption, renal Ca excretion, and bone mineral metabolism to the bird's Ca requirements. The main hormones controlling this balance are PTH, calcitonin, and 1,25-dihydroxycholecalciferol [1, $25(OH)_2D_3$] produced by the renal conversion of 25-hydroxy cholecalciferol [$25(OH)D_3$] through the activity of the enzyme 25-hydroxycholecalciferol-1-hydroxylase (1α-hydroxylase). In laying birds, incresed Ca demands during the laying cycle are accommodated by an appropriate increase in intestinal Ca absorption (Bar et al., 1978) and a decrease in renal Ca excretion (DeLuca et al., 1990). Both renal 1α-hydroxylase activity and plasma 1, $25(OH)_2D_3$ concentrations are significantly higher during the active stage of eggshell calcification than in other stage (Abe et al., 1979). Elaroussi et al. (1994) demonstrated that when birds stopped laying eggs, the dietary Ca needs were less and thus 1α-hydroxylase activity decreased. During reproduction activity in the female chicken, endogenous estrogen mediates changes in the function of the kidney that involve the two major Ca-regulating hormones, PTH and $1,25(OH)_2D_3$ (Elaroussi et al., 1993). The low value of serum calcium concentration is possibly explained by these hormonal function which regulates calcium absorption from the diet and calcium reabsorption blood calcium level.

The significant difference in CaBP activity among the groups (table 3) suggests that it affected egg shell formation and thus shell quality. The occurrence of CaBP on the small intestine is highly correlated with calcium absorption from the diet.

Calbindin-D 28k (calbindin) is the 28,000-Da vitamin D-dependant calcium-binding protein present in relatively large quantities in the avian duodenum (Wasserman and Taylor, 1966) and eggshell gland (Fullmer et al., 1976). Declining eggshell calcification in aging hens may be a manifestation of reduced calbindin concentration in calcium transporting tissues. Conversely, the well known improvements of eggshell quality following induced molting may be due to improved, restored, or increased calbindin expression. Berry and Brake (1991) reported that eggshell quality and calbindin-D28k content in shell glands and duodena of old hens increased following an induced molt. Scheideler et al. (1993) reported that shell gland calcium transport rates were increased after molt.

Strong evidence for the role of intestinal CaBP in calcium absorption was reported by Emtage et al. (1974), who detected CaBP in intestinal supernatants at 12 hours, but not at 8 hours, after administration of vitamin D_3 to vitamin D deficient chicks. An increase in calcium absorption was first observed after 12 hours of vitamin D_3 administration. Furthermore, a relationship of intestinal CaBP with eggshell formation was shown in the studies of Bar and Hurwitz (1972; 1973). CaBP increased at the onset of egg production and decreased when egg production was arrested by nicarbazine administration.

On the other hand, questions about the role of CaBP in calcium absorption have been raised by some workers (Harmeyer and DeLuca, 1969; Spencer et al., 1978) in the light of increased Ca^{2+} transport before CaBP biosynthesis in the intestine of vitamin D deficient chicks and rats. Bar and Hurwitz (1972; 1973; 1975) did not find any significant difference in duodenal CaBP activity between thick shell producers and thin shell producers, and did not find any change in CaBP activity during the shell formation cycle. These reports suggested an existence of other factors in the short-term regulation of calcium absorption. However, the postulation of important role of duodenal CaBP in maintaining good shell quality in eggs produced by aged hens can be supported by the proposal of Spencer et al. (1978) in which a high rate of Ca^{2+} absorption is not maintained without CaBP synthesis even though the stimulation of Ca^{2+} transport is initiated by other 1,25-$(OH)_2D_3$-dependent factors.

The mechanism of calcium transfer across the avian shell gland is not well understood (Simkiss, 1975). The presence of CaBP in the shell gland mucosa of laying hens (Corradino et al., 1968), its increase with the onset of egg production, and its decrease as egg production ceases (Bar and Hurwitz, 1973) suggest an important role for CaBP in calcium transport in the shell gland.

The results of the current study on the uterine CaBP shown in table 3 suggest an involvement of CaBP in shell calcification. Like the duodenal CaBP activity, the uterine CaBP activity of the high quality shell group was much higher than that of the low quality shell group and non-layer group. Slightly higher, but non-significant, mean values of uterine CaBP activity expressed as units per mg protein were obtained from the low quality shell group than from the non-layer group. Nys and De Laage (1984) observed that uterine CaBP was reduced in hens laying shelless egg. Bar et al. (1984) reported that uterine CaBP was reduced in hens laying poorly calcified eggs and that a significant correlation existed between uterine CaBP and shell calcium. This observation implies that birds of the low quality shell group possess a very low ability to transport Ca^{2+} across the shell gland. Further it suggests that the degree of uterine CaBP activity is a better barometer than that of intestinal CaBP activity in determining eggshell quality. According to Bar et al. (1976), laying quail have higher uterine CaBP activity than non-laying quail, and higher uterine CaBP activity was observed during shell formation. Researchers observed a significant correlation between uterine CaBP and shell Ca in hens laying shelless (caused either by a Ca-deficient diet or uterine thread insertion) (Nys and De Laage, 1984; Bar et al., 1984) and hard-shelled eggs (Nys and De Laage, 1984). Rabon et al. (1991) detected two forms of uterine CaBP, both of which were significantly greater in hens laying hard-shelled and shelless eggs than in nonlaying hens. Significantly more of the higher molecular weight CaBP was present 14 h after oviposition in hens laying hard-shelled eggs, but not in hens laying shelless eggs.

However, the essential role of CaBP in calcium translocation in the uterus is still in argument. It has been found that the appearance of CaBP in the shell gland lags about 16 to 18 hours behind the initiation of calcification (Bar and Hurwitz, 1973). A higher uterine CaBP activity during shell calcification was not observed in laying hens (Bar and Hurwitz, 1973; 1975). Further, there is relatively low discrimination between Sr and Ca by the shell gland (Simkiss et al., 1973). These inconsistencies, and those for duodenal CaBP mentioned above, turned our attention to measuring some other biochemical parameters, including uterine CA activity.

Uterine CA activities of the three groups were different significantly (table 4). CA plays an important role in eggshell calcification through the hydration of carbon dioxide produced in uterine tissue to carbonic acid. Pearson et al. (1977) showed that the activity of uterine CA was greater in laying than in nonlaying quails and suggested that its activity might be related to calcium secretion in the uterus. This latter suggestion was supported by Eastin and Spaziani (1978), who worked with White Leghorn hens. Similarly, hens laying shelless eggs have a lower uterine CA activity than hens laying hard-shelled eggs (Nys and De Laage, 1984). However, Nys et al. (1986) found no consistent relationship between eggshell formation and uterine CA activity. Yoselewitz and Balnave (1989) concluded that differences in eggshell quality in single population of hens was not related to variations in uterine CA activity. The above studies indicate that CaBP and CA activity differ when nonlaying hens or hens laying shelless eggs are compared with hens laying hard-shelled eggs.

Pearson and Goldner (1973; 1974) proposed that

calcium transport across the quail uterus was an active process, requiring oxidative aerobic metabolism and depended partly upon the presence of HCO_3^- for maximum transfer. Pearson et al. (1977) suggested an intimate relationship between uterine CA activity and uterine calcium secretion on the ground that *in vitro* studies showed an active calcium transfer across uterine tissue of non-laying quail occurred without performed HCO_3^- in the medium. Furthermore, a high degree of difference in uterine CA activity was observed between laying, molting, and non-laying quails. The results of the present study on uterine CA activity support the work of Pearson et al. (1977) and suggest that the CA activity of the shell gland of laying hens has an important role in determining eggshell quality of aged hens. These results confirm the observation of Gutowska and Mitchell (1945) in which good layers, producing strong shelled eggs, showed significantly higher CA activity than non-layers and hens laying poor shelled eggs.

However, these data are not in agreement with that reported by Mueller (1962) and Heald et al. (1968) who did not obtain a significant correlation between the enzyme activity and eggshell quality. This inconsistency implies that other factors play a role along with the enzyme in shell quality. Different techniques in measuring shell strength and/or enzyme activity might, however, be involved. Further, recently a Mg^{2+}-dependent bicarbonate stimulated ATPase has been identified in the uterine mucosa of the domestic hen by Schwartz (1974). He postulated a functional relationship between the ATPase and HCO_3-translocation across the shell gland. Pike and Alvarado (1975) found Ca^{2+}-Mg^{2+} activated ATPase in the microsomal fraction of quail uterus. The activity was higher in the shell gland of calcifying adults than in the shell gland of immature or precalcifying adults. Possibilities for the involvement of these ATPase in shell calcification are conceivable.

The results of this study, nevertheless, suggest that the poor eggshell quality is in large part the result of decreased CaBP and CA activity in the shell gland. In addition, the decreased CaBP activity in the absorption from the diet is an additional contributory factor.

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