1978

Deterioration of Eggshell Quality and Calcium Metabolism in Laying Hens

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DETERIORATION OF EGGSHELL QUALITY AND CALCIUM METABOLISM IN LAYING HENS

BY

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A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science, Major in
Animal Science, South Dakota
State University
1978

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This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ACKNOWLEDGEMENTS

I wish to express my deep and heart-felt appreciation and gratitude to my two advisers, Dr. C. W. Carlson in Department of Animal Science and Dr. O. E. Olson in Experiment Station Biochemistry, for their unfailing sympathy, kindness, patience, and encouragement during the course of this study and the preparation of this manuscript. Without their able guidance and expert assistance, this work could not have been completed. I am also grateful to Mrs. Elaine M. Olson for her encouragement and moral support.

I wish to extend my appreciation to Dr. W. L. Tucker for his assistance in analyzing statistical data.

I also thank Mr. R. A. Nelson, Superintendent of the Poultry Research Center, and to other field workers for their help in the research work.

I extend my sincere thanks to my friends, Lawrence Novotny and Jong Ha, for their help in preparing the manuscript and moral support.

I also especially want to thank Becky Kramer who drew the beautiful figures in this thesis, Mrs. Elsa Wood for her sense of humor while typing this thesis, and to all the other members of Experiment Station Biochemistry for providing an enjoyable and workable environment.

C. W. Kang

CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The functions of an eggshell are various and efficient. It protects the egg from physical damage, prevents the loss of water, prevents the invasion of microorganisms and provides calcium to the embryo for skeletal formation.

In past decades, development of scientific knowledge of breeding, nutrition and environmentally controlled management has resulted in markedly increased egg production per hen. However, the development of the eggshell quality has not kept pace with the development of egg production, and economic losses from egg breakage present a serious problem to the poultry industry throughout the world.

One recent field study conducted in eight northeastern states showed that shell damage from the hen to the carton reached 12.15% of the eggs produced, and that 50% of the farms in the states suffered more than 10% of shell breakage (Anonymous, 1975).

Many factors are responsible for the shell damage, including malnutrition, high summer temperatures, rough handling, age of hens, as well as others. It is well accepted that the decline of shell quality as the hen ages is normal and significant. Furthermore, its prevention is considered very difficult. Hens fed adequate diets usually produce eggs with shells of good quality during the first 9 or 10 months of lay (Scott, 1977). Then the shell quality declines to the point where inferior eggshells present a major problem to producers.

Much research has been conducted on the prevention of shell thinning due to age during the past several decades. Some workers have considered juvenation of old hens and regeneration of their reproductive organs by forced molting. Forced molting has improved shell quality significantly after the molt period. However, the improvement has not been consistent and has usually disappeared rapidly (Wolford and Tanaka, 1970; Sunde, 1971).

A large amount of research work has been concerned with the role of calcium in shell quality (Roland, 1976). Unfortunately, this work along with research on the role of phosphorus and vitamin D_3 frequently has failed to produce satisfactory results. None of the literature reported so far has clearly shown the mechanism for the shell thinning as hens age.

The present study was undertaken to: (1) develop a reliable method of measuring eggshell quality, (2) determine whether diet alterations that cause a somewhat reduced rate of egg production could influence shell quality, and (3) determine whether certain biochemical parameters involved in calcium metabolism are related to shell quality.

REVIEW OF LITERATURE

Structure and Quality of Eggshell

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).

The ultra structure of the hen eggshell has been examined using electron microscopy (Simons, 1971; King and Robinson, 1972; Meyer et al., 1973). These investigations of the shell structure have shown three different structural layers; the mammillary layer, the palisade layer and the cuticle.

The mammillary layer, about 90 µm thick (Meyer et al., 1973), makes up the base of the calcite. It is attached to the outer membrane by polygonal shaped structures known as mammillae (Simons, 1971). Cores of the mammillae consist of a protein-mucopolysaccharide complex rich in acid groups. Anchoring fibers run from the outer membrane into the mammillary core (Taylor, 1970).

The major portion of the shell, accounting for two thirds of its thickness (Stewart, 1935), is made up of the palisade layer, which extends from the mammillary layer to the outermost layer known as the cuticle. The palisade layer is composed of columns of tightly packed almost pure calcite.

The cuticle covers the surface of the whole shell and is approximately 90% protein (Baker and Balch, 1962).

Recently two groups of workers (King and Robinson, 1972; Bunk and Balloun, 1978) conducted ultrastructural comparisons between strong and weak eggshells, and they found a number of abnormal structural alterations in poor quality eggshells. However, Meyer et al. (1973) observed little or no difference in the mammillary region in eggs with different shell qualities, and thus concluded that different shell breaking strengths are mainly due to thickness of the palisade layers.

Measurement of Eggshell Quality

Carter (1970) defined the strength of the eggshell as its ability to absorb strain energy. This strength derives from two sources, namely: the nature of the material from which it is made, and its shape (including thickness). Thus he distinguished in principle between material strength and structural strength. However, in practice the two terms frequently are combined into the term shell strength.

Recently various techniques or methods have been used to evaluate the eggshell quality, resulting in a number of different units of measurement. Shell thickness, specific gravity, static loading, and dynamic loading are commonly used. Hammerle (1969) characterized these various techniques into two categories, those that measure physical properties and those that measure mechanical properties. Shell thickness and egg specific gravities were classified as the most measured physical properties. He divided mechanical properties into two categories; static (quasi-static) loading which is the response (distortion or cracking) to loads applied very slowly,

and dynamic loading which measures response (cracking) to loads applied very rapidly.

The relationship of shell thickness to shell strength was well reviewed by Tyler (1961). From the majority of cases reviewed, he found a significant correlation between shell strength and thickness. Stewart (1936) reported that taking four thickness readings around the equatorial region gave a better correlation with breaking strength than an individual reading. Some workers (Almquist and Burmester, 1934; Lund et al., 1938) measured thickness after removing membranes, but others measured the shell plus membrane thickness. Godfry (1949) and Vandepopuliere et al. (1974) concluded that the membranes contribute to the shell strength. This was questioned by Frank et al. (1965) in the light of the very low simple correlation coefficient and the standard partial regression coefficient between percentage egg as membrane and breaking strength.

Some have considered shell thickness a poor evaluator of shell quality (Hammerle, 1969; Meyer et al., 1973). Frank et al. (1964) studied the relationships between some physical characteristics of eggshells and shell breaking strength measured using either a static loading or dynamic loading device. They found good correlation between the physical variables and shell strength, although the physical variables (shell thickness, specific gravity, percent shell, shell weight, and egg weight) could explain only 60% of the total variation in breaking strength.

According to Tyler (1961) in his literature review of shell

strength measuring methods, static or quasi-static loading has been done with various devices, including a flat surface, needle, cylinder, etc., and has been expressed as either the force at failure or the deformation at failure. Hammerle (1969) pointed out that variation of shell curvature, the shell thickness at the contact points, and the surface hardness of relating material influenced the results.

The induced deformation under nondestructive forces has been used to predict the force at failure. Brooks and Hale (1955) reported that a high negative correlation ($r = -0.71$) existed between deformation under force applied parallel to the major axis and force at failure. Voisey and Hunt (1967) observed that the error in predicting fracture force from a nondestructive compression test was at a minimum when the force was applied at the egg equator. Richards and Staley (1967) reported that deformation per unit load was more highly correlated with breaking strength than any other physical variables (shell thickness, shell weight, percent shell, and shape index).

Mueller (1957) found no significant correlation between shell thickness and the breaking force measured with a dropping device. On the other hand, Wells (1967) reported that the dropping ball technique was a reliable method for indicating the probability of egg breakage in batteries. Tyler and Geake (1963) compared various dynamic loading methods (impact methods) using a light plummet and ball bearings. All the methods gave highly significant correlation coefficients between thickness and strength of eggshell. They observed also that for a given shell thickness, the waist of an egg had the most

strength compared to the broad and narrow poles, and the narrow pole was the weakest when using the impact methods, while static load methods gave the opposite results.

Meyer et al. (1973) suggested that breaking strength was a better measure than shell thickness because breaking strength includes both the shell thickness and the density of the palisade layer. Potts and Washburn (1974) observed that brown egg strains had poorer values for shell thickness, specific gravity, and shell deformation than white egg strains but higher values for breaking strengths; evidence that poorer physical properties do not necessarily indicate poorer shell strength.

Mechanism of Eggshell Calcification

Three parts of the hen's oviduct are involved in eggshell formation, namely: the isthmus, the tubular shell gland, and the shell gland. Membrane formation takes place in the isthmus (Simkiss and Taylor, 1971). Mammillary cores are formed in either the isthmus or the tubular shell gland (Wyburn et al., 1973; Stemberger et al., 1977), there being controversy as to the exact region where the initiation of calcification takes place. Some recent evidence indicates that the isthmus initiates shell formation (Robinson and King, 1963; Creger et al., 1976; Stemberger et al., 1977). It is well established that the main process of shell calcification occurs in the shell gland.

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). The absorption is considered to be promoted by calcium-binding protein (CaBP) which requires vitamin D_3 for its formation (Wasserman and Taylor, 1966).

There have been controversies about the sources of the carbonate ion. Recent literature has supported the hypothesis that a major source of CO_3^{2-} for eggshell formation is carbon dioxide (CO_2) derived from blood or from metabolism of the cells in the shell gland (Simkiss and Taylor, 1971). The formation of CO_3^{2-} from CO_2 appears to be intimately related to the role of the enzyme carbonic anhydrase, which is present in high concentrations in the shell gland mucosa (Bernstein et al., 1968).

The mechanism of calcium transfer and deposition by the shell gland is not well understood. 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 cell was formation of HCO_3^- from CO_2 and water in the presence of carbonic anhydrase, according to the model. They postulated that the transfer of Ca^{2+} from blood into the lumen of the shell gland is brought about by the columnar cells, and that translocation of the ions may require CaBP. The calcification of eggshell occurs in the lumen of the shell gland from the precipitation of Ca^{2+} by HCO_3^- as $CaCO_3$.

Factors Affecting Eggshell Quality

Wolford and Tanaka (1970) and Speers (1974) reviewed the factors influencing eggshell quality. Nutrition, disease, management, age,

temperature and strain were considered as main factors affecting shell quality. Roland (1976) reviewed recent literature on eggshell quality and reached the conclusion that the blame for decline in shell quality was an increase in egg size with the hen's age.

For this study, literature reporting factors relating to dietary protein levels, feed restriction, strain, age of hen and size of egg are reviewed.

Dietary Protein Levels. A number of reports show that, generally, as the level of protein in a layer diet increases, egg production and egg weight increase, but shell quality is not influenced by protein levels. Increasing dietary protein levels from 13 to 17% (Walsh et al., 1963; Moreng et al., 1964), 14 to 16% (Summers et al., 1966), and 15 to 19% (Combs and Helbacka, 1960) did not affect shell quality. On the other hand, Gleaves et al. (1977) reported that hens fed 13 g protein per 120 g of diet produced much weaker shells than those fed 16 and 19 g.

Other workers (Mueller, 1967; Harbaugh and Sanford, 1969; Middendorf et al., 1959) showed that methionine or lysine supplementation did not influence shell quality.

Different protein sources, such as cottonseed meal (Heywang and Vavich, 1965), fish products (Pepper et al., 1968), or hatchery by-products (Wisman and Beane, 1965) did not influence shell quality.

Feed Restriction. Heywang (1940) restricted feed intake of White Leghorn pullets to 75% and 87.5% of the control group fed on an ad libitum basis and reduced egg production, but the restriction did

not influence egg size or body weight. On the other hand, Dronawat and McGinnis (1966) reduced egg size by restricting feed intake of White Leghorn layers to 90% of ad libitum fed controls. Similar results have been reported by other workers (Polin and Wolford, 1971; Bell and Moreng, 1972).

Andrews (1973) conducted a limited-time feeding program as a means of feed restriction and obtained improved shell quality, thus reducing egg breakage. However, limiting feed intake of commercial layers to 100 or 105 g per day did not influence egg size, shell thickness and shell weight according to Kari et al. (1977).

Strain. Taylor and Lerner (1939) obtained evidence that the dam had a relatively more influencing role in determining the character of the shell produced by the daughters than the sire had. They established two lines of chickens, thick shell line and thin shell line, by a selection-breeding method.

Potts and Washburn (1974) evaluated shell characteristics of eggs from five commercial white and brown egg strains using various shell measurement techniques. They stated that white egg strains had higher shell thickness and specific gravity values than brown egg strains. However, breaking strength values were lower for white egg strains. Hyline strains had the highest values in shell thickness and specific gravity, but not in breaking strength. The same workers in 1977 determined the effect of supplemental calcium and vitamin D_3 on line and strain differences in eggshell strength. They reported neither additional dietary calcium nor injection of vitamin D_3 altered

The strain and line effects.

Tijen (1977a, 1977b) reported a series of work on improvement of shell quality by means of breeding, but egg production decreased slightly.

Age and Egg Size. A decline of shell quality with age has been reported by a large number of workers.

Hurwitz and Bar (1969a) studied the effect of age on the calcium reserve in the bones of laying hens. Their observations showed that calcium content of the femur reached its maximum peak on the third month of lay and after that calcium content decreased slightly. According to the report, age was not likely to influence the hen's ability to utilize the skeletal reserves of ends and medullary segments of femur. However, skeletal mobilization of the cortical segment decreased with age.

Charles and Ernst (1974) reported breaking strength and specific gravity of eggs were increased by the addition of 25-OH-D_3 to calcium adequate diets of hens at the age of 144 weeks.

Roland et al. (1975b) suggested that increased egg size with age was the primary reason for shell thinning. They observed that egg weight increased as the hen aged, but total shell weight remains constant throughout the egg production period. Roland (1976) later postulated in the light of this phenomenon along with some other reports, that the total amount of calcium deposition in the eggshell was limited. The hen's ability for calcium absorption and bone mobilization may not increase with age (Roland, 1976).

Roland et al. (1975a) demonstrated that translocation of about 18-month old hens from individual cages to floor pens resulted in increased egg production, serum calcium content, bone strength, and improved shell quality, compared to those retained in cages. Translocated hens layed smaller eggs than cage-housed hens did. They hypothesized that cage-housed hens had suffered stress for a long time, both physiologically and psychologically. Translocation to floor pens brought them psychological and physiological stimulation and contentment.

Hurnik et al. (1977) found that the percent of magnesium in the eggshell started to drop markedly at the age of 52 weeks. This is the time when a shell thinning problem usually occurs.

Biochemical Parameters

Serum Calcium Level. 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 main outlets of the pool are shell deposition and bone formation.

Mueller et al. (1964) reported that 311-day old Leghorn pullets absorbed 78% of the calcium in the diet consumed. Of this they retained 70%, the other 8% was eventually excreted as endogenous calcium.

Calcium entering the blood stream is carried in various forms. Much of it is protein-bound, while other portions are associated with citrate, bicarbonate and phosphate ions. Only a fraction of the total is in ionized form. Plasma calcium is separated into two parts for analytical convenience, a diffusible fraction which contains ionic

calcium and a nondiffusible fraction which contains the protein-bound fraction. These two forms are in equilibrum with each other. The shell gland takes up ionic calcium which is then replaced by the ionization of some of the protein-bound element (Simkiss and Taylor, 1971). The level of total and ionized blood calcium is influenced markedly by parathyroid hormone and vitamin D (Scott et al., 1976).

Bell and Sturkie reviewed the literature on total plasma calcium level in chickens (1965). They concluded that the actively ovulating female bird had a total calcium level 2 or 3 times that observed in the male and the sexually immature female. In the light of other reports, the elevation of the calcium level during the laying state was considered to be controlled by endogenous estrogens.

Hurwitz and Bar (1967) found no significant differences in the calcium content of the bone or of the plasma between thick shell producers and thin shell producers. On the other hand, data presented by Paul and Snetsinger (1969) showed significant, negative correlation between plasma calcium level and shell thickness and negative correlation (but not significant) between plasma calcium level and breaking strength. They observed also a cyclic change in the plasma calcium level during the active shell calcification process. The plasma calcium content peaked at 1 hour post oviposition, falling slowly toward the later part of shell calcification to its lowest point at 23 hours after oviposition. When related to time of day, the highest serum calcium content was at noon and the lowest was at 8 a.m. and 4 p.m. according to Roland et al. (1972). When the serum calcium peaks were at their

highest, the calcium intake peak and the fecal calcium peak were at their lowest, and vice versa.

Miller et al. (1978) reported that serum calcium levels did not vary in relation to whether or not a bird laid an egg the day before or on the day of blood collection. On the other hand, reproductively inactive hens had lower serum values than active ones.

CaBP in the Duodenum. Sine vitamin D_3-induced CaBP in the intestinal mucosa of chicks was identified by Wasserman and Taylor in 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).

Hurwitz and Bar (1969b) reported that dietary calcium did not significantly influence the CaBP level in laying hens, whereas Bar and Hurwitz (1972) demonstrated that duodenal CaBP increased at the onset of egg production and the elevated high level remained fairly constant as long as egg production continued. The same authors (1973a) reported that calcium restriction (1.7% dietary calcium) for 28 days resulted in a significant increase in intestinal CaBP activity. They determined intestinal CaBP activity of two shell quality groups, thick shell group and thin shell group. The thick shell group had slightly but not significantly higher intestinal CaBP activity. In their later work (Bar and Hurwitz, 1975) no change of calcium binding activity in the intestine was observed during the egg formation cycle, even at the end of the clutch.

It was recently reported that CaBP synthesis in the intestine of

vitamin D-deficient chicks was initiated in response to vitamin D_3 (Emtage et al., 1974) or 1,25-$(OH)_2D_3$ (Spencer et al., 1976). On the other hand, according to Harmeyer and DeLuca (1969) the CaBP formation as a function of vitamin D action was not directly related to calcium transport capacity of the intestinal cells in chicks and rats. Spencer et al. (1978) found that initiation of CaBP biosynthesis lagged about 2.5 hours behind an increase in Ca^{2+} transport across the intestine of vitamin D_3-deficient chicks after an injection of 125 ng of 1,25-$(OH)_2D_3$, and they suggested the presence of some other factor to initiate CaBP synthesis. Their reinvestigation into the localization of the protein within the intestine epithelial cells led them to conclude that CaBP was synthesized on free polyribosomes, and thus it was intracellular protein. They postulated that the protein may play a role as a buffer for intracellular calcium and may be required for maintenance of continued Ca^{2+} transport across the mucosal cell.

CaBP in the Shell Gland. The presence of CaBP in the shell gland of hens was observed by Corradino et al. in 1968. According to Bar and Hurwitz (1973b), shell gland CaBP increased within 24 hours of the onset of egg production, and decreased as egg production ceased. Their data also showed no difference in uterine CaBP between two groups of hens, one producing thick-shelled eggs, the other thin-shelled eggs. No changes in the uterine CaBP were observed during the shell formation cycle of layers. Bar et al. (1976) observed higher uterine CaBP levels in laying quail than in non-laying quail and higher CaBP levels during eggshell formation in the shell gland.

Carbonic Anhydrase in the Shell Gland. In 1933, Meldrum and Roughton reported that carbonic anhydrase reversibly catalyzes the hydration and dehydration of carbonic acid. The carbonic anhydrase activity of the shell gland epithelium was reported by Common (1941) to be higher than that of the other tissues of the oviduct. In the light of the finding he postulated that the enzyme might play an important role in eggshell formation.

Heald et al. (1968) failed to observe significant correlation between shell quality and carbonic anhydrase activity of the shell gland of hens.

Gutowska and Mitchell (1945) determined carbonic anhydrase activity in the blood, shell gland and ovary of two different shell quality groups of hens (strong-shell and poor-shell) and of non-laying hens. There were not any differences in the blood carbonic anhydrase activity, whereas the reproductive systems, especially the shell gland, showed definite differences. The good layers had a uterine carbonic anhydrase activity over three times that of the poor layers and that of the non-layers. Reduced shell quality with subcutaneous injections of 0.16 g sulfanilamide per kg body weight of hen again suggested an inhibitory effect of sulfanilamide on carbonic anhydrase activity in shell calcification.

Mueller (1962) found that some diuretic agents which were not strong carbonic anhydrase inhibitors, interfered with shell deposition. He hypothesized that a breakdown of shell formation by carbonic anhydrase inhibitors might result from their diuretic effects rather than

from reduced carbonate ion supply. In addition, he did not obtain a significant correlation between shell thickness and uterine carbonic anhydrase activity. A few years later, Opel (1965) reported that chronic diuresis did not reduce shell quality, and thus questioned the diuretic effect of carbonic anhydrase inhibitors.

Bernstein et al. (1968) suggested an intracellular role for the enzyme in the light of low levels of carbonic anhydrase activity in the mitochondrial and the microsomal fractions. They proposed that the enzyme functions as an intracellular mediator to promote the production of HCO_3^- from the metabolic CO_2 produced in the cells of the epithelium.

Pearson and coworkers reinvestigated the mechanism of calcium transfer across the avian uterus. In their in vitro study on the relationship between the calcium transport and the electrical properties of quail uterus, Pearson and Goldner (1973) observed that calcium movement across the quail shell gland could occur against an electrochemical gradient, and that net calcium transport across the uterine mucosa occurred in the absence of bicarbonate ion in the medium, which, however, was required for maximal calcium transport. The same authors (1974) confirmed the concept of active calcium transport in the avian shell gland. Furthermore, they suggested that net calcium transfer required oxidative aerobic metabolism as an energy source for the maximal calcium transfer in the shell gland, and that the role of bicarbonate ion in calcium transportation across the avian shell gland might be related to shell gland carbonic anhydrase activity. In their later works, Pearson et al. (1977) observed that

the uterine carbonic anhydrase activity of laying quail was twice that of molting birds and five times that of nonlaying birds. Thus they concluded that this enzyme activity might play a role in the active calcium transport in the avian uterus.

MATERIALS AND METHODS

Trial 1

A dropping ball apparatus had been used to evaluate shell strength in our laboratory. However, difficulties and variations in measuring shell strength by means of the impact methods (Tyler, 1961; Tyler and Geake, 1963) made it desirable to develop another method. A device using water as the breaking force was therefore developed.

This experiment was conducted to compare the two methods, the dropping ball and the water loaded pressure method. Furthermore, the relationships between egg size, shell thickness and shell strength as measured by these two methods were studied.

Over 200 eggs were collected from commercial type flocks fed practical diets. The eggshells were observed visually and the cracked eggs were discarded. Of these, 100 eggs were used for shell strength measurement by means of the water loaded pressure method and an equal number for shell strength measurement by means of the dropping ball method.

Data from both methods were analyzed by means of correlation coefficients and multiple regression analyses (Steel and Torrie, 1960).

Water Loaded Pressure Method. After being weighed individually, the 100 eggs were subjected to shell strength measurements using the water loaded pressure device shown in Fig. 1.

The apparatus consisted of a rigid frame supporting a round steel bar 12.7 mm in diameter. A light plastic water container (D) weighing

73.5 grams hung from the end of the bar (C). The water container was connected to a reservoir through very soft, thin rubber tubing (E). The equipment was built in such a way that the equator of the egg was pressed against the breaking bar (B) when the egg platform (I) beneath the egg was adjusted (J) to where the water flow stop (G) just touched the top of the rubber tubing (E). Thus the water flow was not interrupted as long as the egg withstood the pressure. The pressure on the egg was gradually increased by water flowing into the container. Upon shell failure, the water flow stop pinched the tubing and automatically stopped the water flow.

The water was measured with a graduated cylinder and assumed to have a specific gravity of 1.000. The weight of the bar at C with the empty bottle (D) in place was determined at the outset of the work and considered to be constant. Shell breaking strength was determined as follows:

$$\text{Breaking strength in Kg} = \frac{\text{ml water + Weight at C (g)}}{1000} \times \frac{D_1}{D_2}$$

where D_1 is distance from A to C, D_2 is distance from A to B.

From the equator of the cracked eggs, a piece of shell without the membrane was removed. The shell thickness was determined using a micrometer screw gauge with double convex jaws, Starrett Dial Indicator No-1010M.

Dropping Ball Method. A dropping ball device was also used to measure the shell strength. The equipment was a modified version of that described by Mueller (1959).

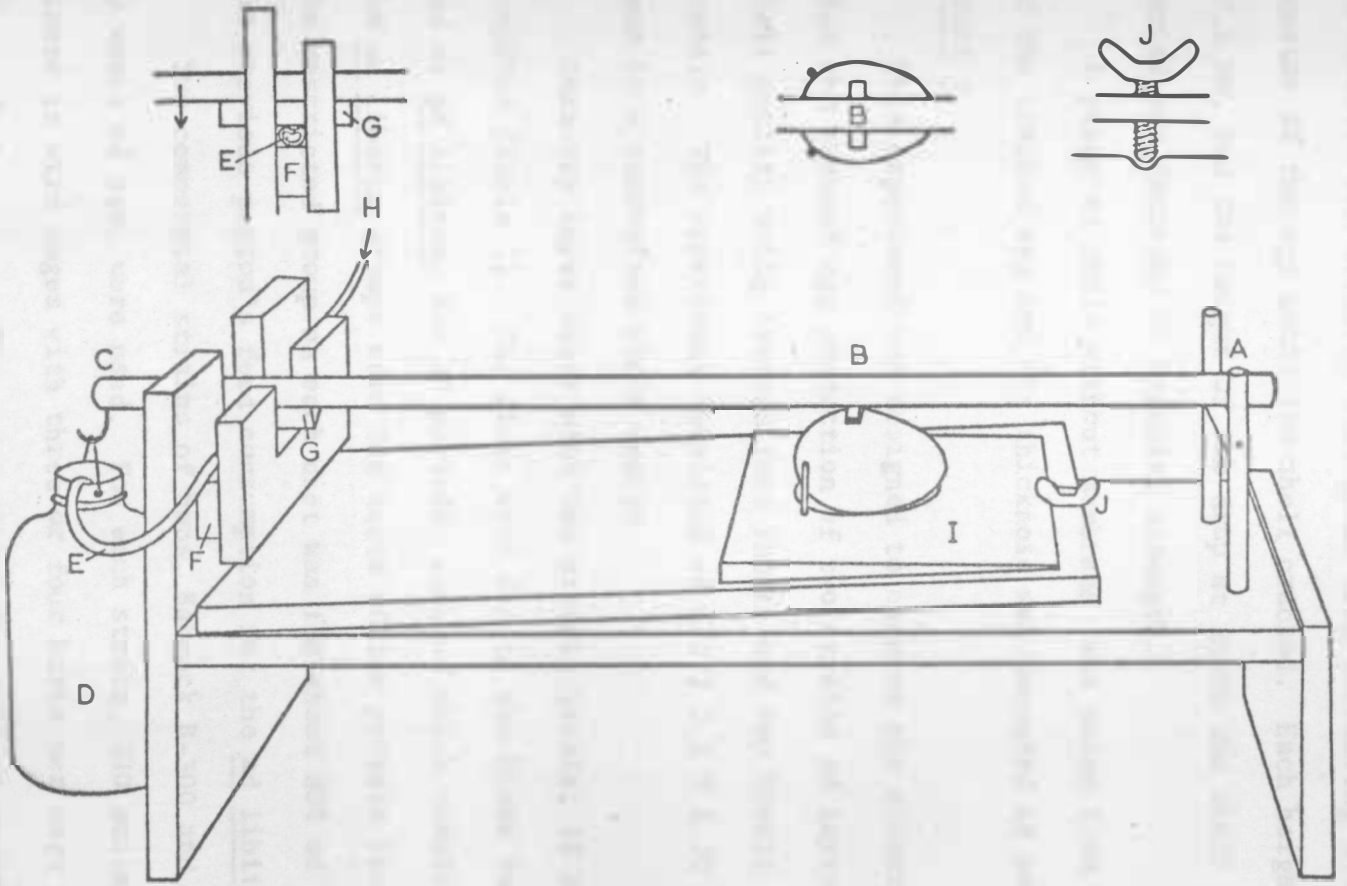

Fig. 1. Shell breaking strength measuring device (water loaded pressure method). A, fulcrum; B, breaking bar; C, hook for water bottle; D, water bottle; E, rubber tubing; F, rubber tubing rest; G, water flow stop; H, water reservoir; I, egg platform; J, egg platform adjusting screw.

It was designed to allow for dropping a steel ball bearing 9.52 mm in diameter and weighing 3.52 grams from increasing heights on the equator of the egg until the shell cracked. Each height increase was 12.5 mm, and the height of the drop at which the shell cracked or was dented was recorded as breaking strength.

A piece of shell without membrane was taken from the equator area of the cracked egg and its thickness was measured as before.

Trial 2

This experiment was designed to examine the effect of reduced egg size and reduced egg production of two strains of laying hens on egg-shell quality using limited feed intake and two levels of dietary protein. The experiment consisted of a 2 X 2 X 2 X 20 factorial arrangement in a randomized block design.

Corn-soy layer diets with two protein levels, 12 and 16%,were prepared (Table 1). The diets were fed to the birds two ways, restricted or ad libitum, for 20 periods, each of which consisted of 4 weeks. The ad libitum groups were fed diets of two protein levels (12 or 16%). The restricted group on each diet was fed about 85% of the amount of the previous period's feed consumption for the ad libitum group.

Two commercial strains of hens, Babcock B-300 and Hyline W-36, 20 weeks of age, were used. For each strain, 240 pullets were randomly placed in wire cages with three or four birds per cage depending upon sizes of the cages. The cages were located in an environmentally controlled layer house. Five replicates of twelve birds from each strain were assigned to one of four dietary treatments: 16% protein-

Table 1. Composition of diets used in Trial 2 and 3.

Ingredients	16% protein layer diet (%)	12% protein layer diet (%)
Corn (ground yellow)	66.0	80.6
Soybean meal, dehulled	20.0	9.0
Alfalfa meal (17% protein)	2.0	2.0
Dicalcium phosphate	2.0	2.0
Limestone	5.0	5.1
Yellow grease	4.0	-
Salt[a]	0.5	0.5
Vitamin mix[b]	0.5	0.5
Methionine	-	0.1
Lysine	-	0.2
Total	100.0	100.0
Calculated analysis		
Crude protein	15.9	12.1
ME_n (kcal/kg)	3061.2	3011.6
Calcium[c]	2.64	2.65
Phosphorus	0.65	0.63
Methionine + cystine	0.52	0.52
Lysine	0.80	0.70

[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 pantothenic acid, 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 (3.25% of diet).

ad libitum (N-16%), 16% protein-restricted feeding (R-16%), 12% protein ad libitum (N-12%), and 12% protein-restricted feeding (R-12%).

Egg production was recorded daily. All eggs from each replication laid during the first 5 days of each period were collected and weighed to determine the changes in egg size as hens aged. Five eggs were selected randomly from those used for egg size measurement, and were subjected to albumen height measurement. Albumen quality was determined by means of the Haugh unit formula.

Shell quality was determined by using the two methods described in Trial 1. Twelve eggs per replicate from each period throughout the experiment were subjected to shell strength measurement using the water loaded pressure device. For the first 16 periods, 25 eggs were used for shell quality evaluation using the ball dropper and thereafter 20 eggs were used.

Data were analyzed statistically by analysis of variance and the Duncan's new multiple-range tests (Steel and Torrie, 1960).

Trial 3

Two laying hens were selected randomly from each replicate in Trial 2 at the age of 102 weeks. A total of 80 birds out of the 40 replicates were placed in individual cages and were fed for 24 days the same 16% protein diet that had been fed in Trial 2.

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 measurements of shell thickness and shell strength as determined by the water loading pressure device. At the end of the

24-day period, 30 birds were selected, based on the eggshell breaking stength 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 the 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 carbonic anhydrase 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 X g for 10 minutes. The total calcium level in the blood serum was assayed using a Perkin-Elmer 303 atomic absorption spectrophotometer.

Duodenal CaBP Activity. Immediately after a bird was sacrificed, the duodenum was removed and rinsed with ice-cold 0.7% NaCl solution. The duodenum was excised and blotted dry with disposable wipes. The mucosa was scraped off from the muscle layers and homogenized with a Potter-Elvehjem homogenizer in tris-buffer, pH 7.4, as described by Wasserman and Taylor (1966). The homogenate was centrifuged at 37,000 X g for 20 minutes. The supernatant liquid was heated at 60° C for 10 minutes and centrifuged again at 12,000 X 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 Taylor

(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 (Lowry et al., 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 (Lowry et al., 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 X g at 4^{o} C for 10 minutes.

The enzyme activity was determined on the supernatant liquid using modifications (Worthington Enzymes Manual, 1977) of the electrometric method of Wilbur and Anderson (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^{o} C.

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

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 AND DISCUSSION

Trial 1

The relationships of shell thickness and egg size to breaking strength as measured with the water loaded pressure device are presented in Table 2. The simple correlation coefficients between shell thickness and breaking strength, egg weight and breaking strength, and shell thickness and egg weight were +0.786, +0.034 and +0.197, respectively. The shell thickness was highly correlated with the eggshell strength ($P<0.001$), but the egg size appears not to be correlated. These results are similar to those reported by some other workers (Frank et al., 1964; Richards and Swanson, 1965; Gaisford, 1965) regardless of the presence of membrane on the eggshell when measuring shell thickness. The correlation coefficient of +0.197 for egg weight and shell thickness is not significant. This value is higher than that of +0.05 to +0.07 reported by Richards and Staley (1967), but somewhat lower than +0.26 to +0.34 reported by Frank et al. (1964).

A measure of the strength of the relationship between variables given by $100 \cdot r^2$ indicates that about 62% of the total variation in breaking strength was accounted for by the shell thickness. Only a negligible portion of variability was explained by the egg weight. Egg size accounted for only 4% of the variation in shell thickness.

The multiple correlation coefficient of +0.797 indicates that about 64% of the total variation in breaking strength was explained by the two variables, shell thickness and egg weight. The two

Table 2. Relationship between shell thickness, egg weight and breaking strength obtained with the water loaded pressure method on 100 eggs.

Parameters		Shell thickness (X_1)	Egg weight (X_2)	Breaking strength (Y)
Means ± SD		33.4 ± 3.0 (10^{-2}mm)	65.2 ± 4.6 (g)	3.12 ± 0.62 (Kg)
Simple correlation coefficients (r)	X_1	1.000		
	X_2	0.197	1.000	
	Y	0.786***	0.034	1.000
$100 \cdot r^2$	X_1	--		
	X_2	3.9	--	
	Y	61.8	0.1	--
Standard partial regression coefficients		$b_1' = +0.815$	$b_2' = -0.126$	
Multiple regression equation		$\hat{Y} = -1.413 + 0.169X_1 - 0.017X_2$		
Multiple correlation coefficient (R)		+0.797		
Coefficient of determination ($100 \cdot R^2$)		63.6		

*** Significantly different ($p<0.001$)

standard partial regression coefficients of +0.81 for shell thickness and -0.13 for egg weight reveal the importance of shell thickness in predicting shell strength.

A multiple regression equation was calculated from the data and presented in Table 2. The equation includes the partial regression of shell strength on shell thickness (b_{y1} = +0.169) and on egg weight (b_{y2} = -0.017).

A significant correlation coefficient of +0.577 between shell thickness and the height of ball drop was obtained with the dropping ball method (Table 3). This is in good agreement with the value of +0.51 reported by Tyler and Geake (1963). Frank et al. (1964) obtained a high value of +0.73 in the first trial of a dropping ball device similar to that used in this study, but obtained a low value of +0.35 in a second trial with the same technique. They considered this variation a large experimental error in measuring shell strength.

The analysis of data disclosed that thickness accounted for only about 33% of the total variation in resistance of the eggshell to cracking. This value is much lower than that of 62% obtained with the water loaded pressure method.

The standard partial regression coefficients for egg weight (-0.126) in Table 2 from the water loaded pressure method indicates the lesser importance of its role in predicting shell strength. On the other hand, negative signs of standard partial regression coefficient and partial regression of egg weight in the multiple regression equation of Table 2 imply that for a given shell thickness a larger

Table 3. Relationship between shell thickness and cracking strength obtained with the dropping ball method on 100 eggs.

Parameters	Shell thickness (X)	Height of ball drop (Y)
Means ± SD	34.9 ± 3.0 (10^{-2}mm)	11.9 ± 2.0 (cm)
Correlation coefficient (r)	+0.577***	
Linear regression equation	$\hat{Y}$ = -1.23 + 0.38X	
$100 \cdot r^2$	33.3	

*** Significantly different ($p<0.001$)

egg has a lower shell strength than a smaller egg. Carter (1970) stated that the structural strength of an eggshell is derived from two variables, the shell thickness and shape, and thus the structural strength of an eggshell is a function of the average curvature and thickness of the eggshell at the loading point. For instance, an increase in radius of shell curvature at the loading point results in reduction of shell strength. According to him, this was considered a partial reason for egg breakage increases with the age of hens. This was also in part an explanation for the narrow pole of the egg having greater breaking strength than the broad pole, as had been reported by Tyler and Geake (1963).

The percentage of the total variation in breaking strength accounted for by the combined effect of shell thickness and egg weight was 63.6%. This result indicates that about 40% of the total variation remains to be explained by factors which are not identified in this experiment. It may be that egg shape and shell membrane thickness might account for a portion of the 40% of the total variation. Richards and Swanson (1965) found that the egg shape index explained 15 to 35% of the variation in crushing strength remaining after shell thickness had been considered. Carter (1970) also considered egg shape as an important factor in determining eggshell strength. Even though Frank et al. (1965) did not find any correlation of membranes to shell failure, a contribution of the membrane to the shell strength has been noted (Godfrey, 1949; Vandepopuliere et al., 1974; Britton, 1977).

Values for the water loaded pressure method have a higher simple correlation coefficient for shell thickness and breaking strength than those for the dropping ball method. This indicates that shell thickness accounts for a higher percentage of the total variation in the shell strength measured with the water loaded method than in the shell strength measured with the dropping ball device. The low value with the dropping ball device is thought to be partly due to poor precision and to variation in visible surface. As described in the Materials and Methods section, 12.5 mm increments in height of drop were used and this allows for less precision than measuring the mass of water in the water loaded pressure method. Furthermore, it was observed that a 3.52 g ball bearing causes various types of damage - a very small circular crack, a little dent, a very small linear crack which is almost invisible with the naked eye or a fairly large linear crack far from the impact spot. It is felt that these two factors are largely responsible for the lower correlation coefficient with the dropping ball method than that with the water loaded method. In addition, Tyler and Geake (1963) pointed out that the cracking strength was affected by a summation of blows of different strength. They previously reported that rotating the egg slightly after each blow did not eliminate the discrepancy unless the egg was rotated at least 90^{o}.

The water loaded pressure method avoids some of the difficulties found in the dropping ball method, but has its own drawbacks. Elasticity of the water tubing at the point of compression (F and G in Fig. 1), water temperature and shape of egg might influence breaking strength.

Further, in studies of various types of shell strength measuring devices, Tyler and Geake (1963) found that when dynamic loading methods were applied to three different parts of an egg (waist, broad pole and narrow pole), the waist was the strongest and the narrow pole was the weakest for a given shell thickness, while when the static loading methods were used, the opposite order was observed. This suggested that different characteristics were observed by the two types of measuring devices. In spite of these shortcomings, these two methods were still used in the following studies.

Trial 2

In this experiment, the effect of four factors - dietary protein levels, restricted feeding, strain and age of hens - on eggshell quality were studied. The two devices tested previously were used to determine shell strength. Some parameters of laying performance of the hens (hen-day egg production and feed conversion) and of egg quality (egg weight and Haugh unit) were examined also to see whether these parameters would be influenced and/or be related to the eggshell strength characteristics.

The results are summarized in Tables 4, 5 and 6. Table 7 shows a summary of the analysis of variance for these data.

Dietary Protein Levels. Protein level influenced hen-day production, feed efficiency, and Haugh units significantly ($p<0.05$), but did not affect mean egg weight or shell strength as measured by either method (Table 4).

Table 4. Effects of dietary protein levels, restricted feeding and strain on laying performance, egg quality and shell strength.

	Protein levels		Feeding		Strains	
	16%	12%	Ad libitum	Restricted feeding	Babcock	Hyline
Hen-day egg production (%)	55.1[a]	51.5[b]	57.6[a]	49.0[b]	53.1[a]	53.5[a]
Feed efficiency (kg feed/dozen)	2.044[a]	2.299[b]	2.161[a]	2.183[a]	2.208[a]	2.135[a]
Haugh units	78.0[a]	80.5[b]	77.9[a]	80.7[a]	79.8[a]	78.9[a]
Egg weight (g)	63.9[a]	63.5[a]	64.0[a]	63.4[a]	63.4[a]	64.0[a]
Shell breaking strength (kg)*	3.110[a]	3.114[a]	3.075[a]	3.150[a]	3.169[a]	3.055[b]
Shell breaking strength (cm)**	13.16[a]	13.12[a]	13.05[a]	13.23[a]	13.05[a]	13.23[a]

[a,b]Any two means within a treatment followed by different superscripts are significantly different ($p \leq 0.05$).

* Water loaded pressure method.

**Dropping ball method.

Table 5. Effects of hen's age on egg production, feed efficiency and Haugh units.

Age (4-week period)	Hen-day egg production (%)	Feed efficiency (kg feed/dozen)	Haugh units
1	53.7^{bcdef}	1.623^{ghi}	93.9^{a}
2	61.6^{ab}	1.393^{i}	89.1^{ab}
3	61.8^{ab}	1.485^{hi}	86.9^{abc}
4	61.6^{ab}	1.783^{fghi}	89.4^{ab}
5	65.6^{a}	1.903^{efghi}	79.1^{cdef}
6	60.3^{abc}	1.945^{efghi}	81.5^{bcde}
7	57.9^{abcde}	2.510^{abcde}	81.6^{bcde}
8	58.7^{abcd}	2.063^{defghi}	81.2^{bcde}
9	57.3^{abcde}	2.200^{cdefg}	80.6^{bcde}
10	57.4^{abcde}	2.138^{cdefgh}	82.5^{bcd}
11	43.3^{gh}	2.783^{abc}	76.9^{defg}
12	51.9^{def}	2.165^{cdefgh}	81.0^{bcde}
13	52.3^{cdef}	2.365^{bcdef}	75.3^{defg}
14	58.6^{abcd}	1.983^{efghi}	76.7^{defg}
15	50.1^{efg}	1.790^{fghi}	75.3^{defg}
16	49.0^{fg}	2.240^{cdefg}	74.7^{defg}
17	49.9^{efg}	2.305^{bcdefg}	66.1^{h}
18	39.9^{hi}	2.710^{abcd}	73.0^{efgh}
19	35.8^{i}	3.085^{a}	71.1^{fgh}
20	39.5^{hi}	2.978^{ab}	70.0^{gh}

^{a,b,c,d,e,f,g,h,i}Means in the same column with different superscripts are significantly different ($p \leq 0.05$).

Table 6. Effects of hen's age on egg weight and shell strength.

Age (4-week period)	Egg weight (g)	Shell Strength	
		Water loading method (kg)	Dropping ball method (cm)
1	53.6^{j}	3.693^{a}	13.24^{cdef}
2	55.1^{i}	3.415^{b}	12.95^{f}
3	57.6^{h}	3.441^{b}	13.09^{def}
4	59.2^{g}	3.454^{b}	13.33^{bcdef}
5	64.1^{ef}	3.292^{b}	13.25^{cdef}
6	64.2^{ef}	3.189^{c}	13.72^{ab}
7	63.8^{f}	3.189^{c}	13.54^{abc}
8	64.8^{cdef}	3.331^{b}	13.89^{a}
9	65.8^{bcde}	3.371^{b}	13.53^{abcd}
10	66.5^{abc}	3.258^{b}	13.48^{abcde}
11	65.6^{bcde}	3.161^{c}	13.16^{def}
12	65.7^{bcde}	2.995^{cd}	13.03^{ef}
13	66.0^{abcd}	2.941^{de}	12.21^{h}
14	66.4^{abc}	2.692^{fg}	13.34^{bcdef}
15	64.5^{def}	2.590^{g}	12.27^{h}
16	64.9^{cdef}	2.850^{def}	12.53^{gh}
17	65.7^{bcde}	2.898^{def}	13.18^{cdef}
18	66.7^{ab}	2.886^{def}	12.92^{fg}
19	66.3^{abc}	2.790^{def}	12.92^{fg}
20	67.6^{a}	2.814^{def}	13.24^{cdef}

^{a,b,c,d,e,f,g,h,i,j}Means in the same column with different superscripts are significantly different ($p \leq 0.05$).

Table 7. Summary of analysis of variance for laying performance and egg characteristics.

Source	df	Hen-day egg production	Feed efficiency	Haugh unit score	Egg weight	Shell strength	
						Water loading method	Dropping ball method
Total	799						
Protein level (P)	1	*	*	*	--	--	--
Feeding method (F)	1	*	--	--	--	--	--
Strain (S)	1	--	--	--	--	**	--
Age (A)	19	**	**	**	**	**	**
P X F	1	*	**	--	--	--	--
P X S	1	--	--	--	--	--	--
F X S	1	--	--	--	--	--	--
P X A	19	**	**	--	--	--	**
F X A	19	**	**	*	**	**	**
S X A	19	*	--	--	*	--	*
P X F X S	1	--	--	--	--	**	*
P X F X A	19	--	--	--	--	--	*
P X S X A	19	--	--	--	--	--	--
F X S X A	19	--	*	--	--	--	--
P X F X S X A	19	*	--	--	--	--	--

* Statistically significant at $p \leq 0.05$.
** Statistically significant at $p \leq 0.01$.

The 12% protein level resulted in a decrease of 3.6% in egg production and an increase of 0.255 kg of feed consumed per dozen eggs produced. A decrease in egg production and feed efficiency with sub-optimal protein levels in the diet were expected in light of the important role of protein in egg production. Further, the protein-by-age interaction (P X A) was highly significant for either egg production or feed conversion (Table 7). This suggests that differences in laying performance between the two dietary protein treatments do not remain the same throughout the experimental periods. The significant interaction between protein levels, restricted feeding, strain, and age (P X F X S X A) for hen-day egg production implies that the magnitude of the protein level effects in egg production depends on the other factors.

The mean Haugh unit scores for all other factors were increased 2.5 units by reducing dietary protein levels to 12% (Table 4). This higher Haugh unit score for the 12% protein treatment than that for the 16% protein treatment is difficult to explain, since so few studies have been done regarding nutritional effects on albumen thickness. Deaton and Quisenberry (1965) obtained lower Haugh unit scores from hens fed a 17% protein diet than from the hens fed a 14% protein diet. Mueller (1956) found no significant differences in Haugh units score among eggs from hens fed corn-soy, corn-meatscrap, and barley-oat-soy diets. However, a barley-oat-meatscrap diet group exhibited higher Haugh unit scores than the others. They were not able to explain the reasons for the difference.

Most of the literature shows that hens fed diets increasing in protein level from 13 to 17% produce significantly heavier eggs. This did not happen in our experiment in that no difference in egg weights between the two different protein level groups (Table 4) were noted. The analysis of variance for egg weight (Table 7) did not show any interaction of protein levels with other factors.

No significant difference between eggs from hens fed the two different protein levels were noted in mean shell strength using either measuring device. Similar results have been shown in most of the reports mentioned previously in the review of literature. Significant protein X age (P X A), protein x feeding method x strain (P X F X S), and protein level X feeding method X age (P X F X A) interactions were found for shell strength measured with the dropping ball method (Table 7). However, this result may have little meaning because of the inconsistency of the dropping ball device in measuring shell strength which will be discussed later.

Feed Restriction. The reduction of feed intake of the experimental groups to about 85% of the ad libitum intake of the control groups resulted in a decrease of 8.6% in mean hen-day egg production averaged over periods and protein levels (Table 4). A significant interaction between protein levels and feeding (P X F) (Table 7) was found for egg production. Table 8 shows that the decrease in egg production of the restricted feeding group was much more severe with the 12% protein diet group (a decrease of 16%) than for the 16% protein diet group (a decrease of 7.4%). It has been shown, in most of the

Table 8. Interactions of protein levels by feed restriction (P X F) for hen-day egg production and feed efficiency.

Hen-day egg production

Protein level in diet	Feeding methods		Simple effect (restricted - ad libitum)
	Restricted (%)	Ad libitum (%)	(%)
12%	42.8	58.8	-16.0
16 %	52.2	59.6	- 7.4
Simple effect (12% - 16%)	-9.4	-.08	P X F = - 4.3

Feed efficiency

Protein level in diet	Feeding methods		Simple effect (restricted - ad libitum)
	Restricted (kg feed/dozen)	Ad libitum (kg feed/dozen)	(kg feed/dozen)
12%	2.577	2.180	+0.397
16%	2.002	2.131	-0.129
Simple effect (12%-16%)	+0.575	+0.049	P X F = 0.263

literature reviewed, that if restricting feed intake decreases egg production it is due to insufficient nutrient intake.

Data presented in Table 4, when averaged over all factors, show no effect of feed restriction on the mean values for feed efficiency, Haugh unit scores, egg weight and shell quality. However, significant feeding method-by-age interactions (F X A) were obtained for the laying performance and egg characteristics (Table 7).

Feeding method-by-age interactions presented in Table 9 do not show any consistent trend for the restricted feeding effect on feed conversion rate of hens throughout the experiment. The observation of no significant difference in feed efficiency between the treatments is not in agreement with results reported by other researchers (Watkins et al., 1973; Snetsinger and Zimmerman, 1975; Kari et al., 1977). These workers demonstrated an improvement of feed efficiency by means of limited feed intake. This disagreement appears to be due to variations among experiments in the extent of restriction, methods of limiting feed intake and protein levels of the diet used. As an example, the statistical analysis of the data of this experiment revealed a significant interaction between protein levels and feeding (P X F) as well as feeding-by-age interaction (F X A) for feed efficiency as shown in Table 7. The protein-by-restricted feeding interaction (P X F) presented in Table 8 indicates that the 12% protein-restricted fed (R-12%) group had a higher feed intake per dozen eggs (2.577 kg/dozen) than the 12% protein-ad libitum (N-12%) group (2.180 kg/dozen), but the 16% protein-restricted fed group (R-16%)

Table 9. Examination of the interaction of restricted feeding with age (F X A) for hen-day egg production, feed efficiency, Haugh unit score and egg weight.*

Age (4-week period)	Hen-day egg production (%)	Feed efficiency (kg feed/dozen)	Haugh unit score	Egg weight (g)
1	- 5.2	+0.080	+2.8	-1.1
2	-11.4	-0.115	-0.7	+0.6
3	-21.1	+0.130	+0.6	-1.0
4	-15.6	+0.110	-.07	+0.5
5	-12.8	-0.19	+2.4	+0.5
6	-22.9	+0.750	+2.3	+0.5
7	-24.9	+1.300	+4.1	-0.5
8	-14.8	+0.105	+3.8	-0.7
9	-15.0	+0.195	+1.9	+0.8
10	-10.4	-0.015	+4.0	+0.5
11	- 3.4	-0.320	+3.9	+1.4
12	-14.4	-0.035	+3.5	+0.4
13	-22.8	+0.410	+5.6	-1.1
14	- 4.2	+0.100	+6.2	+0.4
15	- 6.8	-0.230	+8.2	-0.4
16	- 9.3	+0.440	+7.5	-2.2
17	+ 0.4	-0.275	+3.3	-1.1
18	-10.4	+0.145	+7.3	-1.6
19	- 6.8	+0.450	+5.5	-0.1
20	- 2.6	-0.060	+1.5	-0.6

*Values represent simple effects of restricted feading at each period (deviation from values for the ad libitum group).

had a lower feed intake per dozen eggs (2.002 kg/dozen) than the 16% protein-ad libitum (N-16%) group (2.131 kg/dozen).

The significant ($p<0.05$) interaction between feeding method and age for Haugh unit score (Table 7) is summarized in Table 9. Chickens of the restricted fed group laid eggs with slightly higher Haugh units than those of the ad libitum groups after 16 weeks of lay. It was felt that lower egg production of the restricted fed groups might alleviate a rapid aging of the avian shell gland, which may play an important role in determining the albumen thickness of the fresh eggs (Austic, 1977).

Feeding method-by-age interaction summarized in Table 9 shows no consistent difference in egg weight between the two treatments from the first to fifteenth period, but thereafter the restricted fed groups had a slightly lower mean egg weight than the ad libitum groups.

The effect of feed restriction on shell strength was not significant (Table 4). However, the graphical illustration of the feeding-by-age interaction (Fig. 2 and 3) revealed that with one exception, chickens of the limited feeding group laid eggs with slightly stronger shell than chickens of the ad libitum group after 28 weeks (7 periods) of lay. These results suggest that heavy egg production might accelerate the aging of the shell formation mechanism, and thus cause a more rapid decline of shell quality.

Strain Differences. No significant differences were observed between the two strains for egg production, feed efficiency, Haugh unit scores and egg weight when values were averaged over the other factors (Table 4).

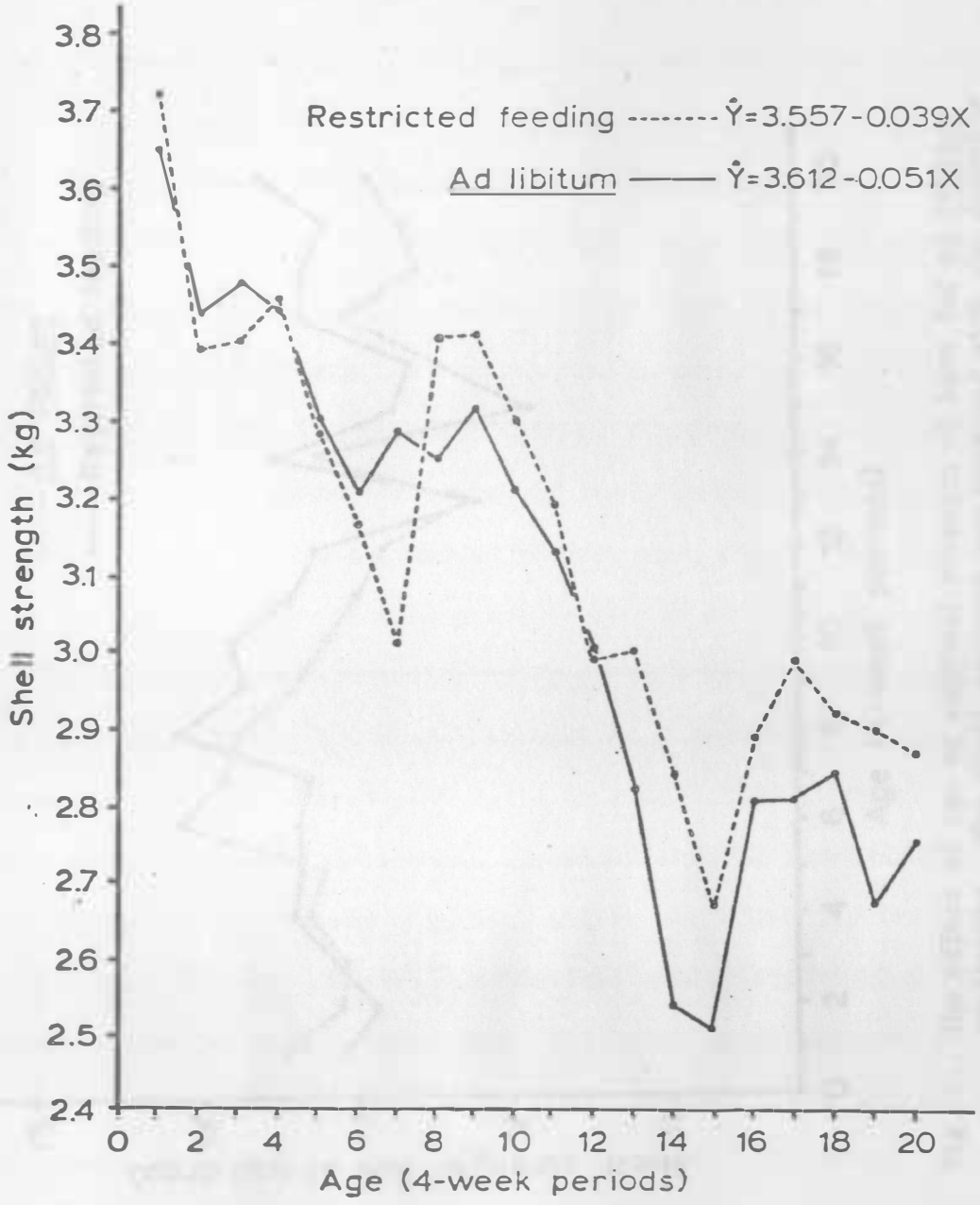

Fig. 2. The effect of age on eggshell strength of hens fed ad libitum or limited. The shell strength was measured with the water loaded pressure method.

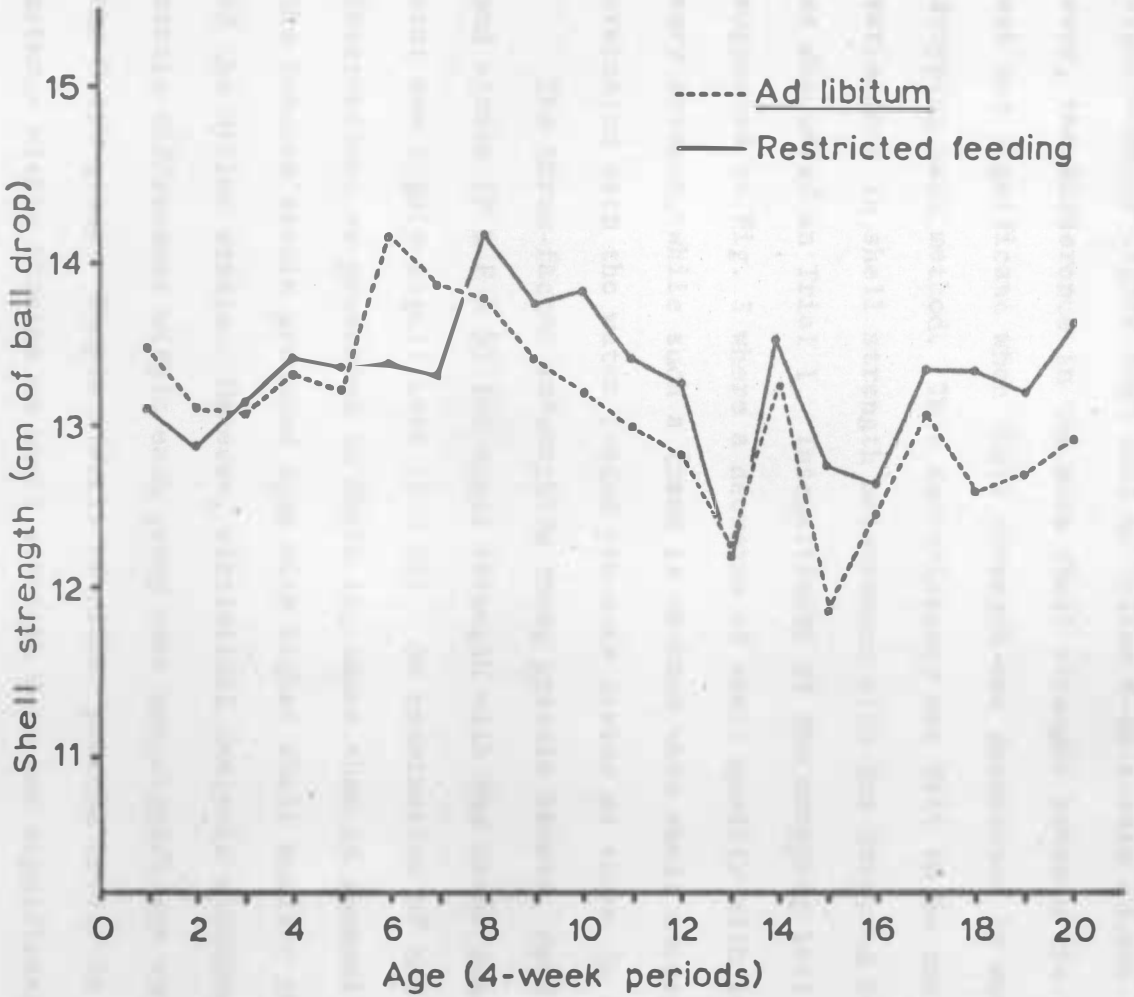

Fig. 3. The effect of age on eggshell strength of hens fed ad libitum or limited. The shell strength was measured with the dropping ball method.

The strain effect on eggshell quality was found significant only when shell strength was measured using the water loaded pressure method. The mean breaking strength (3.169 kg) of the Babcock B-300 strain was significantly higher than that of Hyline W-36 strain (3.055 kg). However, the difference in the mean shell strength between the two strains was not significant when shell strength was determined by means of the dropping ball method. This inconsistency was felt to be due to large variations in shell strength measurement with the dropping ball method as discussed in Trial 1. Insensitivity of the dropping ball device is suggested in Fig. 3 where a decrease of shell quality with age is not very evident, while such a trend is obvious when shell strength was evaluated with the water loaded pressure device as shown in Fig. 2.

The three-factor interaction among protein levels, feeding method and strain (P X F X S) for shell strength with the water loaded measurement was highly significant ($P<0.01$). An examination of the P X F X S interaction, as presented in Table 10, shows that in general birds of the Babcock strain produced eggs with higher shell quality than those of the Hyline strain. However, statistical analysis disclosed that strain differences within each group were not significant except for the R-16% group. Simple effects of either protein levels or feeding methods within strains on shell strength were not significant, when averaged over all the ages.

The higher shell strength of the Babcock strain observed in this study is not in accordance with the findings of Potts and Washburn (1974), who obtained lower mean values of breaking strength from the

Table 10. Three-factor interaction among protein levels, feeding method and strain for shell strength measured with the water loaded pressure device.

Protein	Feeding*	Strain		Simple effect
		Babcock	Hyline	Hyline - Babcock
	N	3.108^{ab}	3.043^{b}	-0.065
16%	R	3.300^{a}	2.990^{b}	-0.310
Simple effect	R - N	+0.192	-0.053	F X S = -0.123
	N	3.107^{ab}	3.039^{b}	-0.068
12%	R	3.160^{ab}	3.149^{ab}	-0.011
Simple effect	R - N	+0.053	+0.110	F X S = +0.029
(N-12%) - (N-16%)		-0.001	-0.004	
(R-12%) - (R-16%)		-0.140	+0.159	

^{a,b}Means followed by different superscripts are significantly different ($p<0.05$).

* N = hens fed ad libitum; R = hens fed restricted.

Babcock strain than from the Hyline strain when shell strength was determined for a one-week period after 7 months of lay. This discrepancy might be attributed to a lack of inheritability of the shell quality trait in commercial strains or the strain by age (S X A) interactions. An examination of data of the current study revealed that for the N-16% group the Hyline strain birds exhibited greater shell strength than the Babcock strain birds for some time during the first 24 weeks of egg production even though after that they consistently produced weaker shelled eggs than the Babcock strain birds.

Age Effect. The rate-of-lay performance and the egg quality traits examined in this experiment and presented in Tables 5 and 6 have been significantly ($P<0.01$) influenced by the hens age (Table 7). Average egg production reached its maximum after about 16 weeks of lay, and thereafter it declined slowly. A significant four-factor interaction among protein levels, feeding method, strain and age indicates that all of the four factors are dependent upon each other in influencing egg production. The feed intake per dozen eggs tended to increase with time, but fluctuated widely. The fluctuations might be in part due to changes in environmental temperature.

Figure 4 demonstrates that the albumen quality in terms of Haugh units decreased as the hens aged. As discussed previously, the 12% protein level had a consistently favorable effect on Haugh units from the fourth period on. The estimated linear regression equations show that during the course of the experiment Haugh units decreased at a rate of 0.95 per 4-week period for the birds of the 12% protein group, and

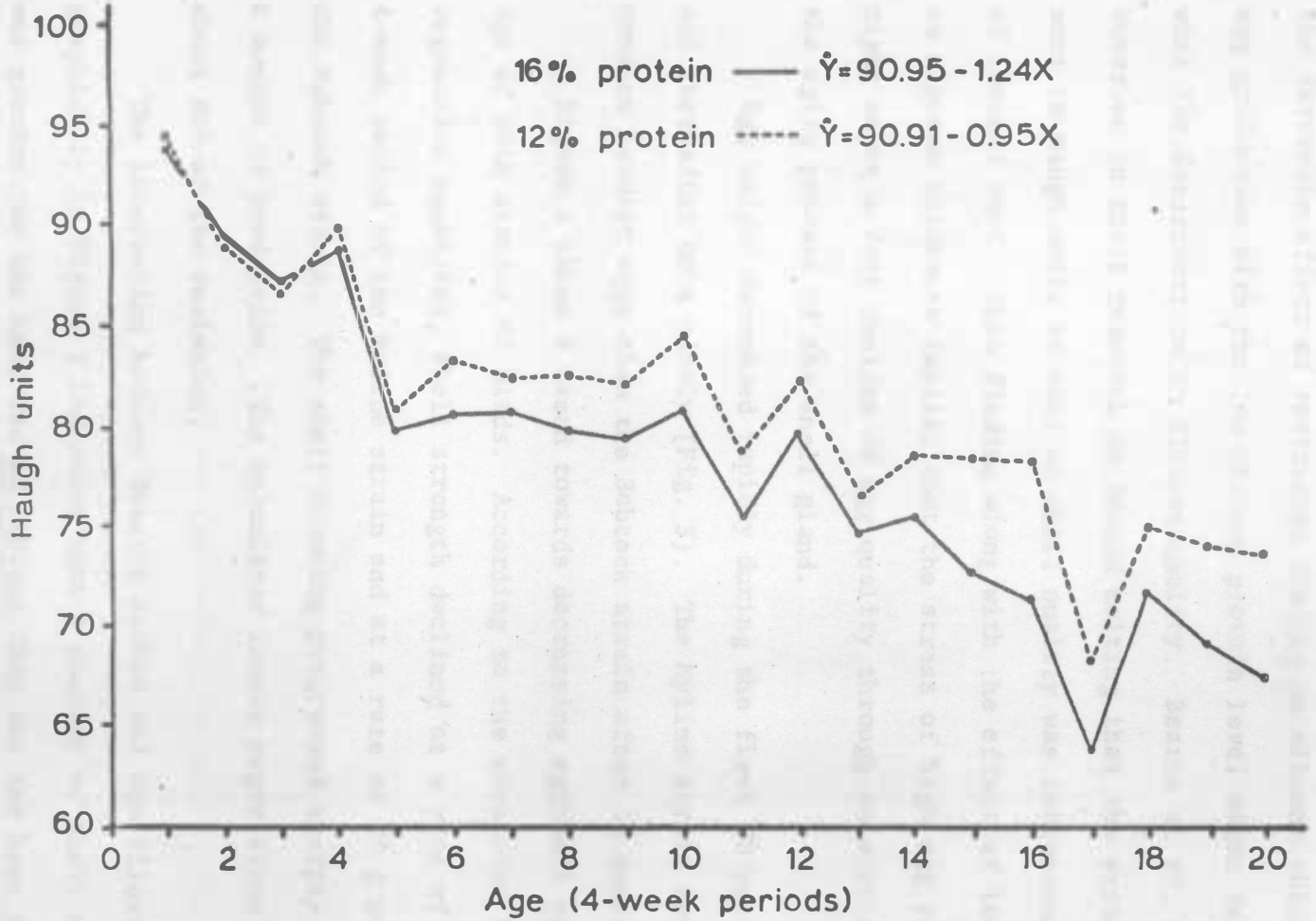

Fig. 4. The effect of age on Haugh unit score of eggs produced by hens fed two dietary protein levels (16 and 12%).

at a rate of 1.24 for the birds of the 16% protein group. Along with the favorable effect of restricted feeding on albumen thickness, low egg production with the low dietary protein level might reduce somewhat the deterioration in albumen quality. Bearse et al. (1967) observed in their research on forced molting that the extent of improvement in Haugh units as well as shell quality was influenced by the length of induced rest. This finding along with the effect of low production on albumen thickness implies that the stress of high egg production might cause a fast decline of egg quality through an acceleration of the aging process of the shell gland.

Egg weight increased rapidly during the first 20 weeks of lay, and thereafter more slowly (Fig. 5). The Hyline strain appeared to produce heavier eggs than the Babcock strain after 36 weeks of production.

Figure 6 shows a trend towards decreasing eggshell strength with age of both strains of birds. According to the estimated linear regression equations, shell strength declined at a rate of 40 g per 4-week period of the Hyline strain and at a rate of 49 g per 4-weeks for the Babcock strain. The shell thinning progressed sharply after about 8 months of production. The calculated linear regressions accounted for about 80% of the variation.

The interaction between feeding method and age illustrated graphically in Figure 2 indicates that a decline of shell strength was greater for the hens fed ad libitum than for the hens fed on a limited basis. The shell quality response to feed limiting along with the albumen quality response suggests that physiological stress from

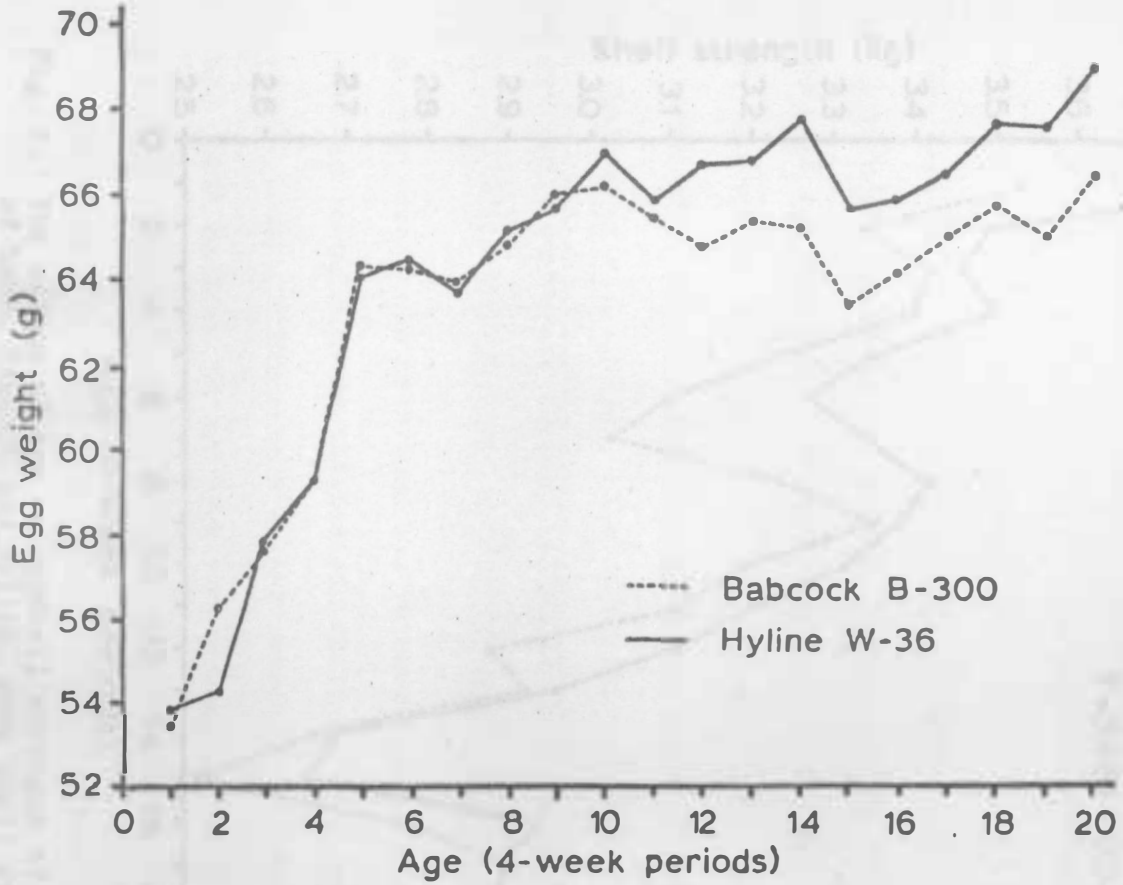

Fig. 5. The effect of age on the egg weight of two strains of hens.

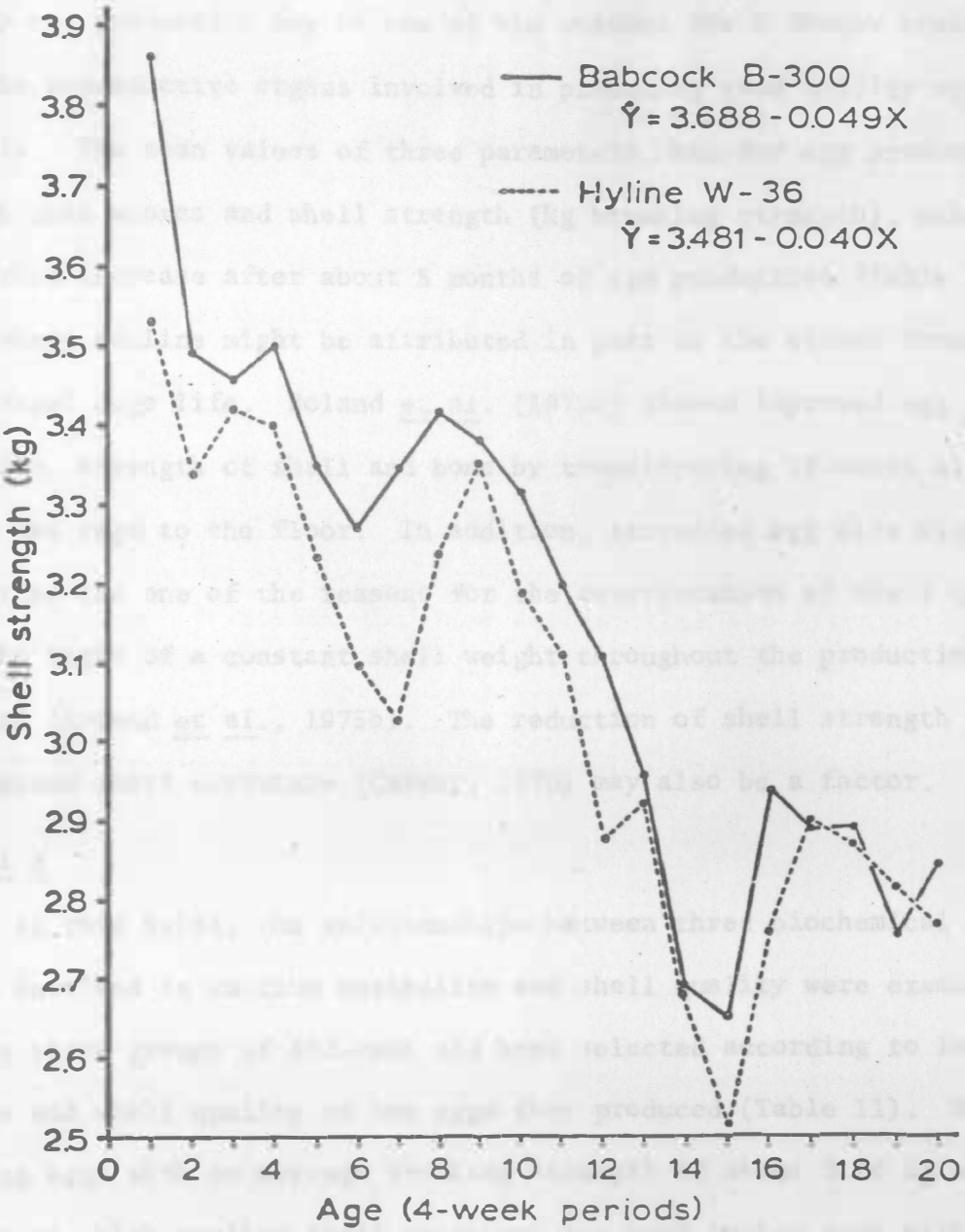

Fig. 6. The effect of age on eggshell strength of two strains of hens, Babcock and Hyline, when shell strength was measured with the water loaded pressure method.

heavy egg production may be one of the reasons for a faster breakdown of the reproductive organs involved in producing good quality eggs or shells. The mean values of three parameters, hen-day egg production, Haugh unit scores and shell strength (kg breaking strength), exhibited a marked decrease after about 8 months of egg production (Table 5, 6). The sharp decline might be attributed in part to the stress from a prolonged cage life. Roland et al. (1975a) showed improved egg production, strength of shell and bone by translocating 18-month old hens from the cage to the floor. In addition, increased egg size with age might be the one of the reasons for the deterioration of shell quality in the light of a constant shell weight throughout the production period (Roland et al., 1975b). The reduction of shell strength with increased shell curvature (Carter, 1970) may also be a factor.

Trial 3

In this trial, the relationships between three biochemical parameters involved in calcium metabolism and shell quality were examined using three groups of 102-week old hens selected according to laying state and shell quality of the eggs they produced (Table 11). Hens laying eggs with an average breaking strength of about 3.00 kg 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 11, the serum calcium level of the low quality shell producers (27.8 mg%) was not significantly

Table 11. Serum Ca level 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 x 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]

[a,b]Means within rows with different superscripts are significantly different ($p<0.05$).

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.

The nonsignificant difference in serum calcium concentrations between the two different shell quality groups 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 significant statistically. 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 11 is similar to that reported by Miller et al. (1978) for non-layers which exhibited regressed reproductive organs. This low value is possibly explained by some hormonal function which regulates calcium absorption from the diet and calcium resorption from the bone. The elevation of total blood calcium level as birds begin laying has been observed by many workers. This phenomenon is considered to be mainly the result of an estrogenic effect in which parathyroid hormone promotes higher blood calcium levels (Sturkie, 1965). The role of

the hypercalcaemic hormone, calcitonin, in avian calcium metabolism is a matter of controversy (Simkiss, 1975).

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 12.

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 12). 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 is about one half that for hens from the high quality shell group, 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, 3.87 units per gram of tissue or 0.192 unit per mg of protein.

Uterine CaBP activity, on either the per gram tissue base or per mg protein base, 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 was twice that of the other groups, 6.54 units per gram of tissue or 0.468 unit per mg of protein.

The significant difference among the groups suggests that CaBP activity affects egg shell formation and thus shell quality. As mentioned previously in the literature review section, the occurrence of CaBP in the small intestine is highly correlated with calcium

Table 12. Duodenal and shell gland CaBP activity 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 ($\times 10^{-2}$)	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[b]
Units/mg protein ($\times 10^{-2}$)	14.2±3.3[a]	20.3±9.8[a]	46.8±20.3[b]

[a,b,c]Means within rows with different superscripts are significantly different ($p<0.10$).

absorption from the diet. 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, 1973b). 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, 1973b, 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 results of this current study suggest that duodenal CaBP may play an important role in maintaining good shell quality in eggs produced by aged hens. This postulation is supported by the proposal of Spencer et al. (1978) that a high rate of Ca^{2+} absorption is not maintained without CaBP synthesis, even if 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, 1973b) 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 12 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. This observation implies that birds of the low quality shell group possessed 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.

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, 1973b). A higher uterine CaBP activity during shell calcification was not observed in laying hens (Bar

and Hurwitz, 1973b; 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 carbonic anhydrase enzyme activity.

Uterine Carbonic Anhydrase Enzyme Activity. As shown in Table 13, the carbonic anhydrase enzyme 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, 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, about half of that for the low quality shell group.

Pearson and Goldner (1973, 1974) proposed that calcium transport across the quail uterus is an active process, requiring oxidative aerobic metabolism and depends partly upon the presence of HCO_3^- for maximum transfer. Pearson et al. (1977) suggested an intimate relationship between this enzyme activity and uterine calcium secretion on the grounds that in vitro studies showed an active calcium transfer across uterine tissue of non-laying quail occurred without preformed HCO_3^- in the medium. Furthermore, a high degree of difference in uterine carbonic anhydrase activity was observed between laying, molting, and non-laying quail. The results of the present study on uterine carbonic anhydrase activity supports the work of Pearson et al. (1977) and suggest that the carbonic anhydrase activity of the shell gland

Table 13. Shell gland carbonic anhydrase activity of hens selected on the basis of shell quality.

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

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

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 carbonic anydrase 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 postulates 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 ATPases in shell calcification are conceivable.

The results of this study, nevertheless, suggest that the breakdown in eggshell quality in old hens is in large part the result of decreased CaBP and carbonic anhydrase activity in the shell gland. In addition, the decreased CaBP activity in the intestine with subsequent decreased calcium absorption from the diet is an additional contributory factor.

SUMMARY AND CONCLUSIONS

Three trials were conducted with cage-housed laying hens to study the problem of eggshell thinning with aging hens.

In Trial 1, two methods of shell strength measurement, a water loaded pressure technique and a dropping ball technique, were compared. Further, relationships of shell thickness and weight of eggs to shell strength were also studied.

Values for both of the shell strength measuring methods showed a highly significant correlation ($p<0.001$) with shell thickness. The water loaded pressure method had a much higher simple correlation coefficient for shell thickness ($r = +0.786$) than the dropping ball method ($r = +0.577$). The lower value of the dropping ball method was considered to be due in part to variations in visible surface damage during measurement and less precision in measuring the height of the ball drop.

Shell strength measured by the water loaded pressure method appeared not to be correlated to egg weight. On the other hand, the negative sign of the standard partial regression coefficient and the partial regression coefficient of egg weight in the estimated multiple regression equation implied that for a given shell thickness a larger egg tended to have less shell strength than a smaller egg.

In Trial 2, the effects of four factors - two dietary protein levels (12 and 16%), feeding methods (ad libitum and restricted), strains (Babcock and Hyline), and age of hens - on eggshell strength

were examined. At the same time, the effects of these factors on laying performance, Haugh unit score and egg weight were evaluated.

Use of the 12% dietary protein level resulted in significantly lower ($p<0.05$) hen-day egg production, higher feed intake per dozen eggs, and higher Haugh unit scores than the 16% dietary protein level. No effects of protein level on egg weight and shell strength were noted.

Reduction of feed intake to about 85% of the ad libitum intake decreased mean hen-day production. Significant feeding method-by-age interactions (F X A) were obtained for all parameters. Restricted feeding had favorable effects on shell strength and Haugh unit values after 28 and 16 weeks of egg production, respectively. However, restricted feeding did not prevent the decline of these characteristics with age. The effects of restricted feeding on feed efficiency and egg weight were not consistent.

The Babcock B-300 strain had a significantly ($p<0.01$) higher mean value for shell strength than the Hyline W-36 strain as measured with the water loaded pressure device. Examination of the three-factor interaction among protein levels, feeding method and strain (P X F X S) disclosed that the favorable effect of the Babcock strain on shell strength was significant only with the 16% protein-restricted fed group. The other treatment group did show a trend for greater shell strength of the Babcock strain. No significant differences were observed between the two strains for egg production, feed efficiency or Haugh unit scores. The Hyline strain produced slightly heavier eggs than the Babcock strain after 36 weeks of lay.

Mean values for all the parameters examined changed significantly ($p<0.01$) with the age of hens. Average hen-day egg production reached its maximum at 16 weeks of production, and after that it declined slowly. Feed intake per dozen eggs tended to increase, with some fluctuation, as the hens aged. An increase of egg weight occurred rapidly during the first 20 weeks of production, and thereafter more slowly. There was a linear decrease in Haugh unit scores and shell strength measured with the water loaded pressure method. After about 8 months of production, declines in egg produciton, Haugh unit values, and shell strength were rather severe. The effect of restricted feeding on either shell strength or Haugh unit scores were favorable for the aged chickens. However, feed restriction did not maintain the two egg qualities of aged laying hens as high as that of laying pullets at an early stage of production.

Finally, in Trial 3, studies on the relationships of eggshell quality with biochemical parameters - serum calcium level, duodenal and uterine CaBP activity and uterine carbonic anhydrase activity - were made using 102-week old hens. For this study, three groups of chickens were selected - those showing high quality shells, low quality shells and non-layer activity.

There were no significant differences in the serum calcium levels between the high and the low quality shell groups. This showed that serum calcium level was not a good indicator of a hen's ability to produce different quality eggshells. However, non-laying hens exhibited significantly ($p<0.05$) lower serum calcium levels than laying hens.

Differences ($P<0.10$) in duodenal CaBP activity among the three groups suggested an involvement of duodenal CaBP in determining eggshell quality of aged laying hens. The mean uterine CaBP activity of the low quality shell group was lower ($p<0.10$) than that of the higher quality shell group, but not significantly different from that of the non-layer group. This implies that CaBP plays a role in shell formation in the shell gland, and thus the magnitude of its activity should be related to eggshell strength.

Among the three groups, there was a significantly different ($p<0.01$) carbonic anhydrase activity of the shell gland. This also proposes an intimate relationship of this enzyme to shell calcification in the shell gland, thus relating enzyme activity to the shell quality of eggs laid by aged hens.

REFERENCES

Almquist, H. J., and B. R. Burmester, 1934. Characteristics of an abnormal type of egg shell. Poultry Sci. 13:116-122.

Andrews, D. K., 1973. Limited time feeding of commercial White Leghorn hens - A field trial. Poultry Sci. 52:1995 (abstr.).

Anonymous, 1975. Egg industry sustains heavy losses from shell damage. Feedstuffs, 47(27):7,31.

Austic, R. E., 1977. Role of the shell gland in determination of albumen quality. Poultry Sci. 56:202-210.

Baker, J. R., and Balch, D. A., 1962. A study of the organic material of the hen's-egg shell. Biochem. J. 82:352-361.

Bar, A., D. Dubrov, U. Eisner and S. Hurwitz, 1976. Calcium-binding protein and calcium absorption in the laying quail *(Coturnix coturnix japonica)*. Poultry Sci. 55:622-628.

Bar, A., and S. Hurwitz, 1972. Relationship of duodenal calcium-binding protein to calcium absorption in the laying fowl. Comp. Biochem. Physiol. 41B:734-744.

Bar, A., and S. Hurwitz, 1973a. Calcium restriction and intestinal calcium-binding protein in the laying fowl. Comp. Biochem. Physiol. 45A:571-577.

Bar, A., and S. Hurwitz, 1973b. Uterine calcium-binding protein in the laying fowl. Comp. Biochem. Physiol. 45A:579-586.

Bar, A., and S. Hurwitz, 1975. Intestinal and uterine calcium-binding protein in laying hens during different stages of egg formation. Poultry Sci. 54:1325-1327.

Bearse, G. E., R. C. Jennings and T. R. Morris, 1967. The effects of inducing rest in White Leghorn hens with I.C.I. compound 33828. Poultry Sci. 46:1233(abstr.).

Bell, D. D., and R. E. Moreng, 1972. Intermittent feeding and lighting of mature Leghorn hens. Poultry Sci. 51:1783 (abstr.).

Bell, D. J., and P. D. Sturkie, 1965. Chemical constituents of blood. In: Avian Physiology, ed., P. E. Sturkie. Cornell University Press, Ithaca, N. Y. pp. 32-69.

Bernstein, R. S., T. Nevalainen, R. Schraer and H. Schraer, 1968. Intracellular distribution and role of carbonic anhydrase in the avian *(Gallus domesticus)* shell gland mucosa. Biochim. Biophys. Acta 159:367-376.

Britton, W. M., 1977. Shell membranes of eggs differing in shell quality from young and old hens. Poultry Sci. 56:647-653.

Brooks, J., and H. P. Hale, 1955. Strength of the shell of the hen's egg. Nature 175:848-849.

Bunk, M. J., and S. L. Balloun, 1978. Ultra structure of the mamillary region of low puncture strength avian eggshells. Poultry Sci. 57:639-647.

Carter, T. C., 1970. Why do egg shells crack? World's Poult. Sci. J. 26:549-561.

Charles, O. W., and R. A. Ernst, 1974. Effects of age, calcium level and vitamin D. metabolites on egg shell quality of S.C.W.L. Poultry Sci. 53:1908 (abstr.).

Combs, G. F., and N. V. Helbacka, 1960. Studies with laying hens. 1. effect of dietary fat, protein levels and other variables in practical rations. Poultry Sci. 39:271-279.

Common, R. H., 1941. The carbonic anhydrase activity of the hen's oviduct. J. Agric. Sci. 31:412-414.

Corradino, R. A., R. H. Wasserman, M. H. Pubols and S. I. Chang, 1968. Vitamin D_3 induction of a calcium-binding protein in the uterus of the laying hen. Arch. Biochem. Biophys. 125:378-380.

Creger, C. R., H. Phillips and J. T. Scott, 1976. Formation of an egg shell. Poultry Sci. 55:1717-1723.

Deaton, J. W., and J. H. Quisenberry, 1965. Effects of dietary protein level on performance of four commercial egg production stocks. Poultry Sci. 44:936-942.

DeLuca, H. F., 1974. Vitamin D: the vitamin and the hormone. Fed. Proc. 33:2211-2219.

Dronawat, N. S., and J. McGinnis, 1966. Effect of feed restriction and of limiting feeding time for laying hens on egg production, egg size and feed consumption. Poultry Sci. 45:1081 (abstr.).

Emtage, J. S., D. E. M. Lawson and E. Kodicek, 1974. The response of the small intestine to vitamin D: correlation between calcium-binding protein production and incrased calcium absorption. Biochem. J. 144:339-346.

Frank, F. R., R. E. Burger and M. H. Swanson, 1965. The relationships among shell membrane, selected chemical properties, and the resistance to shell failure of *Gallus domesticus* eggs. Poultry Sci. 44:63-69.

Frank, F. R., M. H. Swanson and R. E. Burger, 1964. The relationships between selected physical characteristics and the resistance to shell failure of *Gallus domesticus* eggs. Poultry Sci. 43:1228-1235.

Gaisford, M. J., 1965. The application of shell strength measurements in egg shell quality determinations. Br. Poult. Sci. 6:193-196.

Gleaves, E. W., F. B. Mather and M. M. Ahmad, 1977. Effects of dietary calcium protein and energy on feed intake, egg shell quality and hen performance. Poultry Sci. 56:402-406.

Godfrey, G. F., 1949. Shell color as a measure of eggshell quality. Poultry Sci. 28:150-151.

Gutowska, M. S., and C. A. Mitchell, 1945. Carbonic anhydrase in the calcification of the egg shell. Poultry Sci. 24:159-167.

Hammerle, J. R., 1969. An engineering appraisal of egg shell strength evaluation techniques. Poultry Sci. 48:1708-1717.

Harbaugh, D. D., and P. E. Sanford, 1969. Effects of zinc-methionine supplement, calcium and phosphorus levels on flock performance, egg size and shell quality. Poultry Sci. 48:1816 (abstr.).

Harmeyer, J., and H. F. DeLuca, 1969. Calcium-binding protein and calcium absorption after vitamin D administration. Arch. Biochem. Biophys. 133:247-254.

Heald, P. J., D. Pohlman and E. G. Martin, 1968. Shell strength and carbonic anhydrase activity of the shell gland of the domestic fowl. Poultry Sci. 47:858-862.

Heywang, B. W., 1940. The effect of restricted feed intake on egg weight, egg production and body weight. Poultry. Sci. 19:29-34.

Heywang, B. W., and M. G. Vavich, 1965. Camparison of performance of layers fed soybean, glandless or glanded cottonseed meals. Poultry Sci. 44:1240-1244.

Hurnik, J. F., J. D. Summers, B. S. Reinhart and E. M. Swierczewska, 1977. Effect of age on the performance of laying hens during the first year of production. Poultry Sci. 56:222-230.

Hurwitz, S., and A. Bar, 1967. Calcium metabolism of hens secreting heavy or light egg shells. Poultry Sci. 46:1522-1527.

Hurwitz, S., and A. Bar, 1969a. Calcium reserves in bones of laying hens: their presence and utilization. Poultry Sci. 48:1391-1396.

Hurwitz, S., and A. Bar, 1969b. Relation between the lumen-blood electrochemical potential difference of calcium, calcium absorption and calcium-binding protein in the intestine of the fowl. J. Nutr. 99:217-224.

Hurwitz, S., and A. Bar, 1970. The sites of calcium and phosphate absorption in the chick. Poultry Sci. 49:324-325.

Kari, R. R., J. H. Quisenberry and J. S. Bradley, 1977. Egg quality and performance as influenced by restricted feeding of commercial caged layers. Poultry Sci. 56:1914-1919.

King, N. R., and D. S. Robinson, 1972. The use of the scanning electron microscope for comparing the structure of weak and strong egg shells. J. Microscopy 95:437-443.

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall, 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

Lund, W. A., V. Heiman and L. A. Wilhelm, 1938. The relationship between egg shell thickness and strength. Poultry Sci. 17:372-376.

Meldrum, N. U., and F. J. W. Roughton, 1933. Carbonic anhydrase. Its preparation and properties. J. Physiol. 80:113-141.

Meyer, R., R. C. Baker and M. L. Scott, 1973. Effects of hen egg shell and other calcium courses upon egg shell strength and ultrastructure. Poultry Sci. 52:949-955.

Middendorf, D. F., N. V. Helbacka and G. F. Combs, 1959. Effect of protein levels and kelp ash on performance of laying hens. Poultry Sci. 38:1229 (abstr.).

Miller, E. R., H. R. Wilson and R. H. Harms, 1978. The relationship of production status to serum calcium and phosphorus in hens. Poultry Sci. 57:242-245.

Mongin, P., and N. W. Carter, 1977. Studies on the avian shell gland during egg formation: Aqueous and electrolytic composition of the mucosa. Br. Poult. Sci. 18:339-351.

Moreng, R. E., H. L. Enos, W. A. Whittet and B. F. Miller, 1964. An analysis of strain response to dietary protein levels. Poultry Sci. 43:630-638.

Mueller, W. J., 1956. The influence of energy source, energy-fiber concentration and protein source of the diet on certain egg quality characteristics. Poultry Sci. 35:1074-1078.

Mueller, W. J., 1957. The relationship between selected egg characteristics and the resistance of the shell against puncture by pressure and impact. Poultry Sci. 36:1144 (abstr.).

Mueller, W. J., 1959. Shell strength - Important egg quality factor. Sci. for the Farmer, 6(1):4. Penn. S. Univ.

Mueller, W. J., 1962. Carbonic anhydrase, diuretics and egg shell formation. Poultry Sci. 41:1792-1796.

Mueller, W. J., 1967. The effect of two levels of methionine on the biological performance of laying pullets in controlled environments. Poultry Sci. 46:82-88.

Mueller, W. J., R. Schraer and H. Schraer. 1964. Calcium metabolism and skeletal dynamics of laying pullets. J. Nutr. 84:20-26.

Opel, H., 1965. Failure of neurohypophysectomy to reduce egg shell thickness in chickens. Poultry Sci. 44:1134-1136.

Paul, H. S., and D. C. Snetsinger, 1969. Dietary calcium and phosphorus and variations in plasma alkaline phosphatase activity in relationship to physical characteristics of egg shells. Poultry Sci. 48:241-250.

Pearson, T. W., and A. M. Goldner, 1973. Calcium transport across avian uterus. 1. Effects of electrolyte substitution. Am. J. Physiol. 225(6):1508-1512.

Pearson, T. W., and A. M. Goldner, 1974. Calcium transport across avian uterus. 2. Effects of inhibitors and nitrogen. Am. J. Physiol. 227(2):465-468.

Pearson, T. W., T. J. Pryor and A. M. Goldner, 1977. Calcium transport across avian uterus. 3. Comparison of laying and nonlaying birds. Am. J. Physiol. 232(4):E437-E443.

Pepper, W. F., J. D. Summers and J. D. McConachie, 1968. The effect of high levels of calcium, fish products and sodium bicarbonate on eggshell quality. Poultry Sci. 47:224-229.

Pike, J. W., and R. H. Alvarado, 1975. Ca^{2+}-Mg^{2+}-activated ATPase in the shell gland of Japanese quail (*Coturnix coturnix japonica*). Comp. Biochem. Physiol. 51B:119-125.

Polin, D., and J. H. Wolford, 1971. Egg production by W. L. adult females restricted in feed intake by limiting their time or allotted amount to eat. Poultry Sci. 50:1618 (abstr.).

Potts, L., and K. W. Washburn, 1974. Shell evaluation of white and brown egg strains by deformation, breaking strength, shell thickness and specific gravity. 1. Relationships to egg characteristics. Poultry Sci. 53:1123-1128.

Potts, P., and K. W. Washburn, 1977. The effect of supplemental calcium and vitamin D_3 on line and strain differences in egg shell strength. Poultry Sci. 56:1067-1072.

Richards, J. F., and L. M. Staley, 1967. The relationships between crushing strength, deformation and other physical measurements of the hen's egg. Poultry Sci. 46:430-437.

Richards, J. F., and M. H. Swanson, 1965. The relationship of egg shape to shell strength. Poultry Sci. 44:1555-1558.

Robinson, D. S., and N. R. King, 1963. Carbonic anhydrase and formation of the hen's egg shell. Nature 199:497-498.

Roland, D. A., Sr., 1976. Recent developments in eggshell quality. Feedstuffs, 48(29):31-32, 41.

Roland, D. A., Sr., L. O. Rowland, Jr. and R. H. Harms, 1975a. Reproductive performance of aged layers translocated from cage to floor. Poultry Sci. 54:912-915.

Roland, D. A., Sr., D. R. Sloan and R. H. Harms, 1972. Calcium metabolism in the laying hens. 2. Patterns of calcium intake, serum calcium and fecal calcium. Poultry Sci. 51:782-787.

Roland, D. A., Sr., D. R. Sloan and R. H. Harms, 1975b. The ability of hens to maintain calcium deposition in the egg shell and egg yolk as the hen ages. Poultry Sci. 54:1720-1723.

Schwartz, E., 1974. A magnesium dependent bicarbonate stimulated ATPase in the avian oviduct: Subcellular localization and relationship to bicarbonate transport. Fed. Proc. 33:1314 (abstr.).

Scott, M. L., 1977. Less phosphorus strengthens bones and eggshells. N. Y. Food and Life Sci. 10:8-11.

Scott, M. L., M. N. Nesheim and R. J. Young, 1976. Calcium. In: Nutrition of the Chicken. M. L. Scott & Associates, Ithaca, N. Y. Pp. 285-296.

Simkiss, K., 1975. Calcium and avian reproduction. In: Avian Physiology, ed., M. Peaker. Sympos. Zool. Soc. London 35:307-337. Academic Press, New York, N. Y.

Simkiss, K., S. Hurwitz and A. Bar, 1973. Sr^{85}/Ca^{45} discrimination by the active shell gland of the fowl. Comp. Biochem. Physiol. 44A:201-205.

Simkiss, K., and T. G. Taylor, 1971. Shell formation. In: Physiology and Biochemistry of the Comestic Fowl, ed., D. J. Bell and B. M. Freeman. Academic Press, New York, N.Y. pp. 1331-1342.

Simons, P. C. M., 1971. Ultrastructure of the hen eggshell and its physiological interpretation. No. 175. Central Institute for Poultry Research 'Het Spelderholt', Beekbergen, the Netherlands.

Snetsinger, D., and R. Zimmerman, 1975. Limited feeding of layers. Feedstuffs, 47(4):17-18, 92.

Speers, G. M., 1974. Factors affecting egg shell quality. Land O'Lakes, Inc., Fort Dodge, Iowa.

Spencer, R., M. Charman, J. S. Emtage and D. E. M. Lawson, 1976. Production and properties of vitamin-D-induced mRNA for chick calcium-binding protein. Eur. J. Biochem. 71:399-409.

Spencer, R., M. Charman, P. W. Wilson and D. E. M. Lawson, 1978. The relationship between vitamin D-stimulated calcium transport and intestinal calcium-binding protein in the chicken. Biochem. J. 170:93-101.

Steel, R. G. D., and J. H. Torrie, 1960. Principles and Procedures of Statistics. McGraw-Hill Book Co. Inc., New York.

Stemberger, B. H., W. J. Mueller and R. M. Leach, Jr., 1977. Microscopic study of the initial stages of egg shell calcification. Poultry Sci. 56:537-543.

Stewart, G. F., 1935. The structure of the hen's egg shell. Poultry Sci. 14:24-32.

Stewart, G. F., 1936. Shell characteristics and their relationship to the breaking strength. Poultry Sci. 15:119-124.

Sturkie, P. D., 1965. Parathyroids, thymus, pineal, and pancrease. In: Avian Physiology, ed., P. D. Sturkie. Cornell University Press, Ithaca, N. Y. pp. 649-667.

Summers, J. D., J. D. McConachie, S. J. Slinger and W. F. Pepper, 1966. The value of raw unextracted soybeans for laying hens. Poultry Sci. 45:165-169.

Sunde, M. L., 1971. What's behind our $60 million egg breakage problem. Farm Technol. 27(1):18,23.

Taylor, L. W., and I. M. Lerner, 1939. Inheritance of eggshell thickness in White Leghorn pullets. J. Agr. Res. 58:383-395.

Taylor, A. N., and R. H. Wasserman, 1969. Correlations between the vitamin D-induced calcium-binding protein and intestinal absorption of calcium. Fed. Proc. 28:1834-1838.

Taylor, T. G., 1970. How an eggshell is made. Scientific American 222:88-95.

Tijen, W. F. van, 1977a. Shell quality in poultry as seen from the breeder's viewpoint. 1. Improvement reached after four years of selection and the effect on productivity. Poultry Sci. 56:1107-1114.

Tijen, W. F. van, 1977b. Shell quality in poultry as seen from the breeder's viewpoint. 2. Search for heterosis effects. Poultry Sci. 56:1115-1120.

Tyler, C., 1961. Shell strength; its measurements and its relationship to other factors. Br. Poult. Sci. 2:3-19.

Tyler, C., and F. H. Geake, 1963. A study of various impact and crushing methods used for measuring shell strength. Br. Poult. Sci. 4:49-61.

Vandepopuliere, J. M., H. V. Walton and R. O. Hawes, 1974. Egg shell membrane thickness relationship to age and egg breaking strength. Poultry Sci. 53:1986 (abstr.).

Voisey, P. W., and R. M. G. Hamilton, 1976. Ultrasonic measurement of egg shell thickness. Poultry Sci. 55:1319-1324.

Voisey, P. W., and J. R. Hunt, 1967. Relationship between applied force, deformation of egg shells and fracture force. J. Agr. Eng. Res. 12:1-4.

Walsh, B. M., J. R. Kingan and T. W. Sullivan, 1963. Studies on the interrelationship of vitamin D_3 and protein with calcium in the diet of laying hens. Poultry Sci. 42:1316 (abstr.).

Wasserman, R. H., and A. N. Taylor, 1966. Vitamin D_3- induced calcium-binding protein in chick intestinal mucosa. Science 152:791-793.

Wasserman, R. H., and A. N. Taylor, 1968. Vitamin D-dependent calcium-binding protein. Response to some physiological and nutritional variables. J. Biol. Chem. 243:3987-3993.

Watkins, M., B. C. Dilworth and E. J. Day, 1973. The effect of calcium source, particle size, level and restricted feeding time on shell strength and production parameters of commercial laying hens. Poultry Sci. 52:2100 (abstr.).

Wells, R. G., 1967. Egg shell strength. 1. The relationship between egg breakage in the field and certain laboratory assessments of shell strength. Br. Poult. Sci. 8:131-139.

Wilbur, K. M., and N. G. Anderson, 1948. Electrometric and colorimetric determination of carbonic anhydrase. J. Biol. Chem. 176:147-154.

Wisman, E. L., and W. L. Beane, 1965. Utilization of hatchery by-product meal by the laying hen. Poultry Sci. 44:1332-1333.

Wolford, J. H., and K. Tanaka, 1970. Factors influencing egg shell quality - a review. World's Poult. Sci. J. 26:763-780.

Worthington Enzymes Manual, 1977. Carbonic anhydrase. In: Enzymes, Enzyme reagents, and related biochemicals. Worthington Biochem. Co., Freehold, N. J. pp. 280-282.

Wyburn, G. M., H. S. Johnston, M. H. Draper and M. F. Davidson, 1973. The Ultrastructure of the shell forming region of the oviduct and the development of the shell of *Gallus domesticus*. Quart. J. Exp. Physiol. 58:143-151.