STUDIES ON EGG SHELL CALCIFICATION

BY

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ABSTRACT.

The transport of calcium from the blood of the laying hen (Gallus domesticus) to the shell of the developing egg is a very rapid but discontinuous process. These studies were carried out in order to examine the way in which this process is controlled.

In the initial experiments calcium translocation across the shell gland was studied using an in vitro system similar to that described by Ehrenspeck et al (1967). The magnitude of the observed fluxes was similar to those reported by Ehrenspeck et al, but, in contrast to their results, no clear correlation could be found between the flux and the physiological condition of the shell gland. The two hormones of calcium homeostasis, parathyroid hormone and calcitonin, were also without influence on the system. However the dibutyryl derivative of cyclic AMP increased the calcium flux from the shell gland lumen to the blood but did not affect flux in the normal physiological direction, i.e. blood→lumen. One fundamental criticism of the in vitro system is that the fluxes measured are extremely low. Calculation of the flux which must occur in vivo showed that this was 500-1000 times larger than the fluxes obtained in vitro.

In view of the deficiencies of the in vitro system all subsequent studies were carried out on the intact hen. Two of the most likely mechanisms for control of shell gland function involve either the endocrine system, which the
IN VITRO STUDIES IMPLIED WAS NOT INVOLVED, OR THE AUTONOMIC NERVOUS SYSTEM. THE NEURAL INFLUENCE UPON SHELL FORMATION WAS STUDIED USING NEURAL BLOCKING AGENTS AS THE COMPLEX ANATOMY OF THE SHELL GLAND NERVOUS SUPPLY PRECLUDED NEURAL SECTION. AN EXTENSIVE LITERATURE SURVEY FAILED TO PROVIDE SUFFICIENT DATA ON BLOCKING DRUGS IN CHICKENS SO THAT AN EXTENSIVE SERIES OF EXPERIMENTS WAS PERFORMED IN ORDER TO DEFINE APPROPRIATE DOSES TO PRODUCE 70-80% BLOCKADE IN THE ANESTHETIZED HEN FOR PERIODS UP TO TWO HOURS.

USING THE DRUG DOSAGES SO OBTAINED THE EFFECT OF BLOCKADE OF VARIOUS BRANCHES OF THE AUTONOMIC NERVOUS SYSTEM ON THE INCORPORATION OF INTRAVENOUSLY INJECTED 45Ca INTO THE EGG SHELL, AND ON THE DISAPPEARANCE OF THIS LABEL FROM THE PLASMA, WAS STUDIED IN ANESTHETIZED HENS. THE DATA FROM BOTH TYPES OF EXPERIMENT SHOWED THAT, UNDER THE EXPERIMENTAL CONDITIONS, THE RATE OF CALCIUM TRANSPORT ACROSS THE SHELL GLAND IS INDEPENDENT OF THE NERVOUS SUPPLY.
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"EGGSASPERATING EGGSPERIMENTS"
GENERAL INTRODUCTION.

In the female domestic chicken (Gallus domesticus) only the left ovary and oviduct become functional, while the right ones atrophy, so that only one egg can be produced at a time. There is no ready explanation for this phenomenon. Taylor (1970a) wrote "One can only speculate on the evolutionary reason for the disappearance of the right ovary and oviduct. A reasonable guess is that two ovaries were disadvantageous because of the problem of providing enough calcium for the shells of two eggs at once".

Post-embryonic development of the ovum can be divided into three stages. The two major events which occur during the long initial phase are a slow deposition of yolk (mainly neutral fats), which may continue for many months, and formation of the follicle around the vitelline membrane. During the following 60 days the yolk proteins are laid down in vacuoles within the ovum. The developing follicle is not the site of yolk formation. Yolk precursors are synthesized in the liver and transported to the ovary in the plasma. Because of this the blood of the laying hen is characterized by a high content of lipid, free fatty acids and phosphoprotein, Gilbert (1967). About 9-14 days before ovulation, the deposition of phospholipids causes the ovum to enlarge rapidly,
SO THAT, TYPICALLY THE WEIGHT RISES FROM 1 G TO 18 G.

**Characteristic of this period is a high plasma calcium level which occurs in response to estrogen,** Urist (1959). **This elevated plasma calcium is not involved in egg shell calcification as it occurs in many vertebrates which lay yolky eggs with uncalcified shells.** Simkiss (1961) suggested that it is concerned with the transport of yolk precursors and that the phosphoprotein and lipoprotein components of the yolk are soluble in plasma only as long as there is sufficient calcium present.

The gonadotropin directly responsible for ovulation is apparently leutinizing hormone (LH), Fraps (1955). The mature ovum is shed from the follicle by rupture of the stigma, but the precise events leading up to the disruption of the tissues in response to LH have not yet been elucidated, Gilbert (1967).

The oviduct, which conducts the released ovum to the exterior, can be divided into five regions; the infundibulum, magnum, isthmus, shell gland (uterus) and vagina. Passage of the egg down the oviduct takes approximately 25 hours. The ovum is engulfed by the funnel-like infundibulum very soon after ovulation, and spends about 30 min in this region. It is here that the ovum is fertilized in an inseminated hen. The ovum passes into the magnum where the egg-white proteins are laid down. It remains in the magnum an average of almost three hours, so that, although the magnum is the longest part of the oviduct, the egg traverses this portion relatively quickly. The peristaltic movements of the magnum force the

IN HENS ON AN ADEQUATE CALCIUM DIET, BECAUSE THE RATE OF ABSORPTION OF CALCIUM FROM THE GUT WAS NOT SUFFICIENT TO PROVIDE ALL THE CALCIUM FOR THE EGG SHELL. HOWEVER, HURWITZ (1970) CLAIMS THAT, IN HENS GIVEN A NORMAL CALCIUM DIET (3.5-4.0% Ca), THE ABSORPTION OF CALCIUM FROM THE GUT IS SUFFICIENT TO ACCOUNT FOR ALL THE CALCIUM IN THE EGG SHELL.

IN THE PIGEON, WHICH LAYS TWO EGGS 48 HOURS APART, THE ROLE OF MEDULLARY BONE SEEMS FAIRLY CLEARCUT. CALCIFICATION OF EACH SHELL IS ACCOMPANIED BY MEDULLARY BONE RESORPTION ASSOCIATED WITH INTENSE OSTEOCLASTIC ACTIVITY. SOON AFTER THE FIRST EGG IS LAID THE BONE RESORBING PHASE GIVES WAY TO ONE DOMINATED BY BONE FORMATION, BLOOM ET AL (1941). IN THE DOMESTIC HEN IN WHICH THE TIME INTERVAL BETWEEN CALCIFICATION OF SUCCESSIVE EGGS MAY BE ONLY 5-6 HOURS THE CELLULAR CHANGES IN MEDULLARY BONE ARE LESS WELL DEFINED. BLOOM ET AL (1958) WERE ABLE TO DEMONSTRATE TWO WELL-DEFINED HISTOLOGICAL PATTERNS. AT THE START OF SHELL FORMATION BOTH OSTEOBLASTS AND OSTEOCLASTS WERE PRESENT, WHEREAS WHEN SHELL CALCIFICATION WAS WELL ADVANCED THE SPICULES OF MEDULLARY BONE WERE COVERED WITH HUGE NUMBERS OF OSTEOCLASTS AND THERE WAS A PRECIPITOUS DROP IN THE NUMBER OF OSTEOBLASTS. THE HISTOLOGICAL STUDIES OF BELANGER AND TAYLOR (1967) ON MEDULLARY BONE DURING THE EGG CYCLE IN HENS FED A LOW CALCIUM DIET REVEALED THAT MEDULLARY BONE RESORPTION IS APPARENTLY UNDER THE INFLUENCE OF ENLARGED OSTEOCYTES. THE DATA ON THE LAYING HEN HAS BEEN SUMMARIZED BY TAYLOR (1970b) AS FOLLOWS:

1. GROWTH OF MEDULLARY BONE IS CONTINUOUS, ALTHOUGH THE
Actual rate of growth may vary depending upon the circulatory level of estrogens and androgens.

2. Growth is from the surface of the spicules, which penetrate further into the marrow cavity the longer the bird is in lay.

3. Resorption, as a result of osteocytic osteolysis, accompanied or followed by osteoclasia, is intermittent and associated with calcification of the shell. At the start of shell formation resorption occurs mainly in the spicules nearest the centre of the marrow cavity, but as shell formation proceeds resorption extends towards the cortical bone. At any one time some surfaces of the medullary bone may be undergoing active resorption while adjacent surfaces are growing equally actively.

Direct evidence of the role of medullary bone in egg shell calcification was obtained by Mueller et al. (1964), who fed laying hens a constant specific activity ration for 26 days, after which an identical but 45Ca-free ration was fed for a further 26 days. At the end of the experiment it was found that the percentage of 45Ca in the last egg laid was very similar to that found in the medullary bone. These birds were on a 1.95% calcium diet and hence may have been somewhat calcium deficient thus emphasising the role of medullary bone. Nevertheless the data clearly shows that medullary bone can serve as a labile reservoir for egg shell calcium.

Two major theories on the mechanism of medullary bone resorption have been advanced. Urist (1959) proposed that resorption is brought about by a reduction in the level of estrogen circulating in the blood after ovulation. This theory would explain the events occurring in the bones of pigeons in which there is likely to be a substantial reduction of estrogen after the ovulation of the first follicle, and in which the fall in estrogen following ovulation of the second follicle
MUST BE PRECIPITOUS. HOWEVER, AS TAYLOR (1970b) POINTS OUT, IN THE LAYING HEN THERE ARE A LARGE NUMBER OF FOLLICLES DEVELOPING IN THE OVARY AND THESE ARE ALL PRESUMEABLY SECRETING ESTROGENS SO THAT IT IS PROBABLE THAT THE FALL IN PLASMA ESTROGEN OCCURRING AFTER OVULATION IS RELATIVELY SLIGHT.

TAYLOR & HETELENDY (1961) SHOWED THAT THE LEVEL OF DIFFUSIBLE CALCIUM IN THE PLASMA OF LAYING HENS FELL SIGNIFICANTLY DURING EGG SHELL CALCIFICATION. THEY POINTED OUT THAT DIFFUSIBLE CALCIUM IS A FAIRLY GOOD APPROXIMATION OF IONIC CALCIUM, AND SUGGESTED THAT THIS FALL IN IONIC CALCIUM COULD ACT AS A STIMULUS TO THE PARATHYROID GLAND. THEY PROPOSED THAT THE CONSEQUENT RISE IN THE LEVEL OF PARATHYROID HORMONE (PTH) COULD ACT AS THE STIMULUS FOR MEDULLARY BONE RESORPTION. THERE IS SOME EVIDENCE THAT PTH LEVELS MAY BE HIGH DURING EGG SHELL CALCIFICATION. TAYLOR (1965) AND URIST (1959) STATE THAT THE PARATHYROID GLANDS ARE ENLARGED IN LAYING HENS. MUELLER ET AL. (1969) REPORTED THAT CULTURES OF BONES REMOVED FROM HENS WHILST THEY WERE ACTIVELY CALCIFYING AN EGG SHOWED INCREASED LACTATE PRODUCTION. THIS IS SIMILAR TO THE EFFECT OF ADDITION OF PTH TO MAMMALIAN BONE CULTURES. TAYLOR & BELANGER (1969) LOOKED AT THE EFFECT OF PTH ON MEDULLARY BONE HISTOLOGY. THEY INJECTED HENS WITH 100 U.S.P. UNITS OF LILLY PARATHYROID EXTRACT AT THE TIME AN EGG ENTERED THE SHELL GLAND AND KILLED THEM 2, 4, 6 AND 8 HOURS LATER. WHEN THE BONE HISTOLOGY OF THESE BIRDS WAS COMPARED WITH BIRDS UNDERGOING NATURAL MEDULLARY BONE RESORPTION DUE TO EGG SHELL CALCIFICATION THE HORMONE WAS FOUND TO ENHANCE THE NATURALLY OCCURRING CHANGES.
IN BONE CELLS, THE MINERAL FRACTION AND THE ORGANIC MATRIX. OSTEOLYSIS WAS ALSO STIMULATED IN CORtical BONE. THESE DATA GIVE DIRECT SUPPORT TO THE HYPOTHESIS THAT THE NATURAL RESORPTION OF MEDULLARY BONE DURING EGG SHELL FORMATION IN HENS IS INDUCED BY PTH.


HOWEVER BURMESTER ET AL (1939), BURMESTER (1940) AND BRADFIELD (1951) HAVE SHOWN THAT THE RATE OF CALCIUM DEPOSITION IS LOW FOR THE FIRST FIVE HOURS THE EGG SPENDS IN THE SHELL GLAND SO THAT THE MAIN PERIOD OF SHELL CALCIFICATION ONLY LASTS ABOUT 15 HOURS. THIS MAKES THE RATE OF CALCIUM DEPOSITION IN THE EGG SHELL GREATER THAN THE INCREASED RATE OF CALCIUM ABSORPTION FOUND
DURING EGG SHELL CALCIFICATION. THEREFORE CALCIUM ABSORPTION IS UNLIKELY TO BE THE SOLE FACTOR IN PROVIDING CALCIUM FOR THE EGG SHELL. FURTHERMORE, AS THE MAXIMAL PERIOD OF EGG SHELL CALCIFICATION OCCURS IN THE EARLY HOURS OF THE MORNING, AT WHICH TIME THE HENS ARE NOT EATING, THE SUPPLY OF ABSORBED CALCIUM IS DECREASED AT THIS TIME AS THE CONTENTS OF THE GUT ARE USED UP. THEREFORE, EVEN IN BIRDS ON AN ADEQUATE CALCIUM DIET, THERE IS A VERY STRONG POSSIBILITY THAT BONE MOBILIZATION IS IMPORTANT DURING EGG SHELL CALCIFICATION.


WASSERMAN & TAYLOR (1966) AND TAYLOR & WASSERMAN (1967) SHOWED THAT ADMINISTRATION OF VITAMIN D3 TO RACHITIC CHICKS RESULTED IN THE APPEARANCE OF A CALCIUM BINDING PROTEIN (CaBP) IN THE INTESTINAL TISSUE WHICH WAS NOT PRESENT IN UNTREATED
rachitic chicks. The vitamin D enhanced duodenal absorption of 47Ca in rachitic chicks occurred almost simultaneously with the appearance of the vitamin D3-induced factor, and there was good correlation between the concentration of binding factor and the rate of absorption of 47Ca, Wasserman & Taylor (1969). The vitamin D3-induced production of CaBP could be prevented with actinomycin D, Corradino & Wasserman (1968, 1971), suggesting that vitamin D acts via gene derepression to cause the formation of a specific calcium binding protein (CaBP), which is intimately involved in calcium transport across the chick intestinal wall. If this is correct then the amount of CaBP in the intestinal mucosa should vary with the calcium absorptive capacity of the gut, and the calcium need of the animal under different situations. The findings of Wasserman co-workers substantiate this, see Wasserman & Taylor (1968) for references, and may be summarized as follows:—

1. The CaBP content and calcium absorptive efficiency of various segments of the chick small intestine vary in the same direction i.e. duodenum > jejunum > ileum.

2. Young rapidly-growing chicks which need to absorb more calcium have more mucosal CaBP than older individuals.

3. Laying hens, which increase their intestinal absorption of calcium in response to the stress of egg shell formation, have more mucosal CaBP than non-laying hens of the same age. This difference is not due to estrogens since the latter were not capable of increasing calcium absorption in the ligated duodenum of
RACHITIC CHICKS, NOR DID THEY CAUSE INDUCTION OF 
CABP.

4. CHICKS THAT HAVE ADAPTED TO LOW CALCIUM INTAKES 
ABSORB MORE 47Ca AND HAVE MORE INTESTINAL CABP 
THAN CHICKS MAINTAINED ON A NORMAL CALCIUM DIET.

5. TWO OTHER TISSUES WHICH CONTAIN EPITHELIAL CELLS 
ACROSS WHICH CALCIUM IS TRANSFERRED HAVE ALSO BEEN 
SHOWN TO CONTAIN CABP. THE KIDNEYS OF VITAMIN D3 
TREATED RACHITIC CHICKS CONTAIN CABP, WHILE THEIR 
COLON, LIVER AND MUSCLE DO NOT, AND THE UTERUS OF 
THE LAYING HEN PRODUCES CABP IN RESPONSE TO VITAMIN 
D3. IT IS OF SPECIAL INTEREST THAT CABP IS ONLY 
FOUND IN THOSE TISSUES HAVING A MECHANISM FOR MOVING 
CALCIUM ACROSS THE TOTAL EPITHELIAL CELL, AND HENCE 
ONE CAN CONCLUDE THAT IT IS SPECIFICALLY INVOLVED 
IN THE TRANSMURAL FLUX OF CALCIUM.

THERE ARE TWO SCHOOLS OF THOUGHT ABOUT THE ROLE OF CABP 
IN CALCIUM TRANSLLOCATION. ONE IDEA IS THAT IT IS INVOLVED IN 
ACTIVE TRANSPORT, THE OTHER IS THAT IT IS INVOLVED IN FACIL-
ITATED TRANSPORT. IN VITRO STUDIES BY DOWDLE ET AL (1960) 
AND SCHACHTER ET AL (1961) SUGGEST THAT CALCIUM TRANSPORT IN 
THE RAT GUT IS AN ACTIVE PROCESS. HOWEVER INTESTINAL TRANSPORT 
IN THE CHICKEN APPEARS TO BE A PASSIVE PROCESS, HURWITZ & BAR 
KALLFELZ (1962) AND WASSERMAN ET AL (1966) HAVE SHOWN THAT IN 
CHICKS THE EFFECT OF VITAMIN D IS TO INCREASE BOTH EFFLUX 
(LUMEN TO PLASMA) AND INFLUX (PLASMA TO LUMEN) OF CALCIUM.
They conclude from this that the effect of vitamin D is to increase the passive permeability of the intestine to calcium, which is consistent with the idea that CaBP is a carrier involved in facilitated diffusion of calcium across the intestinal wall.

Vitamin D has other effects on the laying hen besides increasing calcium absorption from the gut. As pointed out above, it causes induction of CaBP in the shell gland and hence increases calcium translocation in this organ. Even if the role of CaBP here is to aid in facilitated diffusion the calcium transported is deposited as insoluble calcium carbonate on the egg shell so that back diffusion is negligible. Thus the effect of vitamin D is to increase calcium transport to the egg shell. Because, in the absence of vitamin D, laying hens produce very few eggs (the eggs which they do produce are soft-shelled), but no other changes occur in the secondary sex characteristics of the birds, Turk & McGinnis (1964) concluded that vitamin D is involved in the regulation of ovulation and/or follicular growth. This effect of vitamin D may be mediated by changes in plasma calcium levels influencing the secretion of pituitary gonadotropins, which, in turn, regulate ovarian activity, Taylor (1965, 1970b). However not all data is consistent with this view, see Gilbert (1967), so that, until further data is obtained, the relationship between vitamin D, plasma calcium levels and ovarian function must remain obscure.

The average egg shell weighs 5 g and consists almost entirely of calcium carbonate, as calcite crystals, Simkiss
As these crystals are not hydrated calcium ions only make up 40% of the egg shell, the remaining 60% consisting of carbonate ions. Since the bicarbonate-carbonic acid system is an important buffer system in the body the laying hen has to make adjustments in her acid-base balance.

As soon as the egg enters the shell gland the pH of the blood begins to fall causing acidosis. This is partially compensated by a fall in pCO2 due to increased respiration, Mongin & Lacassagne (1966a), and a decrease in bicarbonate ion excretion by the kidneys, Anderson (1967). However the bicarbonate ion level of the blood still falls 30% and the pH remains low until just before oviposition, Mongin & Lacassagne (1966b), indicating that the bird is unable to adjust completely.

Several studies on the effect of perturbations in the acid-base status of laying hens on shell formation have shown that acidosis causes an increase in shell strength while alkalosis causes a decrease in shell strength, Hall & Helbacka (1959), Frank & Burger (1965) and Mueller (1966).

A certain amount of controversy exists in the literature on the mechanism of formation of the carbonate fraction of the egg shell, but it is generally accepted that carbonic anhydrase is involved. Common (1941) demonstrated the presence of carbonic anhydrase (C.A.) in shell gland tissue by biochemical means, whilst Diamanstein & Schlüns (1964) have histochemically localized its position to the apices of the uterine tubular glands.
Gutowski & Mitchell (1945) suggested that two bicarbonate ions reacted to form a carbonic acid molecule and a carbonate radicle. The latter would be used in the calcification reaction while C.A. would cause the acid to dissociate into carbon dioxide and water and so serve to shift the equilibrium in favor of the formation of more carbonate ions.

\[
2\text{HC}O_3^- = \text{H}_2\text{CO}_3 + \text{CO}_3^{2-} \\
\downarrow \text{C.A.} \\
\text{H}_2\text{O} + \text{CO}_2
\]

Gutowski and Mitchell's theory of the action of C.A. has been refuted by Diamantstein & Schlüns (1964), who calculated that, if all the carbon dioxide of the shell gland secretion was present as carbonate, the solubility product of calcium carbonate would be exceeded and crystalline calcium carbonate would be precipitated within the tubular glands of the shell gland epithelium. Since this does not occur they concluded that the anion secreted into the shell gland lumen was bicarbonate not carbonate ion. Diamantstein (1966) suggested that the shell gland derives the shell carbonate from its own metabolic carbon dioxide.

\[
\begin{array}{ccc}
\text{BLOOD} & \text{SHELL GLAND} & \text{LUMEN} \\
\text{METABOLIC} & \text{CO}_2 + \text{H}_2\text{O} \\
\downarrow \text{C.A.} \\
\text{H}_2\text{CO}_3 \\
\downarrow \text{H}^+ \text{ INTO BLOOD} & \text{H}^+ + \text{HC}O_3^- \rightarrow \text{HC}O_3^- \rightarrow \text{H}^+ + \text{CO}_3^{2-} \\
\text{DEPOSITED} & \text{AS CA}_3\text{CO}_3
\end{array}
\]
The idea that the free H+ liberated in the lumen in some way returns to the blood is supported by the finding of El Jack & Lake (1967) that the pH of uterine fluid is about 7.7. This mechanism would account for the presence of C.A. in the glandular cells and also, by secretion of H+ ions into the blood stream, would account for the drop in blood pH during shell formation. Furthermore Diamantstein suggested that the fall in the pH of the blood could cause the dissociation of calcium from its protein complex in the blood and hence facilitate calcium secretion by the shell gland. Diamantstein's theory is supported by the data of Lörcher & Hodges (1969), who showed that 57% of a 45Ca tracer entering the shell gland did not emerge in the venous effluent, while none of a 14C-labelled sample of sodium bicarbonate was lost.

From the above discussion one can see that the laying hen makes many adaptations to the process of egg laying, and shows changes not only in her calcium metabolism, but also in her acid-base balance in order to facilitate egg shell formation.

This study was undertaken to examine the manner in which these metabolic changes are regulated and coordinated to allow the regular production of fully-formed, calcified eggs.
CHAPTER I: CALCIUM TRANSPORT BY THE SHELL GLAND IN VITRO.

INTRODUCTION.

The avian shell gland provides a model system in which to study the translocation of relatively large amounts of calcium by biological membranes, and possesses several features which facilitate the study of this phenomenon. They can be summarized as follows:

1. It secretes large quantities of calcium (2g in a period of approximately 15 hours).
2. It has regular periods of shell deposition, implying that the "pump" must be turned on and off fairly specifically.
3. There is a distinct physical separation of the cellular site of calcium translocation from the site of mineral deposition.

Ehrenspeck et al. (1967) developed an in vitro preparation of the chicken shell gland in which they could show calcium transfer to the mucosal or lumen side of the tissue, as traced with 45Ca, without an external transmural concentration gradient. The transfer was dependent on the presence of an egg in the shell gland at the time of its excision and required energy derived from oxidative metabolism.

As discussed in the general introduction, the levels of parathyroid hormone (PTH) are thought to increase during egg shell calcification. Urist (1967) points out that the ultimotobranchial bodies of laying hens are enlarged and contain
EASILY EXTRACTABLE LARGE AMOUNTS OF CALCITONIN (CT), SUGGESTING THAT THIS HORMONE MIGHT ALSO BE EXPECTED TO INFLUENCE EGG SHELL FORMATION IN SOME WAY. THIS FIRST SERIES OF EXPERIMENTS WAS UNDERTAKEN TO EXAMINE THE MANNER IN WHICH THE TWO CALCIUM REGULATING HORMONES MIGHT INFLUENCE MOVEMENT OF CALCIUM ACROSS THE SHELL GLAND IN VITRO.
<table>
<thead>
<tr>
<th>SOLUTION</th>
<th>NaCl (mM)</th>
<th>Tris Cl (mM)</th>
<th>KCl (mM)</th>
<th>CaCl₂ (mM)</th>
<th>MgCl₂ (mM)</th>
<th>Glucose (mM)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock Buffer No. 1</td>
<td>130</td>
<td>20</td>
<td>5</td>
<td>2.5</td>
<td></td>
<td></td>
<td>7.2</td>
</tr>
<tr>
<td>Stock Buffer No. 2</td>
<td>130</td>
<td>20</td>
<td>20</td>
<td>2.0</td>
<td>1.0</td>
<td>15.0</td>
<td>7.2</td>
</tr>
</tbody>
</table>
METHODS.


TWO TYPES OF EXPERIMENT, SINGLE AND DUPLICATE, WERE PERFORMED. THE SHELL GLAND WAS TIED ONTO THE END OF A GLASS TUBE (DIAMETER 2.1 CM) USING TWO POLYETHYLENE STRAPS APPLIED WITH A PRESSURE GUN (TYPE GS-2B, PANDUIT CORP., TINLEY PARK, ILL.); IN ORDER TO MINIMIZE VARIATIONS IN THE STRETCH OF THE MEMBRANES. IN THE SINGLE EXPERIMENTS THE SEROSAL SIDE OF THE GLAND FACED THE INTERIOR OF THE TUBE WHILE THE VILLI ON THE MUCOSAL SIDE FACED OUTWARDS, SEE FIGURE 1. EXCESS TISSUE AROUND THE EDGES WAS TRIMMED AWAY. IN THE DUPLICATE EXPERIMENTS THE SHELL GLAND WAS CUT IN HALF LONGITUDINALLY AND EACH HALF WAS MOUNTED ON A SEPARATE GLASS TUBE. TWO BASIC TYPES OF DUPLICATE EXPERIMENT WERE PERFORMED, SEE TABLE II. IN TYPES A AND C BOTH SAMPLES WERE MOUNTED AS IN THE SINGLE EXPERIMENTS, I.E. MUCOSAL SIDE FACING OUTWARDS. IN TYPE B ONE MEMBRANE WAS MOUNTED IN THE OPPOSITE MANNER, I.E. MUCOSAL SIDE FACING INWARDS.

THE APPARATUS USED IS SHOWN IN FIGURE 1. EACH TUBE WAS CLAMPED SO THAT THE END CLOSED BY THE SHELL GLAND COULD BE LOWERED INTO A 100 ML BEAKER CONTAINING 50 ML OF BUFFER (THE
FIGURE 1. THE APPARATUS USED IN THE IN VITRO SHELL GLAND EXPERIMENTS.
The solution within the tube (the inner solution) was 10 ml of the same buffer as was used for the outer solution. Hydrostatic pressure gradients were avoided by adjusting the position of the tube to keep the inner and outer liquid surface levels the same. Both the inner and outer solutions were aerated with 95% O2, 5% CO2; the continuous stream of bubbles also served to stir the solutions. The whole apparatus was maintained at 37.0 ± 0.5 °C with a circulating water bath.

Either inner or outer solution could be labelled by adding 45Ca (obtained from New England Nuclear, Boston Mass.) to the stock buffer; while samples could be removed from either chamber and counted to determine calcium fluxes. Sample size was 50 μl. The outer chamber sampling system, as shown in Figure 1, comprised a 10 ml syringe into which 1-2 ml of solution were drawn via the bent glass tube. A 50 μl aliquot was removed for counting and the rest of the sample was returned to the outer chamber.

A summary of the different types of experimental arrangement is presented in Table II.

An incubation was started with addition of the label and the samples were maintained at 37.0 ± 0.5 °C for approximately seven hours. 50 μl aliquots of the appropriate solution were counted every half hour. Each 50 μl sample was applied to one edge of 4 x 5 cm piece of Whatman No. 1 filter paper folded into a concertina. The micropipet in which the sample was measured was rinsed with distilled water and the washing applied to the
<table>
<thead>
<tr>
<th>Type of Experiment</th>
<th>Sub-Group</th>
<th>Sample No.</th>
<th>Shell Gland Mounting - Mucosa of Shell Gland Pointed</th>
<th>Stock Buffer Used</th>
<th>Soln. to which 45Ca was Added</th>
<th>Vol. of Soln. (mL)</th>
<th>Vol. of 45Ca Added (μL)</th>
<th>Activity of the Final Solution (μcuries/μL)</th>
<th>Solution Sampled</th>
<th>Flux Measured *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single</td>
<td></td>
<td></td>
<td>Outwards 1</td>
<td>1</td>
<td>Inner</td>
<td>10</td>
<td>50</td>
<td>3.65</td>
<td>Outer</td>
<td>Influx</td>
</tr>
<tr>
<td>Duplicate A</td>
<td>A</td>
<td>1</td>
<td>Outwards 1</td>
<td>1</td>
<td>Inner</td>
<td>10</td>
<td>100 (in 25 mL)</td>
<td>2.92</td>
<td>Outer</td>
<td>Influx</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>Outwards 1</td>
<td>1</td>
<td>Inner</td>
<td>10</td>
<td>10 (in each inner)</td>
<td></td>
<td>Outer</td>
<td>Influx</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>1</td>
<td>Inwards 1 or 2</td>
<td>Outer</td>
<td>50</td>
<td>100</td>
<td>1.46</td>
<td></td>
<td>Inner</td>
<td>Influx</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>Outwards</td>
<td>Outer</td>
<td>50</td>
<td>100</td>
<td>1.46</td>
<td></td>
<td>Inner</td>
<td>Efflux</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>1</td>
<td>Outwards</td>
<td>1</td>
<td>Inner &amp; Outer</td>
<td>10</td>
<td>50, 250</td>
<td>3.65</td>
<td>Inner</td>
<td>Net Efflux</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>Outwards</td>
<td>1</td>
<td>Outer</td>
<td>50</td>
<td>250</td>
<td>3.65</td>
<td>Inner</td>
<td>Efflux</td>
</tr>
</tbody>
</table>

* Influx = Blood → Lumen
Efflux = Lumen → Blood
FILTER PAPER, WHICH WAS THEN DRIED UNDER A HEAT LAMP AND PLACED, SPOTTED EDGE DOWN, IN A GLASS VIAL CONTAINING 15 ML OF SCINTILLATION FLUID (4G PPO, 50 MG POPOP*, LITRE OF TOLUENE). COUNTING WAS DONE ON A BECKMAN LS-233 SCINTILLATION COUNTER.

A PLOT OF TIME VERSUS CPM IN 50 μL OF THE SOLUTION GAVE A MEASURE OF CALCIUM FLUX AND SLOPES OF THE LINES SO OBTAINED WERE USED TO CALCULATE THE FLUXES OF CALCIUM IN NMOLES OF Ca/CM2 HR.

ALL DRUGS AND HORMONES WERE ADDED TO THE INNER SOLUTION OF THE SAMPLE AFTER SUFFICIENT TIME HAD ELAPSED FOR A CONTROL FLUX TO BE ESTABLISHED. IN THE CASE OF PTH THE VEHICLE WAS ALSO TESTED FOR POSSIBLE EFFECTS. THE VOLUME OF SOLUTION ADDED WAS SMALL, GENERALLY BETWEEN 20 AND 100 μL, TO MINIMIZE VOLUME CHANGES IN THE INNER CHAMBER.


THE DIBUTYRYL CYCLIC AMP WAS OBTAINED FROM SIGMA CHEMICAL CO., LOS ANGELES, CAL.; IT WAS THE ANHYDROUS FORM OF MONOSODIUM DIBUTYRYL CYCLIC 3', 5'- ADENOSINE MONOPHOSPHATE.

*PPO 2,5- DIPHENYLOXAZOLE
POPOP 1,4-BIS- 2(5-PHENOXAZOLYI)-BENZENE
BOTH SCINTILLATION GRADE OBTAINED FROM FRAZER MEDICAL SUPPLIES, VANCOUVER, B.C.
### TABLE III

**45Ca Fluxes in the In Vitro Shell Gland System with Tissue Obtained From Hens in Various Physiological Conditions**

<table>
<thead>
<tr>
<th>Physiological State of Hen</th>
<th>Calcium Fluxes* in nmoles Ca/cm² hr</th>
<th>Type of Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Influx</td>
<td>Efflux</td>
</tr>
<tr>
<td>Egg in Isthmus</td>
<td>21.79 ± 1.0</td>
<td>32.22 ± 1.4</td>
</tr>
<tr>
<td>Plumping Egg in Shell Gland</td>
<td>81.58 ± 1.65</td>
<td>77.81 ± 0.99</td>
</tr>
<tr>
<td>Active Calcification of Egg Shell</td>
<td>53.69 ± 2.06</td>
<td>94.45 ± 3.20</td>
</tr>
<tr>
<td></td>
<td>23.16 ± 0.68</td>
<td>40.81 ± 0.62</td>
</tr>
<tr>
<td>Egg Just Laid</td>
<td>38.2 ± 4.9</td>
<td>Single</td>
</tr>
<tr>
<td></td>
<td>57.8 ± 2.3</td>
<td>Single</td>
</tr>
<tr>
<td></td>
<td>178.2 ± 4.0</td>
<td>Single</td>
</tr>
<tr>
<td></td>
<td>59.00 ± 1.75</td>
<td>65.55 ± 0.41</td>
</tr>
</tbody>
</table>

* Influx = Blood → Lumen  
  Efflux = Lumen → Blood
RESULTS & DISCUSSION.

CHARACTERIZATION OF THE IN VITRO SYSTEM.

The dramatic changes in secretion levels exhibited by each part of the oviduct as the egg passes from the ovary to the exterior suggest that the translocation of calcium by the shell gland is a discontinuous process. The calcium transporting capacity of the tissue might well be low when the egg is not in the gland and become much greater during shell formation. Although these variations may be exhibited in vitro there is no a priori reason why they should still be evident in excised strips of shell gland tissue. The initial experiments in this section were planned to examine the relationship between the physiological state of the hen and the calcium translocating capacity of excised shell gland.

The values for influx of calcium into the lumen of the shell gland which were obtained in the various physiological states (i.e. egg in different parts of the oviduct) overlapped, with low values being obtained both during active shell calcification and when the egg was in the isthmus, whilst the highest value was obtained in a preparation taken after the egg was laid. For example (see Table III), when the egg was in the isthmus, where egg white proteins were being incorporated, the influx measured was 22 nmoles Ca/cm² hr, whereas when the egg was in the plumping stage the influx was approximately 80 nmoles Ca/cm² hr. A wide variation in fluxes was obtained even with birds in the same physiological condition. The influxes
Measured during active shell calcification were 94, 54 and 23 nmoles Ca/cm² hr, with the first two values being obtained from the two halves of the same shell gland. Gland preparations taken after the egg had been laid showed influx rates of between 38 and 178 nmoles Ca/cm² hr. Thus, under the experimental conditions used, the absolute size of the influx was independent of the physiological state of the shell gland.

The data obtained here do not agree with that of Ehrenspeck et al. (1967), who found the flux to be 30 nmoles Ca/cm² hr in both directions when no egg was present, while during active calcification influx into the shell gland lumen was 50 nmoles Ca/cm² hr and efflux was 20 nmoles Ca/cm² hr. Thus, although the fluxes measured in this study and those of Ehrenspeck et al. were of a similar magnitude, the results obtained here showed that influx was independent of the physiological state of the shell gland and was very variable from preparation to preparation, whilst the results of Ehrenspeck et al. (1967) showed that influx and efflux were equal when no egg was present in the oviduct, but that influx was twice efflux when the shell gland was excised during active shell calcification.

No matter what the physiological state of the gland the efflux obtained was always greater than the influx in these experiments, see Table III. When the egg was in the isthmus the efflux was approximately 1.5 times as large as influx, whereas during active calcification it was twice as large as influx. This data is thus in direct conflict with that of
Figure 2. A graph showing net efflux of 45Ca (lumen→blood) in a shell gland excised during active shell calcification. Labelled calcium was present on both sides of the membrane.
Ehrenspeck et al. (1967).

In order to check that these results were not due to physical drawbacks in the experimental system a double label experiment was performed on a shell gland excised during active shell calcification (duplicate type C sample no. 1). This experiment eliminated any spurious results due to exchange diffusion of non-labelled calcium for 45Ca, since the specific activity of the solutions on either side of the membrane was the same. The results are shown in figure 2.

The actual parameter measured here was net efflux. From zero to three hours the net efflux was $-75.3 \pm 21.7$ nmoles Ca/cm² hr. Thus in fact a net influx of $+75.3 \pm 21.7$ nmoles Ca/cm² hr was taking place. However from 3.5 to 7 hours the net efflux became practically nil, i.e. $-1.77 \pm 5.2$ nmoles Ca/cm² hr, implying that the two fluxes had come to equilibrium and influx was approximately equal to efflux. When one considers sample no. 2 in this experiment which measured efflux in the other half of the shell gland, see figure 3, one finds that from zero to three hours efflux was increasing non-uniformly and at this time was not very great. However from 3.5 to 7 hours the efflux was linear and was equal to $126.9 \pm 4.5$ nmoles Ca/cm² hr. During 3.5 to 7 hours the net efflux was from $-7$ to $+3$ nmoles Ca/cm² hr. Therefore the influx must have been somewhere from 116 to 136 nmoles Ca/cm² hr, i.e. $126 \pm 10$ nmoles Ca/cm² hr. Thus the duplicate label experiment indicated that the influx was only greater than the efflux during the first three hours of the experiment when the individual fluxes were small and non-linear so that they were
Figure 3. Efflux (lumen $\rightarrow$ blood) of $^{45}$Ca in a shell gland preparation taken during active shell calcification. (The material was from the same gland as that used in Figure 2.)
UNMEASUREABLE. Once the fluxes became linear influx and efflux had come to equilibrium and no net exchange of calcium was taking place. Again this does not agree with Ehrenspeck et al. (1967), but, as this group carried out no experiments with label on both sides of the membrane, a direct comparison of data is difficult to make.

In the duplicate type B experiments efflux was always found to be greater than influx, see tables III and V, while the double label experiment implied that the fluxes were equal after the initial three hours. This apparent paradox may be explained in the following manner: In the efflux setup the mucosal surface of the shell gland was pointing outwards, whereas in the influx setup it was pointing inwards; but, in both cases, the label was added to the outer solution while the inner solution was sampled. Since the mucosal surface of the shell gland has many invaginations and microvilli it has a larger total surface area than the serosal surface. Therefore, in the efflux case, there was a larger surface area of the shell gland in contact with the 45Ca solution, giving rise to a larger measured efflux than influx. However, the difference is a result of the geometry of the experimental arrangement rather than a physiological effect. Ehrenspeck et al. (1967) used a similar experimental design to the one used in the duplicate type B experiments, so that it is difficult to explain why they did not see this effect. Quite possibly, as the surface area of the membrane used in their experiments (2.96 cm²) was smaller than that used here
TABLE IV

THE EFFECT OF MAGNESIUM CHLORIDE AND GLUCOSE ON THE IN VITRO 45Ca FLUXES ACROSS A SHELL GLAND EXCISED DURING ACTIVE EGG SHELL CALCIFICATION

<table>
<thead>
<tr>
<th>MEDIUM*</th>
<th>FLUXES (nmoles Ca/cm² hr)</th>
<th>FLUX DIRECTION</th>
<th>% RESPONSE °</th>
<th>FLUX RATIO</th>
<th>% CHANGE IN FLUX RATIO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>INFLUX</td>
<td>EFFLUX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stock buffer No. 1</td>
<td>23.16 ± 0.68</td>
<td>40.81 ± 0.62</td>
<td></td>
<td>0.5675</td>
<td></td>
</tr>
<tr>
<td>+MgCl₂ (1mM)</td>
<td>27.44 ± 0.55</td>
<td>52.88 ± 4.0</td>
<td>INFLUX</td>
<td>+18.48</td>
<td>0.5189</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EFFLUX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Glucose (1mM)</td>
<td>28.40 ± 0.70</td>
<td>60.39 ± 1.33</td>
<td>INFLUX</td>
<td>+3.49</td>
<td>0.4703</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EFFLUX</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* REAGENTS WERE ADDED TO BOTH INNER AND OUTER SOLUTIONS.

° % CHANGE IN THE SLOPE OVER THAT IMMEDIATELY PRIOR TO THE ADDITION OF THE REAGENT.

* FLUX RATIO = INFLUX / EFFLUX = BLOOD → LUMEN / LUMEN → BLOOD
(3.47 cm²), the geometry effect in their experiments could have been reduced.

There were a number of other minor experimental differences between the system used here and that employed by Ehrenspeck et al. (1967). For example, stock buffer no. 1, which was used as the medium in most of the experiments, differed from the medium of Ehrenspeck et al. in that it lacked both magnesium and glucose, see Table 1. Although it appeared unlikely that either was having a major effect on the system, as the overall fluxes observed here and those of Ehrenspeck et al. were similar, the influence of both chemicals on the fluxes was tested. As expected there was no major change in flux rates, see Table IV and Figure 4. 1 mM MgCl₂ increased influx 18% and efflux 30%, while 1 mM glucose only increased influx 3% and efflux 14%.

Despite these results several more duplicate type B experiments were carried out using stock buffer no. 2, which was very similar to the medium used by Ehrenspeck et al. (1967). As can be seen from Table V, the only change in the results was that the influx was lower than when buffer no. 1 was used, it dropped from approximately 22 to 9 nmoles Ca/cm² hr. In a recent paper, published after this series of experiments was completed, Ehrenspeck et al. (1971) also found that omission of glucose from their medium had no effect on their fluxes.

In these experiments the buffers were aerated with 95% O₂, 5% CO₂. The carbon dioxide was included because Lörcher & Hodges (1969) showed that the carbonate fraction of the egg
**Figure 4.** The effect of addition of magnesium chloride and glucose to the incubation medium on 45Ca fluxes in a shell gland excised during active shell calcification.
Shell was formed from carbon dioxide, and if the calcium pump was linked in some way to the carbonate transfer, lack of carbon dioxide could be inhibitory. In one experiment aeration with 100% O2 was tried, see Table V. There was no change in influx but efflux dropped from about 48 to 20 nmoles Ca/cm² hr. An increase in efflux would tend to decrease egg shell calcification. However, in vivo, any calcium transported into the shell gland is deposited as insoluble calcium carbonate so that any increase in efflux would have negligible effects on egg shell calcification.

Ehrenspeck et al. (1967) used 100% O2 to aerate their system. However in their (1971) paper they report that substitution of bicarbonate for tris buffer and aeration with 95% O2, 5% CO2 had no effect on their in vitro fluxes. Furthermore, inhibition of carbonic anhydrase with 50 mM acetazolamide increased both influx and efflux in their system, but had no significant effect on the flux ratio. They interpreted these results as an indication that calcium and carbonate translocation were not tightly coupled. Thus these results tend to agree with the data found here.

In their 1971 paper Ehrenspeck et al. repeated the flux ratio experiments which they had done in 1967 and obtained essentially similar data. They also substantiated their label experiments by showing that total calcium changed. They found that when paired membranes were incubated in solutions which contained 75±1 µg Ca/ml after four hours of incubation the calcium concentrations of the solutions
TABLE V

The Effect of Changing the Incubation Buffer and Aeration Gas on In Vitro 45Ca Fluxes in the Shell Gland

<table>
<thead>
<tr>
<th>Physiological State of Gland</th>
<th>Stock Buffer</th>
<th>Aeration</th>
<th>Calcium Fluxes nmoles Ca/cm² HR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Influx</td>
</tr>
<tr>
<td>Egg in Isthmus</td>
<td>1</td>
<td>95% O₂, 5% CO₂</td>
<td>21.79 ± 1.0</td>
</tr>
<tr>
<td>Active Shell Calcification</td>
<td>1</td>
<td>95% O₂, 5% CO₂</td>
<td>23.16 ± 0.68</td>
</tr>
<tr>
<td>Active Shell Calcification</td>
<td>2</td>
<td>95% O₂, 5% CO₂</td>
<td>8.029 ± 0.108</td>
</tr>
<tr>
<td>Active Shell Calcification</td>
<td>2</td>
<td>100% O₂</td>
<td>9.99 ± 0.13</td>
</tr>
</tbody>
</table>

* Flux = \( \frac{\text{Influx}}{\text{Efflux}} = \frac{\text{Blood} \rightarrow \text{Lumen}}{\text{Lumen} \rightarrow \text{Blood}} \)
had increased to $81 \pm 1 \mu g \text{Ca}/\text{ml}$ on the mucosal side and decreased to $66 \pm 2 \mu g \text{Ca}/\text{ml}$ on the serosal side.

At present there seems no explanation for the disagreement between the results found here and those of Ehrenspeck et al. Their fluxes were of the same order of magnitude as those reported here. However they found that influx and efflux were equal when no egg was present in the oviduct but that influx was twice efflux when the shell gland was excised during active shell calcification, while these experiments were unable to show any relationship between egg shell calcification and the sizes of the fluxes. Although the fluxes obtained here were very variable, see Table III, the double label experiment implied that influx and efflux were equal even in a shell gland removed from a hen during active calcification.

Hormones.

Despite the obvious drawbacks to the experimental system, the effects of parathyroid hormone (PTH) and calcitonin (CT) on the in vitro fluxes was tested. A summary of the results is shown in Table VI.

In the first single experiment reported the PTH appeared to cause a large increase in influx, but this early result was never repeated and may well have been due to a leak in the membrane. The second single experiment in Table VI is shown in Figure 5. Although the hormone appears to have been responsible for a marked increase in influx the control application
<table>
<thead>
<tr>
<th>Type of Experiment</th>
<th>Physiological State of Hen</th>
<th>PTH Vehicle</th>
<th>CT (M.R.C. Milli-units)</th>
<th>Influx nmoles Ca/cm²·hr</th>
<th>Treatment</th>
<th>% * Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>SINGLE</td>
<td>Egg Just Laid</td>
<td>0.01M NH₄COOCH₃ 0.001M MERCAPTOETHANOL PH 5.3</td>
<td>350</td>
<td>38.2 ± 4.9 821.91 ± 33.3</td>
<td>PTH</td>
<td>+2051.5</td>
</tr>
<tr>
<td>SINGLE</td>
<td>Egg Just Laid</td>
<td>0.01M NH₄COOCH₃ PH 5.3</td>
<td>25</td>
<td>57.8 ± 2.3 112.1 ± 1.97 203.8 ± 4.5</td>
<td>PTH VEHICLE</td>
<td>+82 +94</td>
</tr>
<tr>
<td>DUPLICATE Type A</td>
<td>Plumping Egg in Shell Gland</td>
<td>Stock Buffer No. 1</td>
<td>40</td>
<td>81.58 ± 1.65 132.79 ± 2.94</td>
<td>PTH VEHICLE</td>
<td>+24.8</td>
</tr>
<tr>
<td>DUPLICATE Type A</td>
<td>Egg Just Laid</td>
<td>Stock Buffer No. 1, 0.125M IN CYSTEINE HCL</td>
<td>40</td>
<td>59.00 ± 1.75 65.55 ± 0.41 65.97 ± 2.35 73.01 ± 2.36</td>
<td>PTH VEHICLE</td>
<td>+23.7</td>
</tr>
<tr>
<td>DUPLICATE Type A</td>
<td>Calcifying Egg in Shell Gland</td>
<td>Stock Buffer No. 1, 0.125M IN CYSTEINE HCL</td>
<td>200 400</td>
<td>53.69 ± 2.06 94.45 ± 3.20 90.42 ± 0.52 67.41 ± 0.92 98.32 ± 8.74</td>
<td>PTH VEHICLE CT</td>
<td>+25.6 -5.27</td>
</tr>
</tbody>
</table>

* % INCREASE OF THE SLOPE OVER THAT IMMEDIATELY PRIOR TO THE ADDITION OF THE CHEMICAL LISTED IN THE TREATMENT COLUMN.
OF VEHICLE INDICATES THAT THE EFFECT IS MINIMAL. IN FACT THE INCREASE IN INFLUX DUE TO VEHICLE ALONE IS SLIGHTLY GREATER THAN THAT CAUSED BY PTH; I.E. % RESPONSE TO VEHICLE WAS +95% WHILE % RESPONSE TO PTH WAS ONLY +84%. THEREFORE FROM THIS EXPERIMENT ONE WOULD CONCLUDE THAT PTH HAS NO EFFECT ON CALCIUM INFLUX.

THE DUPLICATE TYPE A EXPERIMENTS, TABLE VI, IN GENERAL SUPPORT THIS CONCLUSION, ALTHOUGH THE LAST TWO SHOW A SLIGHT TENDANCY FOR PTH TO INCREASE CALCIUM INFLUX, SEE FIGURE 6. IN VIEW OF THE VARIATION IN THE SIZE OF FLUXES FROM MEMBRANE PREPARATION TO PREPARATION, AND BETWEEN THE TWO HALVES OF THE SAME MEMBRANE, THIS TENDANCY CAN HARDLY BE CONSIDERED SIGNIFICANT.

THE IN VIVO STUDIES OF THE EFFECTS OF PTH DEPRIVATION OR EXCESS IN LAYING HENS ARE NOT DEFINITIVE. WHILE TOTAL PARATHYROIDECTOMY IN HENS LEADS TO DEATH WITHIN 48 HOURS MANY ANIMALS HAVE ACCESSORY PARATHYROID TISSUE EMBEDDED IN THE ULTIMOBANCHIAL OR THYROID GLANDS AND THESE ARE ABLE TO SURVIVE, URIST (1967). THE PRESENCE OF THIS ACCESSORY TISSUE COMPLICATES THE INTERPRETATION OF THE EXPERIMENTAL RESULTS. POLIN & STURKIE (1957) FOUND THAT PARATHYROIDECTOMY OF LAYING HENS CAUSED PREMATURE EXPULSION OF THE EGG 3-7 HOURS AFTER THE OPERATION, WHICH THEY SUGGESTED COULD PROTECT THE HEN AGAINST HYPOCALCEMIA IN THE ABSENCE OF THE PARATHYROIDS, SINCE HENS WHICH DID NOT EXPEL THEIR EGGS PREMATURELY GENERALLY DIED. THE HENS STOPPED LAYING FOR ABOUT SIX DAYS AND THEN RESUMED LAYING NORMAL EGGS. THEY SHOWED A MARKED DROP IN BOTH THE DIFFUSIBLE AND NON-DIFFUSIBLE CALCIUM LEVELS IN PLASMA 18 TO 24 HOURS AFTER THE OPERATION. THE DROP IN NON-DIFFUSIBLE PLASMA CALCIUM WAS PROBABLY DUE TO
Figure 5. The effect of PTH on 45Ca influx (blood→lumen) in a shell gland excised just after the egg had been laid.
STARVATION AND SURGICAL TRAUMA SINCE SHAM-OPERATED AND STARVED 
HENs SHOWED A SIMILAR DECREASE IN THIS PLASMA CALCIUM FRACTION. 
A FEW OF THE PARATHYROIDECTOMIZED HENS IN THIS STUDY SHOWED A 
MUCH LONGER PAUSE BEFORE RESUMING EGG PRODUCTION, AND OTHERS 
DIED A FEW DAYS AFTER THE OPERATION; THESE MAY HAVE BEEN BIRDS 
WHICH DID NOT POSSESS ACCESSORY PARATHYROID TISSUE. THUS 
PARATHYROIDECTOMY IN BIRDS DECREASES THE DIFFUSIBLE PLASMA 
CALCIUM LEVEL AND MAY ALSO CAUSE AN INTERRUPTION IN EGG PRODUC­ 
TION, ALTHOUGH THE RESULTS ARE NOT DEFINATIVE BECAUSE OF THE 
PRESENCE OF ACCESSORY GLAND TISSUE.

STUDIES OF THE EFFECT OF EXOGENOUS PTH ON LAYING HENS ALSO 
DO NOT GIVE VERY CLEARCUT RESULTS. HENS REQUIRE A VERY LARGE 
DOSE OF MAMMALIAN PTH AND EFFECTS ARE ONLY PRODUCED IN VERY 
YOUNG ANIMALS OR HENS IN ACTIVE LAY WITH VERY ACTIVE BONES. 
CANDLISH & TAYLOR (1970) FOUND THAT THE HYPERCALCEMIC EFFECT OF 
LILLY PARATHYROID EXTRACT WAS VERY RAPID IN LAYING HENS, THE MEAN 
RESPONSE TIME BEING 7-8 MINUTES. URIST ET AL (1960) FOUND THAT 
PARATHYROID EXTRACT INJECTIONS CAUSED A MODERATE DISTURBANCE OF 
EGG PRODUCTION IN LAYING HENS. THUS THE RESULTS WITH EXOGENOUS 
PTH SHOW THE SAME GENERAL EFFECTS OF PTH ON LAYING HEN PHYSIOLOGY 
AS THE PARATHYROID ABLATION EXPERIMENTS DISCUSSED ABOVE.

THE ABSENCE OF A RESPONSE TO PTH IN VITRO MAY NOT NECESSARILY 
MEAN THAT PTH HAS NO ACTION IN VIVO. LIFSHITZ ET AL (1969) WERE 
UNABLE TO SHOW ANY RESPONSE OF INTESTINAL LOOPS FROM NORMAL RATS 
TO PTH, BUT FOUND THAT WHEN THE LOOPS WERE TAKEN FROM PARA— 
THYROIDECTOMIZED RATS THEY DID GET AN INCREASED ABSORPTION OF 
CALCIUM AND PHOSPHATE. THEREFORE RESPONSES IN AN IN VITRO
Figure 6. The effect of PTH and CT on calcium influx (blood→lumen) in a shell gland excised during active egg shell calcification.
SYSTEM MAY DEPEND UPON THE PARATHYROID STATUS OF THE ANIMAL FROM WHICH AN ORGAN IS EXCISED. AS THE LAYING HEN IS KNOWN TO HAVE HYPERTROPHIED PARATHYROID, URIST (1967), TAYLOR (1965), IT IS POSSIBLE THAT THE PRESENCE OF A LARGE AMOUNT OF ENDOGENOUS PTH, POSSIBLY BOUND TO CELLULAR RECEPTORS, PRECLUDES THE ACTION OF EXOGENOUS PTH IN VITRO.

WHEN THE EFFECT OF SALMON CALCITONIN (CT) WAS TESTED IN THE IN VITRO SYSTEM IT WAS FOUND TO HAVE VERY LITTLE EFFECT ON CALCIUM INFLUX; THE INCREASE IN INFLUX WAS ONLY 8.7%, SEE FIGURE 6, AND TABLE VI.

THIS ABSENCE OF A RESPONSE TO CT IN VITRO WAS NOT UNEXPECTED SINCE ATTEMPTS TO SHOW AN IN VIVO EFFECT OF CT IN BIRDS HAVE BEEN LARGELY UNSUCCESSFUL. URIST (1967) POINTS OUT THAT THE ULTIMOB RANCHIAL GLANDS IN THE LAYING HEN ARE HYPERPLASTIC AND CONTAIN EASILY-EXTRACTABLE LARGE AMOUNTS OF CT. HOWEVER WITTERMAN ET AL. (1969), USING THE RAT BIOASSAY, SHOWED THAT THE CONCENTRATION OF CT IN THE ULTIMOB RANCHIAL TISSUE OF NINE MONTH OLD LAYING HENS WAS NO DIFFERENT FROM THAT IN THREE WEEK OLD CHICKS OR THREE MONTH OLD PULLETS, WHICH HAD A NORMAL PLASMA CALCIUM AND NOT THE ESTROGEN-INDUCED HYPERCALCEMIA FOUND IN LAYING HENS. CANDLISH & TAYLOR (1970) WERE UNABLE TO SHOW AN EFFECT OF PORCINE CT ON THE PLASMA CALCIUM OF THE LAYING HEN. SPEERS ET AL. (1970) ULTIMOB RANCHIALECTOMIZED CHICKS AT ONE TO TWO DAYS OF AGE AND REARED THEM TO MATURITY THROUGH SEVERAL MONTHS OF NORMAL EGG PRODUCTION. THE ULTIMOB RANCHIALECTOMIZED CHICKS ATE LESS AND FORMED SMALLER EGGS WITH A TREND TOWARDS REDUCED SHELL THICKNESS IN THE EARLY LAYING PERIOD BUT NOT DURING SUBSEQUENT WEEKS. THUS NO VERY GREAT DISTURBANCE OF CALCIUM
Figure 7. The effect of dibutyryl cyclic AMP on 45Ca fluxes in a shell gland excised while the egg was in the isthmus.
METABOLISM was observed in the absence of CT. The only positive response of chickens to CT to date is that of Kraintz & Intsher (1969), who showed that porcine CT had no effect on five day old chicks or intact cocks, but both porcine and avian CT caused a drop in plasma calcium in partially parathyroidectomized cocks. This implies that CT may have a role in normal calcium homeostasis in the hen but it is, at best, a minor one.

Cyclic AMP.

The concept that many hormones act by way of a two messenger system is now generally accepted. Such hormones may be regarded as first messengers which travel from their cells of origin to the cells of their target tissues to stimulate the formation therein of a second messenger. The only second messenger identified to date is cyclic 3'5'-adenosine monophosphate (cyclic AMP), and many hormones are thought to act via this messenger, Robison et al (1968, 1971). The dibutyryl derivative was used since many workers have shown that, in this form the cyclic AMP is more active than the parent compound in mimicking hormone actions, and in several cases it has been effective where cyclic AMP itself was virtually inactive, Robison et al (1968). Posternak et al (1962) showed that dibutyryl cyclic AMP is resistant to the inactivation caused by phosphodiesterase catalysing the opening of the cyclic phosphate ring. This led to the suggestion that the increased potency of the dibutyryl derivative could be due to its resistance to degradation by phosphodiesterase.
In the present series of experiments dibutyryl cyclic AMP had no effect on influx but increased efflux, see Figure 7. Influx remained at about 20 nmoles Ca/cm² hr, while efflux increased from 30 to 75 nmoles Ca/cm² hr, see Table VII. When stock buffer no. 2 was used as the incubation medium the influx remained about 8 nmoles Ca/cm² hr; the effect on efflux was not tested in this experiment.

The lack of response of influx to dibutyryl cyclic AMP does not completely rule out the involvement of adenyl cyclase in this calcium translocation. Lack of reaction could be due to poor entry into cells, Robison et al. (1967), or it could be due to the inability of the cells to remove the 2'-0-acyl group of dibutyryl cyclic AMP which Posternak et al. (1962) have shown is necessary before the dibutyryl derivative can show biological activity.

The physiological meaning of the increase in efflux is obscure since the in vivo calcium flux is obviously from serosal to mucosal side, i.e. influx. It would therefore seem superfluous to have a hormonally mediated flux in the opposite direction. However there is some in vivo data which lends support to the increase in efflux found here. Mueller et al. (1969) added aminophylline to the diet of laying hens, at a concentration of 0.05%, and found that it caused the hens to lay eggs with shells of reduced thickness. Aminophylline is the solubilized form of theophylline which inhibits the cytoplasmic phosphodiesterase and hence causes an intracellular buildup of cyclic AMP. They also found a significant increase in
TABLE VII
THE EFFECT OF DIBUTYRYL CYCLIC AMP ON 45Ca FLUXES IN THE IN VITRO SHELL GLAND

<table>
<thead>
<tr>
<th>State of Gland</th>
<th>Stock Buffer No.</th>
<th>Calcium Fluxes in nmoles Ca/cm²·hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Influx</td>
</tr>
<tr>
<td>Egg in Isthmus</td>
<td>1</td>
<td>21.79 ± 1.0</td>
</tr>
<tr>
<td>Active Egg Shell Calcification</td>
<td>2</td>
<td>8.029 ± 1.08</td>
</tr>
</tbody>
</table>

* Flux Ratio = \( \frac{\text{Influx}}{\text{Efflux}} \) = \( \frac{\text{Blood} \rightarrow \text{Lumen}}{\text{Lumen} \rightarrow \text{Blood}} \)
THE PH AND BICARBONATE CONCENTRATION OF THE SHELL GLAND FLUID. IF THE CALCIUM WAS TRANSPORTED AWAY FROM THE MUCOSAL SIDE OF THE SHELL GLAND DUE TO THE INCREASE IN CYCLIC AMP, AS THE IN VITRO DATA TEND TO SUGGEST, THEN THE BICARBONATE CONCENTRATION, AND HENCE PH, WOULD RISE BECAUSE THE REACTION OF CALCIUM IONS WITH THE BICARBONATE IONS TO PRODUCE THE SOLID CALCIUM CARBONATE OF THE EGG SHELL WOULD BE REDUCED DUE TO LACK OF CALCIUM.

IF IT IS ACCEPTED THAT AN INCREASE IN INTRACELLULAR CYCLIC AMP CAUSES AN INCREASE IN EFFLUX THEN ONE MUST ASK WHICH HORMONE, IF ANY, COULD BE THE EXTERNAL MEDIATOR IN THIS SYSTEM; I.E. WHICH HORMONE BY REACTING WITH A SPECIFIC RECEPTOR SITE ON THE CELL MEMBRANE COULD ACTIVATE A MEMBRANE-BOUND ADENYL CYCLASE AND HENCE INCREASE THE CYCLIC AMP CONCENTRATION.


MURAD ET AL (1970) PRESENT EVIDENCE THAT CT INCREASES CYCLIC AMP IN KIDNEY AND BONE CELLS. THUS THE EXTERNAL MEDIATOR IN THE SHELL GLAND SYSTEM COULD BE CT. THIS WOULD SEEM A MORE LIKELY CANDIDATE SINCE AT THE END OF EGG SHELL CALCIFICATION ONE COULD ENVISAGE A TRANSIENT INCREASE IN PLASMA CALCIUM WHICH
could trigger the release of CT, and any excess calcium which had been pumped into the shell gland lumen would be returned to the blood. In this regard it would be of interest to test the effect of CT on efflux, an experiment which was not done here.

Comparison between the in vitro and in vivo system.

It is possible to calculate the approximate flux which must take place in vivo in order to calcify the shell at the observed rate. In order to do this one must know the area of the shell gland in contact with the shell. This can be approximated fairly well by assuming that it is equal to the surface area of the egg shell. Mueller & Scott (1940) calculated the surface area of 50 white Leghorn pullet eggs, using three different methods, and found that the average surface area was about 70 cm². Taylor (1965) states that, on a low calcium diet, the hen mobilizes approximately 120 mg Ca/hr from the skeleton for egg shell calcification. Therefore one can calculate that the in vivo flux should be 42.8 μmoles Ca/cm² hr. The in vitro fluxes obtained in these experiments ranged from 8 to 180 nmoles Ca/cm² hr, while those of Ehrenspeck et al. (1967) ranged from 20 to 50 nmoles Ca/cm² hr. On average the in vitro rates were about 500-1000 times slower than the calculated in vivo rate. Obviously there is some factor present in the intact hen which is absent in the in vitro system.

One of the most obvious differences between the in vivo system and this experimental one is lack of blood flow. Gilbert
(1967) calculated that during egg shell calcification about 1/6th to 1/8th of the cardiac output must go through the shell gland for the amount of calcium found in the egg shell to be deposited in the fifteen hours of active shell calcification. Since the blood is constantly renewed a concentration gradient (e.g. for calcium) can always be maintained between the serosal and mucosal sides of the shell gland. But, in the in vitro system, the blood is represented by a stagnant chamber so that the concentration of calcium on the serosal side can fall, thereby allowing back diffusion to occur. Furthermore, in vivo the calcium is precipitated as the carbonate, making the pump functionally irreversible. Obviously the in vitro system does not approximate this situation.

The shell gland is an extremely thick and muscular organ which must present a very great barrier to gas diffusion. Although the in vitro system was constantly bubbled with 95% O2, 5% CO2, there is the possibility that these gases did not diffuse fast enough through the shell gland to reach the values they would have attained in the intact hen. This might well limit the rate at which an energy dependent pump could operate.

Before the active period of egg shell calcification, during the first five hours in the shell gland, the egg imbibes water and swells, a process known as plumping, Burmester (1940). This greatly increases the size of the egg and the walls of the shell gland are therefore stretched. This stretching process could be necessary for the functioning of the pump, especially
since Gilbert & Lake (1963) have shown that the shell gland contains an extensive intrinsic nervous supply, so that stretch could set up local reflex activity which would then activate the pump. In the experiments performed here an attempt was made to subject all the preparations to the same amount of stretch, but there was no way to ascertain how closely this stretch approximated the in vivo situation. Also the in vitro preparation used small pieces of the shell gland and the whole intrinsic nervous system could be necessary for activity. However, Gilbert (1967) has pointed out that the fairly common production of miniature eggs (usually small quantities of albumen surrounded by a shell) does not support the hypothesis that distension of the shell gland per se is necessary for calcification. He suggests that the stimulus for shell formation may occur in the isthmus with neural coordination between this region and the shell gland.

The in vitro preparation obviously lacks an extrinsic nervous supply and, since Freedman & Sturkie (1963a) have shown that the shell gland has an extensive extrinsic nervous supply containing both sympathetic and parasympathetic nerves, it is reasonable to postulate that these could be involved. This appears even more probable when one considers that birds possess an ion pump which is dependent on an intact nerve supply, viz the salt gland, Schmidt-Nielson (1960). The dependence of the shell gland calcium pump on an intact nerve supply could explain the very low fluxes obtained in vitro compared with the in vivo flux. Therefore it was decided that the in vitro
PREPARATION WOULD BE ABANDONED AND POSSIBLE NERVOUS EFFECTS ON THE SHELL GLAND PUMP IN *VIVO* WOULD BE EXAMINED.
CHAPTER II: OBSERVATIONS ON THE EFFECT OF CERTAIN DRUGS UPON THE ANESTHETIZED CHICKEN.

INTRODUCTION.

As an adaptation of elimination of excess salt ingested in their food and drink marine birds have a highly developed salt gland, Schmidt-Nielsen (1960). This gland acts to remove sodium chloride from the animal when it is subjected to an osmotic load of any kind. Fange et al. (1958) showed that secretion could be produced by stimulation of a nerve to the gland, and that this nerve was cholinergic because the response to the nerve stimulation was blocked by atropine and the gland was stimulated by acetylcholine and mecholyl. Since the response due to an osmotic load was also blocked almost completely by atropine they concluded that the salt gland secretion was dependent upon parasympathetic nervous activity. This idea is now generally accepted.

The low flux rates observed with the in vitro system suggested that some factor which is normally present in the intact animal has been removed. The most obvious difference between the in vivo and in vitro situations is that, in the latter, the blood and nervous supplies have been severed. In view of the nature of the control of the salt gland described above, a series of experiments were conducted to investigate the relationship, if any, between various branches of the autonomic nervous supply and calcium translocation in the shell gland.

Freedman & Sturkie (1963a) have shown that the shell gland has both sympathetic and parasympathetic innervation. All nerves
AUTONOMIC NERVOUS SUPPLY ON THE CALCIFICATION OF THE EGG SHELL BY THE INTACT HEN'S UTERUS.

There is now general acceptance of the theory of neurohumoral transmission, i.e. nerves transmit their impulses across most synapses and neuroeffector junctions by means of specific chemical agents. The neurohumoral transmittor of all preganglionic autonomic fibres, all postganglionic parasympathetic fibres and a few postganglionic sympathetic fibres is acetylcholine (cholinergic fibres); while in the majority of postganglionic sympathetic fibres the transmittor is norepinephrine (adrenergic fibres), Goodman & Gilman (1965).

Cholinergic junctions can be further differentiated into two receptor types on the basis of the effects of the alkaloids muscarine and nicotine. Muscarine is a parasympathomimetic which activates the cholinergic receptors at the autonomic effector cells and therefore, by convention, these are called muscarinic. Nicotine in low doses stimulates, and in higher doses paralyses autonomic ganglia. The same sequence occurs at the motor end plates of certain types of skeletal muscle. Hence these junctions are known as nicotinic junctions. These two types of cholinergic receptor respond differently to certain blocking agents. Ganglionic nicotinic receptors are blocked by hexamethonium salts while atropine and other related belladonna alkaloids selectively block muscarinic receptors, Goodman and Gilman (1965). Blockade of nicotinic receptors blocks all the autonomic ganglia and hence paralyses both the sympathetic and parasympathetic nervous supplies, whereas muscarinic blockade selectively inhibits the parasympathetic
NERVOUS SUPPLY. Therefore atropine was chosen as the para-
sympathetic blocking agent.

Adrenergic receptors have also been differentiated into
two types. Ahlquist (1948) studied the potency of five
sympathomimetic amines on a variety of tissue responses both
in isolated organ baths and intact animals. He was able to
classify sympathetic receptors into two groups which he called
alpha and beta. The order of potency for stimulation of alpha
receptors was epinephrine, norepinephrine, methylnorepinephrine,
methylepinephrine and isoproterenol; while for the beta
receptors it was isoproterenol, epinephrine, methylepinephrine,
methylnorepinephrine and norepinephrine. In general alpha
receptor stimulation resulted in excitatory responses and beta
receptor stimulation in inhibitory responses. There were two
exceptions, cardiac excitation, which was classified chemically
as a beta receptor response, and inhibition of the gut, which
appeared to be an alpha response. It was shown subsequently
that the gut response was mediated by both alpha and beta
receptors, Ahlquist & Levy (1959).

Ahlquist's classification has since been vindicated by
studies with alpha and beta blocking drugs. Before his
proposal the adrenergic blocking action of the ergot alkaloids
had been demonstrated by Dale (1906), but these were all alpha
blockers. It was not until 1958 that Powell & Slater described
dichloroisoproterenol, the first beta adrenergic receptor
blocking drug. Since then many different adrenergic blocking
drugs have been described. One of the most effective and
SPECIFIC ALPHA BLOCKERS IS THE HALOALKAMINE PHENOXYBENZAMINE (dibenzyline), Nickerson & Hollenberg (1967). This is an irreversible blocker and it takes some time before its effects become maximal. The favoured beta blocker to date appears to be propranalol (Inderal), Fitzgerald (1969).

The experimental protocol which was to be adopted for the calcification experiments required a continuous block for up to two hours so that drug dose levels which gave a uniform maximal blockade over this time period were required. The literature on the pharmacology of the chicken is very scanty and hence it was difficult to find the required doses in the literature.

In their 1962 review article Bunag & Walaszek state that, "the only cholinergic blocking agent which has been investigated in the avian cardiovascular system is atropine". There is some controversy about the dosage required for complete cholinergic blockade. Gibbs (1926) showed that intravenous administration of atropine sulphate 0.5-1.0 mg/kg in the chicken completely blocked vagal slowing of the heart. Lloyd & Pickford (1961) were able to block depressor responses to acetylcholine with 1-2 mg of atropine given intravenously. However Harvey et al (1954) found that intravenous doses of atropine below 5 mg/kg never completely blocked acetylcholine-induced vasodepression, and that doses of 9-12 mg/kg were generally required. With such doses the block lasted for 1-3 hours. Bunag & Walaszek (1962) confirmed that 5-10 mg/kg of atropine given intravenously blocked the depressor response
TO ACETYLCHOLINE BUT LOWER DOSES (0.5-2.0 MG/KG) ONLY ACHIEVED PARTIAL BLOCKADE. Thus it appears that the hen requires more atropine to block the effects of acetylcholine than it does to block parasympathetic neurotransmission, in apparent contradiction to the usual findings, Goodman & Gilman (1965).

The usual intravenous dose of atropine for cholinergic blockade in dogs is 1.0 MG/KG, Barnes & Eltherington (1964), so that the dose required by the chicken appears to be very large. Large doses of atropine can have undesirable non-specific effects such as ganglionic blockade and inhibition of the responses to other transmitters, namely histamine, serotonin and norepinephrine, Goodman & Gilman (1965).

The large blocking doses required in the hen may be due to either the presence of an atropinase, as has been described in the rabbit, Ambache (1955), or because of rapid destruction of the drug as a result of the high metabolic rate in birds, Sturkie (1965). In either case, a continuous intravenous infusion would obviate the necessity for large nonspecific doses.

As preliminary experiments on the blockade of the depressor response to 4 µG/KG of acetylcholine by 0.5 MG/KG of atropine showed that the block did indeed decay very quickly experiments were conducted to find the level of atropine needed for a continuous intravenous infusion. Campos & Urquilla (1969) had developed an intravenous infusion of atropine for the dog in which they used a priming dose of 0.25 MG/KG and gave a continuous infusion of 5 µG/KG/MIN. This was used as a
Starting point for the experiments to find a suitable infusion level of atropine for approximately 100% blockade in the chicken. The continuous infusion of atropine also meant that blockade could be made to last for as long as required and hence the requirements of the protocol for the calcification experiments could be met.

Data on adrenergic blockers in chickens is not as scarce as that on parasympathetic blockers. As with atropine, the doses required in the chicken are quite large compared with those in mammals. Harvey et al. (1954) were the first workers to make a really systematic study of adrenergic blockade in the chicken. At that time only alpha blockers were available. They found that 20 mg/kg of dibenamine failed to induce adrenergic blockade, but the more potent substituted halo-alkamine dibenzyline (phenoxybenzamine), at levels of 20-60 mg/kg, did produce a vasomotor reversal of the response to epinephrine indicating alpha blockade. Bunag & Walaszek (1962) report that they were unable to keep their chickens alive long enough to allow testing of the degree of blockade when they gave such high doses of dibenzyline. They show quite marked reduction of the pressor response to epinephrine and norepinephrine with 20 mg/kg of dibenzyline but no reversal. Bolton & Bowman (1969) also found vasomotor reversal with epinephrine with 20-60 mg/kg of dibenzyline. These experiments were all hampered by the lack of a specific alpha agonist so that complete block had to result in vasomotor reversal as the beta effects were unmasked. With these nonspecific agonists
AN ABSOLUTE MEASURE OF THE LEVEL OF BLOCKADE WAS NOT POSSIBLE. Phenylephrine is a specific alpha adrenergic agonist, Goodman & Gilman (1965). Using this it was possible to determine absolute levels of adrenergic blockade and hence select a suitable dose of dibenzyline to give the required blockade. A continuous infusion was not necessary with this drug because it is an irreversible blocker, Goodman & Gilman (1965).

The first report of the use of a beta adrenergic blocker in chickens was by Bunag & Walaszek (1962), who used dichloroisoproterenol, the first beta blocker discovered. They found that a dose of 10 mcg/kg abolished the depressor response to 10 μg/kg of the specific beta agonist isoproterenol. The blockade was of fairly long duration and was present three hours after the drug administration. Bolton & Bowman (1969) also found that a dose of 5-10 mg/kg dichloroisoproterenol abolished the depressor response to isoproterenol. A dose of 0.2 mg/kg of propranalol abolished the depressor response to isoproterenol, although some hens required as much as 1 mg/kg. However these workers did not state how long the propranalol blockade lasted, and therefore it was necessary to conduct blocking experiments to find the optimal level of propranalol to produce a good blockade for as long as two hours as the protocol of the calcification experiments demanded.

The experiments described in this chapter are the result of a study to find suitable blocking doses of the various drugs discussed above in order to have maximal autonomic blockade over periods as long as two hours, so that the effect of
VARIOUS BRANCHES OF THE AUTONOMIC NERVOUS SYSTEM ON EGG SHELL CALCIFICATION COULD BE EVALUATED.
<table>
<thead>
<tr>
<th>Chemical</th>
<th>Drug Name</th>
<th>Supplier</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium pentobarbital</td>
<td>Somnopentyl (parenteral soln.)</td>
<td>Pittman-Moore, Ontario</td>
<td>Anesthetic</td>
</tr>
<tr>
<td>Acetylcholine chloride</td>
<td></td>
<td>Merck &amp; Co., N.J.</td>
<td>Parasympathetic agonist</td>
</tr>
<tr>
<td>Atropine sulphate</td>
<td></td>
<td>B.D.H., Toronto</td>
<td>Parasympathetic blocker</td>
</tr>
<tr>
<td>Norepinephrine H Cl.</td>
<td>D.L. Arterenol B grade</td>
<td>Calbiochem., Los Angeles</td>
<td>Alpha-sympathetic agonist</td>
</tr>
<tr>
<td>Phenylephrine H Cl.</td>
<td></td>
<td>Winthrop Labs., Aurora, Ontario</td>
<td>Alpha-sympathetic agonist</td>
</tr>
<tr>
<td>Phenoxybenzamine H Cl.</td>
<td>Dibenzyline</td>
<td>Smith Cline &amp; French I.A.C., Montreal</td>
<td>Alpha-sympathetic blocker</td>
</tr>
<tr>
<td>Propranalol H Cl.</td>
<td>Inderal</td>
<td>Ayerst, Montreal</td>
<td>Beta-sympathetic blocker</td>
</tr>
</tbody>
</table>
METHODS.

WHITE LEGHORN HENS, EITHER IN LAY OR IN MOULT, WEIGHING APPROXIMATELY 2 KG WERE USED IN THESE EXPERIMENTS. THEY WERE OBTAINED FROM THE POULTRY FARM OF THE UNIVERSITY OF BRITISH COLUMBIA. THE BIRDS WERE ANESTHETIZED WITH 0.7 ML OF SODIUM PENTOBARBITOL (SOMNOPENTYL, PARENTERAL SOLUTION, PITTMAN-MOORE, ONT.), INJECTED INTO THE SAPHENOUS VEIN IN THE LEG, AND ANESTHESIA WAS MAINTAINED WITH 0.15 ML DOSES GIVEN AS REQUIRED (USUALLY AT 15-20 MIN INTERVALS).

A POLYETHYLENE CANNULA (INTRAMEDIC P.E.50, CLAY ADAMS, DIVISION OF BECTON, DICKENSON & CO., PARSIPPANY, N.J.) WAS INSERTED INTO THE RIGHT MEDIAL WING VEIN FOR ADMINISTRATION OF ANESTHETIC AND DRUGS. IN EXPERIMENTS WHERE A CONTINUOUS INTRAVENOUS INFUSION OF A BLOCKING DRUG WAS USED A SECOND P.E.50 CANNULA WAS INSERTED INTO THE MEDIAL VEIN ON THE OTHER WING. THIS CANNULA WAS ONLY USED FOR THE INFUSION TO ENSURE THAT NO BLOCKING AGENT WAS ACCIDENTLY FLUSHED INTO THE ANIMAL.

AN INTRAMEDIC P.E.10 CANNULA WAS PLACED IN THE RIGHT BRACHIAL ARTERY FOR MEASUREMENT OF BLOOD PRESSURE, WHICH WAS RECORDED USING A PRESSURE TRANSDUCER (STATHAM MODEL P23AA, HATO REY, PUERTO RICO) ATTACHED TO A PEN RECORDER (GILSON MEDICAL ELECTRONICS, MIDDLETON WIS.). IN EXPERIMENTS WHERE HEART RATE WAS MEASURED THIS WAS DONE, EITHER BY COUNTING THE PULSES IN THE BLOOD PRESSURE TRACE, OR BY USE OF A HEART RATE METRE (EKEG ELECTRONIC CO., VANCOUVER, B.C.) ATTACHED TO THE CUMULATIVE CHANNEL OF THE GILSON RECORDER.

ALL CANNULAE WERE FILLED WITH HEPARINIZED SALINE (10 MG
<table>
<thead>
<tr>
<th>Drug</th>
<th>Type of Agonist</th>
<th>Dose Used for Challenge</th>
<th>Determination of Dose Level</th>
<th>Response Measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>Parasympathetic</td>
<td>4 µg/mg/kg</td>
<td>Low range used by Bunag &amp; Walaszek (1962) (B.P. ↓ 54 mm Hg.)</td>
<td>Decrease in B.P.</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>Alpha-sympathetic</td>
<td>4 µg/mg/kg</td>
<td>See Bolton &amp; Bowman (1969) (B.P. ↑ 80 mm Hg.)</td>
<td>Increase in B.P.</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>Alpha-sympathetic</td>
<td>40 µg/mg/kg</td>
<td>From log-dose response curve (see Figure 8)</td>
<td>Increase in B.P.</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>Beta-sympathetic</td>
<td>0.4 µg/mg/kg</td>
<td>From log-dose response curve (see Figure 8)</td>
<td>Increase in H.R.</td>
</tr>
</tbody>
</table>

**Abbreviations**  
B.P. Blood Pressure  
H.R. Heart Rate
HEPARIN / 100 ML OF 0.9% SALINE).

Table VIII lists all the drugs used in these experiments and their suppliers. Drugs were routinely dissolved in 0.9% saline. The only exception was dibenzyline which produced a milky suspension in saline; it was made up as follows: 50 mg of dibenzyline were dissolved in 0.8 ml of propylene glycol and then acidified with 0.2 ml of 0.1 N HCl and 4 ml of 0.9% saline were added. The resulting clear solution was used for injection.

The agonist drugs and the responses measured are given in the accompanying table (Table IX). The dose levels of acetylcholine and norepinephrine were based on doses used in the literature; examples of the actual responses obtained in these experiments are shown in parentheses. The phenylephrine and isoproterenol dose levels were taken as the midpoint of the linear portion of their respective log-dose response curves. The latter were constructed by finding the responses to various doses of the drugs in a chicken and plotting the log of the dose versus the response as shown in the results.

Several challenge doses of the agonist drug were given to the hen prior to the addition of the blocking agent in order to establish the response when no blockade was present. After the blocking agent had been given the response to the agonist drug was tested every 10 minutes.

In the infusion experiments a priming dose was given and then the infusion was started immediately and continued for two hours. The blockade was challenged every 10 minutes during the
INFUSION AND FOR A FURTHER 90 MINUTES AFTER THE INFUSION. ALL INFUSIONS WERE CARRIED OUT USING A MULTISPEED TRANSMISSION INFUSION PUMP (HARVARD APPARATUS CO. INC., DOVER, MASS.) AT A RATE OF 0.035 ML/MIN FOR THE ATROPINE INFUSIONS AND A RATE OF 0.02 ML/MIN FOR THE PROPRANALOL INFUSIONS.
Figure 8. Log-dose curves to Phenylephrine (top) and Isoproterenol (bottom).

Regression line for experimental points.
--- 95% confidence limits on regression line.
Challenge dose used in the blocking drug experiments.
RESULTS AND DISCUSSION.

Figure 8 shows the log dose response curves to phenylephrine and isoproterenol in an anesthetized chicken. The arrows indicate the dose level selected for use as a challenge dose in the experiments with the blocking drugs.

In the blocking experiments, % blockade at any time t was calculated as follows:

\[
\% \text{blockade} = \left(100 - \frac{\text{Observed response to agonist drug at time } t}{\text{Response when no blocking agent was present}} \times 100\right)
\]

All slopes were calculated using the least mean squares method as described by Steele & Torrie (1960), and all calculations were done on a desk computer (Olivetti-Underwood Programma 101, New York).

Table X gives a summary of all the types of drug experiments performed.

Atropine.

From Table X it can be seen that a single dose of atropine (0.5 mg/kg) gave a very short blockade which would have decayed to 30% in an hour. The actual experiment is depicted at the top of Figure 9. The protocol used in this experiment was based on
### TABLE X

**A Summary of all the Drug Experiments. The Effect of Different Doses of Blocking Drugs on the Responses to Various Agonists**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Agonist</th>
<th>Priming Dose (mg/kg)</th>
<th>Infusion Dose (μg/kg/min)</th>
<th>% Blockade During Infusion</th>
<th>After Infusion (one hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Atropine</strong></td>
<td>Acetylcholine</td>
<td>0.5</td>
<td>8.75</td>
<td>75%</td>
<td>100% down to 30%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.25</td>
<td>10.5</td>
<td>80%</td>
<td>70% down to 15%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>17.5</td>
<td>95%</td>
<td>95% down to 25%</td>
</tr>
<tr>
<td><strong>Dibenzylamine</strong></td>
<td>Norepinephrine</td>
<td>10 70 min later added another</td>
<td>10</td>
<td>55%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phenylephrine</td>
<td>5</td>
<td></td>
<td></td>
<td>65%</td>
</tr>
<tr>
<td><strong>Propranolol</strong></td>
<td>Isoproterenol</td>
<td>0.5</td>
<td>6</td>
<td>95%</td>
<td>90% down to 50%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.25</td>
<td>5</td>
<td>100%</td>
<td>90% down to 40%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>95%</td>
<td>95% down to 90%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>90%</td>
<td>85% down to 70%</td>
</tr>
</tbody>
</table>
THAT USUALLY USED IN A DOG, WHERE A DOSE OF 0.5 MG/KG IS GIVEN AND 1/10TH OF THIS DOSE IS ADDED EVERY HOUR (DR. F. LIOY, PERSONAL COMMUNICATION). ONE CAN READILY SEE THAT IN A CHICKEN THIS WOULD GIVE A VERY POOR LEVEL OF BLOCKADE BECAUSE THE BLOCK DECAYS SO FAST.

CAMPOS & URQUILLA (1969) USED A PRIMING DOSE OF 0.25 MG/KG AND A INFUSION LEVEL OF 5 μG/KG/MIN IN DOGS FOR PARASYMPATHETIC BLOCKADE. OBVIOUSLY A LARGER DOSE WOULD BE REQUIRED IN A CHICKEN. THE PRIMING DOSE WAS KEPT AT 0.25 MG/KG BUT THE LEVEL OF THE INFUSION WAS INCREASED. THE FIRST INFUSION LEVEL TRIED WAS 8.5 μG/KG/MIN. THIS GAVE A BLOCKADE OF 75%, SEE TABLE X. THE DECAY OF BLOCKADE AFTER STOPPING THE INFUSION WAS NOT TESTED IN THIS EXPERIMENT. INCREASING THE INFUSION LEVEL TO 10.5 μG/KG/MIN GAVE A BLOCKADE OF 80%. AS SOON AS THE INFUSION WAS STOPPED THE BLOCK STARTED TO DECAY AND WAS ONLY 15% AFTER ONE HOUR. WHEN THE INFUSION LEVEL WAS 17.5 μG/KG/MIN AN ESSENTIALLY 100% BLOCK WAS OBTAINED. AGAIN AS SOON AS THE INFUSION WAS STOPPED THE BLOCK RAPIDLY DECAYED. THIS EXPERIMENT IS SHOWN AT THE BOTTOM OF FIGURE 9. SINCE THE BLOCK WAS CONSTANT AND COMPLETE WITH THIS DOSAGE THIS WAS THE DOSE CHOSEN FOR USE IN THE CALCIFICATION EXPERIMENTS.

A 17.5 μG/KG/MIN INFUSION WOULD ONLY ADMINISTER 1.08 MG/KG OVER A PERIOD OF ONE HOUR, SO THAT THE TOTAL DOSE OF ATROPINE GIVEN TO THE CHICKEN IN THIS TIME WOULD ONLY BE 1.33 MG/KG. THE DECAY OF THE BLOCKADE IMMEDIATELY AFTER STOPPING THE INFUSION SHOWED THAT IN FACT THE ATROPINE WAS NOT REMAINING IN AN ACTIVE FORM FOR VERY LONG SO THAT THE TOTAL DOSE ACTUALLY IN THE BODY AFTER ONE HOUR WAS MUCH LESS THAN THIS. THEREFORE THE REPORTS
Figure 9. The effect of time on % blockade of the hypotensive response to acetylcholine by atropine. (Top: single dose, Bottom: continuous intravenous infusion)

95% confidence limits on regression line.

Regression line for experimental points.
IN THE LITERATURE THAT VERY HIGH DOSES OF ATROPINE ARE NECESSARY TO BLOCK THE VASODEPRESSOR RESPONSE TO ACETYLCHOLINE WERE NOT BORNE OUT BY THESE RESULTS.

BUNAG & WALASZEK (1962) STATED THAT DEPRESSOR RESPONSES TO 5-50 µG DOSES OF ACETYLCHOLINE WERE ONLY PARTIALLY BLOCKED BY 0.5-2.0 MG/KG DOSES OF ATROPINE AND THAT 5-10 MG/KG OF ATROPINE WERE REQUIRED BEFORE COMPLETE BLOCKADE WAS ATTAINED. HARVEY ET AL (1954) REPORTED THAT IT REQUIRED 9-12 MG/KG OF ATROPINE TO BLOCK ACETYLCHOLINE-INDUCED VASODEPRESSION, AND THAT DOSES BELOW 5 MG/KG NEVER COMPLETELY BLOCKED THIS RESPONSE. THESE WORKERS DO NOT SAY WHAT DOSE OF ACETYLCHOLINE THEY USED AS AN AGONIST OR HOW LONG THEY WAITED BEFORE THEY TESTED THE BLOCK. IT IS CONCEIVABLE THAT IF THEY WAITED MORE THAN 15 MIN THE BLOCK HAD ALREADY DECAYED. THEY DO POINT OUT THAT WITH 9-12 MG/KG DOSES THE BLOCK LASTED 1-3 HOURS. IN VIEW OF THE RAPID DECAY OF BLOCKADE WITH SMALL DOSES OF ATROPINE WHICH HAS BEEN DEMONSTRATED HERE, FIGURE 9, IT APPEARS THAT THESE WORKERS WERE GIVING VAST QUANTITIES OF ATROPINE IN ORDER TO HAVE A LONG LASTING BLOCK. AS POINTED OUT IN THE INTRODUCTION THESE CAN BE NON-SPECIFIC AND HENCE ARE UNDESIRABLE.

THE RESULTS PRESENTED HERE ARE MORE IN ACCORD WITH THE EARLY REPORT OF GIBBS (1926) THAT 0.5-1.0 MG/KG OF ATROPINE WAS SUFFICIENT TO PREVENT CARDIAC SLOWING DUE TO VAGAL STIMULATION COMPLETELY. LLOYD & PICKFORD (1961) REPORTED THAT IN THREE HENS THEY WERE ABLE TO BLOCK DEPRESSOR RESPONSES TO ACETYLCHOLINE WITH 1-2 MG OF ATROPINE ADMINISTERED INTRAVENOUSLY. THEY DID NOT GIVE THE EXACT DOSE OF ACETYLCHOLINE USED TO TEST THE BLOCK,
Figure 10. The effect of time on %blockade of the hypertensive response to norepinephrine (top) or phenylephrine (bottom) by single doses of Dibenzyline.

--- Regression line for the experimental points.
--- 95% confidence limits on regression line.
HOWEVER SINCE THEY WERE USING HENS APPROXIMATELY 2 KG IN WEIGHT
THE DOSE OF ATROPINE WAS 0.5-1.0 MG/KG, WHICH IS IN GOOD AGREEMENT WITH THE RESULTS FOUND HERE.

DIBENZYLINE (PHENOXYBENZAMINE).

TWO LEVELS OF DIBENZYLINE WERE TESTED IN THE CHICKEN. BOTH EXPERIMENTS ARE ILLUSTRATED IN FIGURE 10 AND SUMMARIZED IN TABLE X.

THE FIRST DOSE USED WAS 10 MG/KG, DISSOLVED IN SALINE. EVEN WHEN WARMED THIS DID NOT DISSOLVE VERY WELL BUT GAVE A MILKY SUSPENSION, SO THAT IT IS POSSIBLE THAT NOT ALL THE DRUG WAS ASSIMILATED BY THE CHICKEN. THE AGONIST USED TO TEST BLOCKADE IN THIS EXPERIMENT WAS NOREPINEPHRINE, WHICH IS NOT A SPECIFIC ALPHA AGONIST. FOR TRUE 100% BLOCKADE ALL THE ALPHA RECEPTORS WOULD BE BLOCKED AND A VASODEPRESSION WOULD RESULT AS THE Beta RECEPTORS BECAME UNMASKED. IF ONE REALLY ACHIEVED THIS ABSOLUTE LEVEL OF BLOCKADE THE BLOCKING DRUG WOULD GENERALLY BE PRESENT IN EXCESS, THEREBY PROBABLY CAUSING NONSPECIFIC EFFECTS. THEREFORE AS A TEST SUBSTANCE FOR ALPHA ADRENERGIC BLOCKADE NOREPINEPHRINE WAS NOT A GOOD CHOICE. IT WAS USED BECAUSE IT WAS ONE OF THE TEST SUBSTANCES USED BY OTHER WORKERS IN EXPERIMENTS ON CHICKENS, SEE HARVEY ET AL. (1954) AND BUNAG & WALASZEK (1962).

% BLOCKADE IN THIS EXPERIMENT WAS CALCULATED ASSUMING THAT AT 100% BLOCK NOREPINEPHRINE PRODUCED NO EFFECT ON BLOOD PRESSURE, I.E. DISREGARDING VASOMOTOR REVERSAL. THIS WOULD GIVE VALUES OF BLOCKADE WHICH WOULD BE TOO HIGH. EVEN WITH THIS ERROR THE
blockade obtained was only 55% and it only increased to 65% when another 10 mg/kg dose was added, see table X. This experiment tends to agree with the data in the literature, namely that it takes 20-60 mg/kg of dibenzyline to produce vasomotor reversal with epinephrine or norepinephrine and hence presumably to cause complete adrenergic blockade in the chicken, Harvey et al. (1954) and Bolton & Bowman (1969).

When phenylephrine was used as the agonist a different result was obtained. Phenylephrine is a specific alpha agonist with almost no beta effects, Goodman & Gilman (1965). Hence 100% block is obtained when the vasopressor response is abolished, allowing meaningful calculations of % blockade.

In this experiment the dibenzyline was dissolved in acidified propylene glycol and diluted with saline in a manner similar to that described by Harvey et al. (1954). No solubility problems were encountered.

The dose used was 5 mg/kg, half the dose given in the previous experiment. The results are shown at the bottom of figure 10. There was incomplete blockade for the first thirty minutes but after that a steady block was observed which decayed from 80% to 70% over a period of two hours. The incomplete block for the first half hour was not surprising since dibenzyline is an irreversible blocker and it takes time for the reaction with the receptor site to occur. The molecular configuration directly responsible for blockade is probably a highly reactive carbonium ion formed when the three-membered ring breaks, and the relatively slow onset of action is probably
DUE TO THE TIME FOR THE FORMATION OF THIS REACTIVE INTERMEDIATE WHICH THEN ACTS AS AN ALKYLATING AGENT, GOODMAN & GILMAN (1965).

These writers also point out that the presence of a catecholamine during the development of blockade by a haloalkamine can decrease the degree of blockade attained. They explain this as due to competition between the two drugs for the same population of receptors. Once blockade is complete it is unaltered by the presence of a sympathetomimetic amine, indicating that the drug is no longer in equilibrium with the receptors. Thus the fact that blockade was tested with the agonist during the development of blockade probably meant that the observed blockade was less than that which would have developed if no testing had been carried out for the first hour. It was therefore decided that during the calcification experiments the dibenzyline would be added an hour before the 45Ca so that full blockade would have time to develop. As the 5 mg/kg dose of dibenzyline gave sufficient blockade it was chosen as the dose for use in the calcification experiments.

It appears as though the large doses of dibenzyline required for vasomotor reversal to epinephrine and norepinephrine in the chicken were due to the nature of the agonist used. GOODMAN & GILMAN (1965) point out that since these two agonists have both alpha and beta effects their effects on blood pressure are complex, and failure to appreciate this has led to frequent misinterpretations of the properties of adrenergic blocking agents.

From the experiments reported here it is clear that a
Table XI
A Summary of the Cardiovascular Responses in the Propranalol Experiments

<table>
<thead>
<tr>
<th>Propranalol Dose</th>
<th>Slope of H.R. vs Response to 0.4 µg/kg of Isoproterenol (Before Propranalol)</th>
<th>Slope of H.R. vs Time (Before Propranalol)</th>
<th>Mean Blood Pressure*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before Propranalol</td>
<td>After Propranalol</td>
</tr>
<tr>
<td>PRIMING MG/KG</td>
<td>INFUSION µG/KG/MIN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>6</td>
<td>-0.699 ± 0.072</td>
<td>0.592 ± 0.057</td>
</tr>
<tr>
<td></td>
<td></td>
<td>141.76 ± 1.46</td>
<td>116.18 ± 1.42</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>-1.877 ± 0.359</td>
<td>0.277 ± 0.060</td>
</tr>
<tr>
<td></td>
<td></td>
<td>135.77 ± 1.28</td>
<td>111.36 ± 0.87</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>-0.963 ± 0.169</td>
<td>0.757 ± 0.075</td>
</tr>
<tr>
<td></td>
<td></td>
<td>133.50 ± 1.16</td>
<td>97.71 ± 2.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-0.479 ± 0.176</td>
<td>0.1375 ± 0.078</td>
</tr>
<tr>
<td></td>
<td></td>
<td>156.74 ± 1.13</td>
<td>118.71 ± 1.19</td>
</tr>
<tr>
<td>0.5</td>
<td>6</td>
<td>-0.897 ± 0.125</td>
<td>-0.3312 ± 0.091</td>
</tr>
<tr>
<td></td>
<td></td>
<td>125.21 ± 1.85</td>
<td>115.00 ± 1.53</td>
</tr>
</tbody>
</table>

* In the infusion experiments a paired t-test on the mean blood pressures before and after the addition of propranalol, regardless of the level of infusion, showed that the drop in blood pressure was significant at the 0.5% level (P < 0.005).
DOSE OF 5 mg/kg of dibenzyline gives 70-80% blockade in the chicken which in most cases is sufficient to elucidate the effect of alpha adrenergic blockade on various physiological processes.

Propranalol.

Although a single dose of 0.5 mg/kg of propranalol gave a 90% block of the increase in heart rate caused by 0.4 µg/kg of isoproterenol, the level of blockade was not constant, so that, at the end of one hour, the block had decayed to 50% (Table X). A continuous infusion was chosen in order to maintain a high level of blockade for up to two hours as required in the calcification experiments.

In combination with a priming dose of 0.25 mg/kg, two infusion levels were tried, 5-6 µg/kg/min and 10 µg/kg/min. Both levels gave 90-95% blockade during the infusion, but, when the infusions were stopped, the lower infusion level showed a faster decay of blockade (Table X). The 10 µg/kg/min infusion level was selected for use in the calcification experiments as it gave a good block for at least two hours.

A summary of the cardiovascular changes occurring in the propranalol experiments is given in Table XI. From this table one can see that in four out of five experiments the heart rate increased with time before the propranalol was given. This was probably due to an increase in sympathetic activity. An anesthetized animal is no longer able to maintain a proper homeostasis. Immobilization of an animal in the supine position
Figure 11. The effect of a single dose of propranolol on the blood pressure (left) and heart rate (right) of an anesthetized chicken.

--- Regression line for the experimental points.
--- 95% confidence limits on regression line.
CAN CAUSE POOLING OF BLOOD IN THE EXTREMITIES AND THE ANIMAL CAN ALSO LOSE BODY HEAT. SURGICAL TRAUMA CAN ALSO CAUSE INCREASED SYMPATHETIC FIRING. BARBITURATE ANESTHESIA TENDS TO DEPRESS RESPIRATION AND THIS COULD CAUSE ACIDOSIS WHICH WOULD ACTIVATE THE CHEMORECEPTORS. ON THE OTHER HAND THE BLOOD PRESSURE IS GENERALLY STEADY UNDER BARBITURATE ANESTHESIA DUE TO INCREASES IN PERIPHERAL RESISTANCE SO THAT BARORECEPTOR RESPONSES ARE PROBABLY NOT IMPORTANT, CHENWETH & VAN DYKE (1969).

IN THE EXPERIMENT WHERE THE HEART RATE DID NOT INCREASE WITH TIME, BUT DECREASED, THERE WAS STILL A CHANGE IN HEART RATE AND THIS ALLOWED THE TESTING OF THE EFFECT OF THE SAME DOSE OF ISOPROTERENOL AT DIFFERENT HEART RATES. IT WAS FOUND THAT, WHETHER THE HEART RATE INCREASED OR DECREASED WITH TIME, THE PLOT OF HEART RATE VERSUS HEART RATE RESPONSE TO 0.4 μG/KG OF ISOPROTERENOL WAS ALWAYS LINEAR AND ALWAYS HAD A NEGATIVE SLOPE (TABLE XI). THIS IMPLIES THAT AT HIGHER HEART RATES FEWER BETA RECEPTORS WERE AVAILABLE FOR REACTION WITH ISOPROTERENOL. IF THE CHANGE IN HEART RATE WAS DUE TO A CHANGE IN SYMPATHETIC ACTIVITY AN INCREASE IN HEART RATE WOULD INDEED MEAN THAT FEWER BETA RECEPTORS WERE AVAILABLE FOR REACTION WITH EXOGENOUS ISOPROTERENOL SINCE THEY WOULD ALREADY BE ACTIVATED BY ENDOGENOUS TRANSMITTOR. THE NEGATIVE LINEAR RESPONSE OF HEART RATE VERSUS HEART RATE RESPONSE TO 0.4 μG/KG OF ISOPROTERENOL OCCURRED IN EVERY EXPERIMENT. THE SLOPES VARIED MARKEDLY (TABLE XI) BUT THIS WOULD BE EXPECTED SINCE THE RECEPTOR POPULATIONS IN THE HEART AND THE SYMPATHETIC
FIGURE 12. THE EFFECT OF A CONTINUOUS INTRAVENOUS INFUSION OF PROPRANOLOL ON THE BLOOD PRESSURE (LEFT) AND HEART RATE (RIGHT) OF AN ANESTHETIZED HEN.

--- REGRESSION LINE FOR THE EXPERIMENTAL POINTS.
--- 95% CONFIDENCE LIMITS ON REGRESSION LINE.
ACTIVITY NECESSARY TO MAINTAIN A GIVEN HEART RATE WOULD VARY FROM INDIVIDUAL TO INDIVIDUAL.

THIS VARIATION OF RESPONSE TO ISO PROTERENOL WITH HEART RATE WAS IMPORTANT BECAUSE ADDITION OF PROPRANALOL INVARIABLY DROPPED THE HEART RATE (SEE FIGURES 11 AND 12 FOR EXAMPLES) AS THE ENDOGENOUS SYMPATHETIC ACTIVITY ON THE HEART WAS BLOCKED. THEREFORE IT WAS NECESSARY TO USE THIS LINE AS A CORRECTION FACTOR WHEN CALCULATING THE % BLOCKADE BY PROPRANALOL. FROM THE PLOT IT WAS POSSIBLE TO PREDICT WHAT THE RESPONSE TO ISO PROTERENOL WOULD BE AT ANY PARTICULAR HEART RATE, SO THAT THE UNBLOCKED RESPONSE TO ISO PROTERENOL COULD BE FOUND BY TAKING THE HEART RATE JUST BEFORE THE CHALLENGE DOSE OF ISO PROTERENOL WAS GIVEN AND READING THE EXPECTED INCREASE IN HEART RATE FROM THE GRAPH. SINCE THE ACTUAL RESPONSE TO THE CHALLENGE DOSE OF ISO PROTERENOL WAS KNOWN % BLOCKADE COULD BE CALCULATED. AN EXAMPLE OF THE LINEAR PLOT OF HEART RATE VERSUS HEART RATE RESPONSE TO ISO PROTERENOL IS GIVEN IN FIGURE 13 AND BESIDE THIS IS THE EFFECT OF AN INTRAVENOUS INFUSION OF PROPRANALOL ON BLOCKING THE RESPONSE TO ISO PROTERENOL IN THE SAME HEN. THE HEART RATE VERSUS HEART RATE RESPONSE LINE WAS USED AS A CORRECTION FACTOR TO CALCULATE THE % BLOCKADE IN THE SECOND GRAPH.

PROPRANALOL NOT ONLY HAS A PROFOUND EFFECT ON HEART RATE BUT ALSO CAUSES A MARKED DROP IN BLOOD PRESSURE, SEE FIGURES 11 AND 12 AND TABLE XI. THIS DROP IN BLOOD PRESSURE IS PROBABLY PRIMARILY DUE TO THE DROP IN HEART RATE SINCE THE BETA VASODILATOR RECEPTORS IN THE PERIPHERAL CIRCULATION ARE OF DOUBTFUL PHYSIOLOGICAL SIGNIFICANCE, GOODMAN & GILMAN (1965), AND
**Discussion**

Challenge by using the graph on the left as explained in the text. The expected response to isoproterenol when no block was present.

**Figure 17.** Left: The effect of heart rate on the heart rate response to isoproterenol. Right: The effect of heart rate on the heart rate response to isoproterenol in an anesthetized chicken.

---

95% confidence limits on regression line.
PRESUMABLY THE ALPHA RECEPTORS ARE NOT AFFECTED BY PROPRANALOL.

The influence of prolonged anesthesia is an important factor in the failure of the blood pressure to return to the level observed before propranolol was added.

The blocking dose of propranolol found here for maximal blockade was in good agreement with the value reported by Bolton & Bowman (1969), who found that 0.2-1 mg/kg doses of propranolol blocked the depressor response to 0.5 μg of isoproterenol in a hen. They were using the depressor effect of isoproterenol as a test, while the effect of isoproterenol on heart rate was used in the above experiments. However as discussed above, the blood pressure response was probably mainly due to changes in heart rate and therefore the two parameters are comparable.
Synopsis of drug experiments.

In essence this series of experiments was conducted to find suitable doses of autonomic nervous system blockers in the chicken to give maximal blockade over periods of up to two hours. The extended periods of blockade were required for the protocol of the egg shell calcification experiments discussed in the next chapter.

The drugs and doses found were as follows:

<table>
<thead>
<tr>
<th>Type of Blockade</th>
<th>Drug</th>
<th>Priming Dose (mg/kg)</th>
<th>Infusion Dose (μg/kg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parasympathetic</td>
<td>Atropine</td>
<td>0.25</td>
<td>17.5</td>
</tr>
<tr>
<td>Alpha-sympathetic</td>
<td>Dibenzyline</td>
<td>5.0</td>
<td>--</td>
</tr>
<tr>
<td>Beta-sympathetic</td>
<td>Propranalol</td>
<td>0.25</td>
<td>10.0</td>
</tr>
</tbody>
</table>
CHAPTER III : CALCIIUM TRANSPORT BY THE SHELL GLAND IN VIVO.

INTRODUCTION.

As the calcium fluxes observed in the in vitro system were very much lower than those which are known to occur in the intact hen the remainder of these studies were carried out on the whole animal. Since birds possess an ion pump which is dependent on parasympathetic nervous activity, i.e. the salt gland, Schmidt-Nielsen (1960), the initial experiments were undertaken to determine the nervous involvement in shell gland function. This was done by studying the effect of blockade of various branches of the autonomic nervous system on the calcification of the egg shell.

Two parameters may be used as a measure of egg shell calcification. The simplest system is to study the incorporation of label into the egg shell. Lörcher & Hodges (1969) showed that 110-170 min after intravenous 45Ca injection into anesthetized hens 45-53\% of the 45Ca dose appeared in the egg shell. When they repeated the experiment in unanesthetized birds they found that 38-45\% appeared in the egg shell after two hours. These workers did not look at incorporation at shorter time periods in any detail although they did mention that 12\% of the dose appeared in the shell in less than 20 min. As these minimal data did not adequately describe the time course for the reaction a preliminary study was undertaken to determine the incorporation of labelled calcium into the egg shell over the period from zero to two hours. This would give some measure of the rate of incorporation of label into
THE EGG SHELL.

Another way in which a picture of the factors involved in egg shell calcification can be obtained is by studying the disappearance of \(^{45}\)Ca from the blood. It is generally accepted that the decrease with time of the tracer concentration in the blood can be expressed mathematically by a series of exponential terms. Generally these are found by curve analysis. The original curve is plotted on semilogarithmic coordinates, a straight line is fitted through the tail of the curve, the absolute values of the differences between the ordinate values of the original curve and the straight line are plotted, and the procedure is repeated until only a final straight line remains. The straight lines so obtained are the exponential components of the curve. In general, a closed system of \(n\) compartments in steady state gives rise to \(n-1\) exponential terms in the disappearance curve, Robertson (1957). Analysis of the plasma \(^{45}\)Ca disappearance curve would give some idea of the number of physiological parameters which are involved in the disappearance of calcium from the blood during egg shell calcification.

Compartmental analysis implies that it is possible to resolve the curve into all its exponential components. This is not always feasible, especially in the case of very fast reactions, since one needs at least three points to define a straight line and this might require sampling at time periods which are too close together to be practical. It also implies steady state conditions, i.e. in any compartment the rate of
REMOVAL OF THE SUBSTANCE BEING STUDIED IS EQUALED BY ITS RATE OF REPLACEMENT SO THAT THE CONCENTRATION AND AMOUNT OF THE SUBSTANCE BEING STUDIED IS CONSTANT. THIS CONDITION IS SELDOM STRICTLY TRUE SO THAT ANY COMPARTMENTAL ANALYSIS IS NECESSARILY AN APPROXIMATION, NEVERTHELESS IT DOES GIVE AN OVERALL PICTURE OF THE SYSTEM UNDER STUDY.

BY COMPARISON OF THE PLASMA $^{45}$Ca DISAPPEARANCE CURVES IN HENS WITH AND WITHOUT AN EGG IN THE SHELL GLAND IT SHOULD BE POSSIBLE TO DETERMINE WHICH EXPONENTIAL COMPONENT CORRESPONDS WITH EGG SHELL CALCIFICATION; WHILE COMPARISON OF THESE CURVES IN HENS WITH ACTIVE AND INACTIVE OVARIES SHOULD GIVE SOME INSIGHT INTO THE CONTRIBUTION OF OTHER PARAMETERS OF CALCIUM METABOLISM WHICH CHANGE IN THE LAYING HEN (SUCH AS BONE REMODELLING, GUT ABSORPTION AND RENAL EXCRETION).

TWO TYPES OF EXPERIMENT WERE PERFORMED. THE PRELIMINARY EXPERIMENTS WERE DESIGNED TO EXAMINE THE EFFECT OF NEURAL BLOCKADE ON INCORPORATION OF TRACER INTO THE EGG SHELL, WHILE ANALYSIS OF THE PLASMA $^{45}$Ca DISAPPEARANCE CURVE IN BIRDS UNDER VARIOUS PHYSIOLOGICAL CONDITIONS GAVE SOME INSIGHT INTO THE PHYSIOLOGICAL PARAMETERS CORRESPONDING TO THE EXPONENTIAL COMPONENTS OBTAINED FROM THE CURVE. THIS PERMITTED INTERPRETATION OF ANY EFFECTS OF THE NEURAL BLOCKING DRUGS ON THE DISAPPEARANCE CURVE IN TERMS OF PHYSIOLOGICAL CHANGES IN THE HEN.
METHODS.

The experimental animals were white Leghorn laying hens, obtained from the poultry farm of the University of British Columbia, weighing 1.5-2 kg. They were maintained on a diet of laying pellets (Buckerfields, Vancouver, B.C.), containing 2.5% calcium, supplemented with flaked oyster shells to ensure maximal calcium intake. Care was taken to select animals which were laying down egg shell at a steady rate. The method of determining the phase of the laying cycle is described in the Appendix.

The birds were anesthetized with 0.6 ml of somnopentyl, given intravenously, and anesthesia was maintained with 0.15 ml aliquots of this anesthetic, given as required.

A P.E. 50 cannula was inserted into the right medial wing vein for administration of anesthetic, 45Ca and drugs. In experiments where a continuous intravenous infusion of a blocking drug was used a second P.E. 50 cannula was inserted into the medial vein on the other wing.

A P.E. 10 cannula was placed in the right brachial artery for blood pressure measurement, which was recorded using a Statham P23 AA pressure transducer attached to a Gilson recorder. In experiments in which the arterial plasma 45Ca levels were monitored a second P.E. 10 cannula was inserted into the left brachial artery and used for collection of blood samples.

All cannulae were filled with heparinized saline (10 mg heparin/100 ml of 0.9% saline).

In the preliminary series of experiments each hen was
GIVEN, INTRAVENOUSLY, 0.2 MCURIES OF 45Ca (SPECIFIC ACTIVITY 0.5 μCURIE OF 45Ca/MG OF Ca), DISSOLVED IN 0.5 ML OF 0.9% saline. THE BIRDS WERE KILLED WITH AN OVERDOSE OF ANESTHETIC AT VARIOUS TIME INTERVALS AFTER INTRODUCTION OF THE TRACER, THE EGG REMOVED FROM THE OVIDUCT, AND THE SHELL ISOLATED. THE EGG SHELL WAS DISSOLVED IN 50 ML OF 11.5 N HCl, THE SHELL MEMBRANE BEING DISCARDED. THE 45Ca IN A 50 μL ALIQUOT OF THE RESULTING SOLUTION WAS COUNTED AS DESCRIBED IN CHAPTER I.

A PLOT OF TIME VERSUS % 45Ca DOSE ADDED TO THE HEN APPEARING IN THE EGG SHELL WAS CONSTRUCTED, SEE FIGURE 14, AND FROM THIS TWO TIME INTERVALS WERE SELECTED FOR FURTHER STUDY. THE EARLY TIME PERIOD (30 MIN) ON THE RISING PORTION OF THE CURVE WAS TAKEN AS A MEASURE OF THE RATE OF EGG SHELL CALCIFICATION, WHILE THE LATE TIME PERIOD (120 MIN) ON THE PLATEAU PORTION OF THE CURVE WAS USED AS A MEASURE OF THE TOTAL AMOUNT OF LABEL INCORPORATED INTO THE EGG BY ANY PARTICULAR BIRD.

USING THE DRUG DOSAGES WORKED OUT IN THE PRECEDEING CHAPTER, THE EFFECT OF BLOCKADE OF VARIOUS BRANCHES OF THE AUTONOMIC NERVOUS SYSTEM ON THE INCORPORATION OF 45Ca INTO THE EGG SHELL AT THESE TWO TIME PERIODS WAS STUDIED. THE EXPERIMENTAL PROTOCOL USED WAS THE SAME AS IN THE PRELIMINARY EXPERIMENTS.

IN THE TWO HOUR INCORPORATION STUDIES THE DISAPPEARANCE OF 45Ca FROM THE ARTERIAL PLASMA WAS ALSO MONITORED BY TAKING 0.4 ML BLOOD SAMPLES FROM THE LEFT BRACHIAL ARTERIAL CANNULA AT FREQUENT INTERVALS. THE BLOOD WAS CENTRIFUGED IN A
Clinical centrifuge to remove the cells and a 50 µl aliquot of the arterial plasma was counted as described in Chapter 1. The rate of disappearance of counts from the plasma was analysed in an attempt to describe the individual events contributing to the overall rate of tracer movement. The method selected was that of Robertson (1957), which is, essentially, the graphical fitting of straight lines to the experimental curve, starting with the slowest rate. Figure 15 illustrates the procedure. The half lives of each exponential rate were calculated from the slopes of the semi-logarithmic plots in the usual manner.

The arterial plasma 45Ca disappearance curve was also examined in hens which had no egg present in the shell gland and compared with the curve obtained during active shell calcification when no drugs were present. Two groups of hens were used. In one group the hens were in active lay but were on the pause day of their cycle, while the hens in the other group were out of lay and had inactive ovaries. The experimental design remained the same as described earlier.
Figure 14. A graph to show the time course of incorporation of 45Ca into the egg shell.
RESULTS AND DISCUSSION.

Figure 14 shows the effect of time on the incorporation of $^{45}\text{Ca}$ into the egg shell. With increasing time more of the $^{45}\text{Ca}$ dose appeared in the egg shell. However the graph was approaching a plateau indicating that at longer time periods than two hours very little more than 50% of the $^{45}\text{Ca}$ dose would appear in the egg shell.

The data of Lürcher & Hodges (1969) fits well onto this curve. These workers showed that after 100 minutes 51.0% of an intravenous $^{45}\text{Ca}$ dose given to an anesthetized hen had been incorporated into the egg shell. After longer time periods, 155-185 min, they found that 45-53% of the $^{45}\text{Ca}$ dose had been incorporated, indicating that the plot of time versus % $^{45}\text{Ca}$ dose appearing in the egg shell did indeed plateau as the results presented here indicated. This was not surprising since the blood disappearance curve shows that the amount of label in the blood drops precipitously in two hours (Figure 15) so that, as time wears on, less and less of the calcium deposited in the shell will be labelled.

Lürcher & Hodges (1969) also looked at the incorporation of intravenously injected $^{45}\text{Ca}$ into egg shells by unanesthetized hens, and found that 38-45% of the dose was present in the egg shell after 120 minutes. Therefore the anesthetized hen appears to calcify eggs at the same rate as an unanesthetized bird.

Examples of the disappearance of $^{45}\text{Ca}$ from the arterial plasma in a bird calcifying an egg shell and in a bird with
**Figure 15.** Graph to show the rate of disappearance of $^{45}$Ca tracer from the arterial plasma of a hen which is actively calcifying an egg.

The curve has been analysed kinetically by manual fitting of straight lines, Robertson (1957).

- $\times$ - Experimental points.
- $\bigcirc$ - Points obtained by subtraction of the slowest exponential component from the curve.
- $\square$ - Points obtained by subtracting the intermediate exponential component from the derived curve ($\bigcirc$). Since these points define a straight line this line is the fastest exponential component of the curve.
- $K_1$ - Rate constant for the slowest exponential component.
- $K_2$ - Rate constant for the intermediate exponential component.
- $K_3$ - Rate constant for the fastest exponential component.
INACTIVE OVARIIES ARE SHOWN IN FIGURES 15 AND 16. IT MAY BE NOTED THAT THE POSITION OF THE CURVE RELATIVE TO THE X-AXIS WAS DIFFERENT IN THESE TWO BIRDS. IN GENERAL IT VARIED FROM BIRD TO BIRD PROBABLY BECAUSE OF DIFFERENCES IN BODY WEIGHT AND HENCE BLOOD VOLUME. HOWEVER IN THE EXAMPLES SHOWN BOTH BIRDS WEIGHED 1.5 KG SO THAT THIS COULD NOT BE THE EXPLANATION HERE. SEVERAL WORKERS REPORT THAT INJECTION OF ESTROGEN INTO CHICKENS INCREASES PLASMA AND BLOOD VOLUME, GILBERT (1967), THUS ONE MIGHT EXPECT LAYING HENS TO HAVE A LARGER BLOOD VOLUME THAN BIRDS OF THE SAME AGE AND WEIGHT WHICH WERE OUT OF LAY.

COMPARISON OF THE BLOOD CURVES IN THE TWO TYPES OF BIRD SHOWS THAT THE CURVE IN THE BIRD OUT OF LAY WAS VERY MUCH SHALLOWER AND FLATTER THAN THAT IN THE BIRD CALCIFYING AN EGG SHELL. THIS WAS BORNE OUT BY THE KINETIC PARAMETERS OBTAINED BY CURVE ANALYSIS, SEE TABLE XII. THREE EXPONENTIAL COMPONENTS WERE OBTAINED FROM THE TWO HOUR PLASMA 45Ca DISAPPEARANCE CURVE. THE SLOWEST COMPONENT HAD A HALF LIFE WHICH WAS TWICE AS LONG WHEN THE BIRD WAS OUT OF LAY AS WHEN IT WAS CALCIFYING AN EGG (300 MIN AS COMPARED TO 150 MIN). THE BIRDS IN ACTIVE LAY ON A PAUSE DAY SHOWED A LARGE VARIATION IN THIS KINETIC COMPONENT. IN ALL CASES THE HALF LIFE WAS LONGER THAN WHEN AN EGG WAS BEING CALCIFIED AND IN GENERAL IT WAS ABOUT 240 MIN AS OPPOSED TO 150 MIN, ABOUT A 60% INCREASE. HOWEVER IN ONE CASE THE HALF LIFE WAS EVEN LONGER THAN THAT IN BIRDS OUT OF LAY, INDICATING THAT INDIVIDUAL VARIATIONS WITHIN THE TWO GROUPS OF BIRDS NOT CALCIFYING AN EGG WERE SO
Figure 16. Graph to show the rate of disappearance of 45Ca from the arterial plasma of a hen which is out of lay.

All legends as in Figure 15.
LARGE THAT THESE TWO GROUPS COULD PERHAPS BE CONSIDERED AS ONE.

There was a slight indication that the intermediate exponential component had a longer half life in the birds out of lay than in those with active ovaries. There was individual variation in the half life of the fastest exponential component but since this kinetic parameter is the one most subject to error due to the nature of the curve analysis, Robertson (1957), no real differences between the groups of birds could be discerned.

The kinetic analysis of the two hour plasma 45Ca disappearance curve gave three exponentials so that the blood can be considered as exchanging with three other compartments. Since changes could be seen in these components in hens under different physiological conditions some idea of which physiological parameters these might represent could be obtained. Figure 17 gives a diagrammatic representation of possible physiological compartments which could exchange with plasma calcium.

The plasma calcium is in equilibrium with calcium in the extracellular fluids and the soft tissues. Since the hen's extracellular fluid volume is approximately five times the volume of the plasma, Sturkie (1965), one would expect rapid loss of calcium into this compartment. This probably gave rise to the fastest component in the blood curve with a half life of 2-4 min. Exchange with the cells would be complex and depend upon active processes; perturbation of the blood
TABLE XII

THE EFFECT OF VARIOUS PHYSIOLOGICAL STATES OF THE HEN ON THE HALF LIVES OF THE EXPONENTIAL COMPONENTS CALCULATED FROM THE ARTERIAL 45Ca DISAPPEARANCE CURVE

<table>
<thead>
<tr>
<th>Egg in Shell Gland</th>
<th>Physiological State of Hen</th>
<th>Half Life in Minutes</th>
<th>% Ca45 Dose in Egg Shell After 2 Hrs</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Fast (k1)</td>
<td>Intermediate (k2)</td>
</tr>
<tr>
<td>Yes</td>
<td>Active Lay</td>
<td>--</td>
<td>--</td>
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<td></td>
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<td>2.85 ± 0.19</td>
<td>17.79 ± 0.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.27 ± 0.06</td>
<td>18.97 ± 0.46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.65 ± 0.01</td>
<td>18.04 ± 0.34</td>
</tr>
<tr>
<td>No</td>
<td>Active Lay</td>
<td>2.44 ± 0.02</td>
<td>18.11 ± 1.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.99 ± 0.01</td>
<td>20.72 ± 0.89</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.74 ± 0.02</td>
<td>13.68 ± 0.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.28 ± 0.01</td>
<td>18.38 ± 0.77</td>
</tr>
<tr>
<td>No</td>
<td>Out of Lay</td>
<td>4.19 ± 0.18</td>
<td>20.66 ± 0.62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.48 ± 0.08</td>
<td>24.44 ± 1.55</td>
</tr>
</tbody>
</table>

* ± S.E. on the least mean squares slope.
Absorption from the gut would dilute the plasma calcium pool with unlabelled calcium. The level of calcium in the diet affects the amount of calcium absorbed, Hurwitz & Bar (1969). Since the hens used in this study had access to grit it seems appropriate to assume that they absorbed calcium at a rate comparable to the hens on the highest calcium diet in the studies of Hurwitz & Bar, i.e. 2.43 g/hen/day. This would mean that during egg shell calcification each hen absorbed on average 1.68 mg/min. The plasma calcium level in a laying hen is generally approximately 25 mg/l and the plasma volume of a 2 kg hen is about 100 ml, Sturkie (1965); therefore the total amount of calcium in the plasma is roughly 25 mg. Thus it follows that approximately 7% of the total amount of calcium in the plasma is absorbed every minute. The birds used in these experiments were taken during their night, see the Appendix. Since absorption decreases during the night, as the contents of the gut are used up, the absorption rate calculated above may be somewhat high, but it gives some idea of magnitude and shows that absorption from the gut would be an important contributor to the plasma 45Ca disappearance curve.

Hurwitz (1964) has shown that bone can be divided into two compartments, exchangeable and stable bone. He concludes from his data that the major portion of exchange is due to physicochemical processes rather than active bone resorption. Mueller et al. (1964) state that from 4.3 to 4.9 g of the skeletal calcium participates in egg shell formation, of which
Figure 17. A diagrammatic representation of the possible fate of intravenously injected 45Ca, showing physiological compartments which could be involved.
1 g is turned over daily. This would mean that 0.7 mg of Ca/min was being exchanged with the bone. Using the same assumptions as above, this means that the bone turnover rate is equivalent to about 2.8% of the total plasma calcium being exchanged per minute. Exchange with bone can therefore be expected to contribute significantly to the plasma 45Ca disappearance curve.

The laying hen also secretes calcium in its urine. Although the level of calcium secretion decreases on laying days the hen still secretes 7.8 meq of Ca/day (equivalent to 0.312 g/day), Taylor & Kirkley (1967). Hurwitz & Griminger (1961) report a slightly lower value of 0.22 g/hen/day. Thus calcium is lost in the urine at a rate of 0.15-0.22 mg/min, or with the same assumptions as before, about 0.61-0.86% of the total plasma calcium is lost in the urine every minute. Thus some contribution to the plasma 45Ca disappearance curve can be expected.

Endogenous fecal excretion could also cause loss of calcium from the blood. According to Mueller et al. (1964) endogenous excretion is responsible for the loss of 7% of the intake on laying days and 12% on nonlaying days, which is about 0.16-0.28 g/day. However, since the hens in this study were not colostomized, endogenous excretion included urine as well as feces, and as the levels of endogenous excretion are very close to those for urine excretion alone, one might infer that endogenous fecal excretion is negligible.

The other compartment into which the plasma loses calcium...
IN THE LAYING HEN IS THE EGG SHELL. FROM THE RESULTS REPORTED
HERE (FIGURE 14) IT IS EVIDENT THAT 50% OF THE BLOOD CALCIUM
LABEL IS LOST TO THE SHELL IN THE SPACE OF TWO HOURS, SO THAT
THIS MUST MAKE A VERY IMPORTANT CONTRIBUTION TO THE DISAPPEARANCE CURVE. IF THERE WAS NO RECYCLING OF TRACER ONE WOULD EXPECT EGG SHELL CALCIFICATION TO PRODUCE AN EXPONENTIAL COMPONENT IN THE BLOOD CURVE WITH A HALF LIFE OF ROUGHLY 120 MIN. DURING ACTIVE SHELL CALCIFICATION THE HALF LIFE OF THE SLOWEST COMPONENT WAS 150 MINUTES AND THIS GREATLY INCREASED WHEN NO EGG WAS PRESENT IN THE SHELL GLAND. THE EXTRACELLULAR FLUID VOLUME IS FIVE TIMES AS LARGE AS THE PLASMA VOLUME AND 50% OF THE 45Ca DOSE APPEARS IN THE EGG SHELL, THEREFORE, IF ONE ASSUMES EQUILIBRIUM BETWEEN THE EXTRACELLULAR FLUID AND THE PLASMA, IT FOLLOWS THAT 45Ca MUST HAVE RETURNED TO THE BLOOD FROM THE EXTRACELLULAR FLUID. THIS WOULD TEND TO LENGTHEN THE HALF LIFE OF THE EGG SHELL CALCIFICATION COMPONENT.

THE OTHER FACTOR WHICH WOULD TEND TO PRODUCE A LONGER HALF LIFE IS THE PRESENCE OF OTHER EXPONENTIAL COMPONENTS WITH LONGER HALF LIVES WHICH DO NOT APPEAR IN THE TWO HOUR BLOOD CURVE. SUBTRACTION OF THESE BY CURVE ANALYSIS WOULD DECREASE THE HALF LIVES OF THE PRECEEDING EXPONENTIALS. HURWITZ (1964) LOOKED AT THE 45Ca DISAPPEARANCE CURVE OVER A MUCH LONGER TIME INTERVAL THAN THE ONE USED HERE. IF HIS CURVE IS SUBJECTED TO THE SAME TYPE OF KINETIC ANALYSIS PERFORMED HERE ONE OBTAINS A MINIMUM OF THREE EXPONENTIAL COMPONENTS WITH HALF LIVES OF 5, 26 AND 111 HOURS. THIS ANALYSIS WAS VERY CRUDE, SINCE THE NUMBER OF POINTS WAS SO SMALL, BUT THE GENERAL PATTERN CAN BE
discerned. Subtraction of these exponential components would have shifted the half life of the slowest component in the two hour curve to a smaller value and hence closer to the expected 120 minutes of the egg shell calcification process. It therefore appears that the slowest component of the two hour blood curve was due to egg shell calcification.

When the stress of egg shell calcification was removed this exponential had a much longer half life, but the individual variation between the birds was much greater than when an egg was being calcified (table XII). This could have been because other underlying kinetic components, such as bone remodelling, were present, but when egg shell calcification caused a major drain on calcium metabolism these non-essential processes would probably have virtually ceased.

Generally in the birds on a pause day the third exponential had a faster half life than in birds out of lay. This is not surprising since one would expect laying birds to have a faster calcium metabolism than birds with inactive ovaries. However one bird on a pause day had an even slower half life than the birds out of lay, so that individual variations were so great that no clear cut conclusions could be drawn.

The fastest exponential was probably mainly due to equilibration with the extracellular fluids and soft tissues while the slowest exponential was due to egg shell calcification. This leaves the second exponential which could be due to calcium absorption, exchange with bone, urine formation, or a combination of any or all of these.
The half life of the second exponential did not change much with egg shell calcification. It was much more variable when the birds were on a pause day and may possibly have been slightly slower when the birds were out of lay (Table XII).

Mueller et al. (1964) showed that the difference between bone accretion and bone resorption was -0.44 g/hen/day on laying days and 1.48 g/hen/day on non-laying days. Therefore one would expect a faster disappearance of label from the blood due to bone deposition on non-laying days than on laying days.

Hurwitz & Sar (1969) showed that the absorption of calcium increased from 40% to 70% when egg shell calcification was taking place. Therefore hens on a pause day would be absorbing less and this would tend to slow the disappearance curve.

Calcium excreted in the urine increases from 7.8 meq/day on laying days to 13.9 meq/day on non-laying days, Taylor & Kirkley (1967). Thus the effect of urine formation on the 45Ca disappearance curve would be to decrease the half life of the second exponential in hens on a pause day.

If only one of these three factors were involved one would expect to see a change in the second exponential between birds actively calcifying an egg and those which were not. However absorption acts to increase the half life of the second exponential, while bone formation and urine formation act in the opposite direction in hens on a pause day. The calculations performed above showed that the changes in total blood
CALCIUM DUE TO THESE THREE FACTORS WERE OF THE SAME ORDER OF MAGNITUDE. THEREFORE, IF THE SECOND EXPONENTIAL WERE DUE TO A COMBINATION OF ALL THREE, THE CHANGES IN EACH CAUSED BY EGG SHELL CALCIFICATION COULD POSSIBLY CANCEL OUT AND NO NET EFFECT ON THE SECOND EXPONENTIAL WOULD BE SEEN.

THE SLIGHT SLOWING OF THE SECOND EXPONENTIAL COMPONENT IN HENS OUT OF LAY COMPARED WITH THOSE IN ACTIVE LAY WAS TO BE EXPECTED BECAUSE, PRESUMABLY, THESE BIRDS LACKED MEDULLARY BONE AND THEREFORE HAD LESS BONE ACCRETION. HURWITZ (1965) STUDIED CALCIUM EXCHANGE IN VARIOUS SEGMENTS OF BONE AND FOUND THAT THE TURNOVER RATE OF CALCIUM IN THE MEDULLARY SEGMENT WAS AT LEAST 10-15 TIMES LARGER THAN THAT OF CORTICAL SEGMENTS. HOWEVER THERE WAS MUCH MORE CALCIUM MASS IN THE CORTICAL BONE AND THE ACCRETION RATE WAS 1.50, 0.23 AND 0.83 MG/HR IN THE FEMUR ENDS, CORTEX AND MEDULLARY SEGMENT RESPECTIVELY. THEREFORE ALTHOUGH THE TURNOVER RATE IN THE MEDULLARY BONE MAY BE GREATER, THE GREATER MASS OF THE OTHER SEGMENTS TENDS TO OVERRIDE THE EFFECT AS IT WOULD APPEAR IN A KINETIC ANALYSIS; THUS ONLY A VERY MINOR EFFECT WAS SEEN.

ONE OF THE FUNDAMENTAL ASSUMPTIONS IN TRACER KINETICS IS THAT THE SUBSTANCE BEING STUDIED IS UNIFORMLY DISTRIBUTED AT ALL TIMES. THIS IMPLIES INSTANTANEOUS AND HOMOGENEOUS MIXING WITHIN A COMPARTMENT. 45Ca WAS INJECTED INTRAVENOUSLY AND ARTERIAL SAMPLING BEGAN TWO MINUTES LATER. SINCE THE CIRCULATION TIME IN A HEN IS 2.8 SECONDS, STURKIE (1965), MIXING IN THE PLASMA WOULD OCCUR RAPIDLY SO THAT THIS ASSUMPTION APPEARED VALID. HOWEVER HURWITZ (1968) HAS DEMONSTRATED THE
<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Drug</th>
<th>Type of Blockade</th>
<th>% Counts in Egg Shell</th>
<th>Number of Hens</th>
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</thead>
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<tr>
<td>30</td>
<td>Atropine</td>
<td>Parasympathetic</td>
<td>32.26 ± 1.94*</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Dibenzyline</td>
<td>Alpha-sympathetic</td>
<td>31.01</td>
<td>1</td>
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<tr>
<td></td>
<td>Propranalol</td>
<td>Beta-sympathetic</td>
<td>33.12</td>
<td>1</td>
</tr>
<tr>
<td>120</td>
<td>Atropine</td>
<td>Parasympathetic</td>
<td>51.36 ± 3.34*</td>
<td>6</td>
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<tr>
<td></td>
<td>Dibenzyline</td>
<td>Alpha-sympathetic</td>
<td>54.06 ± 8.87*</td>
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<tr>
<td></td>
<td>Propranalol</td>
<td>Beta-sympathetic</td>
<td>54.60 ± 3.91*</td>
<td>2</td>
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</tbody>
</table>

* ± S.E. of the mean.
Presence of a slowly-exchanging protein-bound calcium fraction in the plasma of the laying hen which comprises about 22% of the total plasma calcium and exchanges at a rate of 0.11 mg/min/100 ml. The presence of this slowly-exchanging fraction would prevent instantaneous homogeneous mixing in the plasma and hence invalidate one of the basic assumptions used in the kinetic analysis.

Compartmental analysis of the 45Ca disappearance curve presupposes steady state conditions. During egg shell calcification this condition is not strictly true. Taylor & Hertelendy (1961) showed that the diffusible calcium fell 1 mg/100 ml, approximately a 4% fall in total plasma calcium. Winget et al. (1958) showed that the shell gland venous blood had 20% less total calcium than the arterial blood. Therefore strict steady state conditions are not present. The mathematical treatment for non-steady state systems is very much more complex and has been ignored in favour of the less rigorous steady state treatment.

Thus, while the kinetic analysis is an approximation it does give some idea of the relative rates of exchange between compartments and the fate of calcium in the laying hen.

In conclusion it appears that the three exponentials obtained in the kinetic analysis were not all simple functions. The fastest one was probably a result of equilibration with the extracellular fluid and soft tissues and the slowest was due mainly to egg shell calcification. The intermediate exponential was probably due to a combination of absorption,
TABLE XIV

The Effect of Various Neural Blocking Drugs on the Half Lives of the Exponential Components Calculated from the Arterial Plasma 45Ca Disappearance Curve in Hens Actively Calcifying an Egg

<table>
<thead>
<tr>
<th>Drug</th>
<th>Type of Blockade</th>
<th>Fast (k₁)</th>
<th>Intermediate (k₂)</th>
<th>Slow (k₃)</th>
<th>% Ca⁴⁵ Dose in Egg Shell After 2HR</th>
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<td></td>
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<td>165.47 ± 20.72*</td>
<td>53.07</td>
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<td></td>
<td>161.79 ± 14.02</td>
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<tr>
<td></td>
<td></td>
<td>139.79 ± 6.01</td>
<td>51.77</td>
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<td>2.85 ± 0.19</td>
<td>17.79 ± 0.41</td>
<td>133.78 ± 7.49</td>
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<td></td>
<td>3.27 ± 0.06</td>
<td>18.97 ± 0.46</td>
<td>138.01 ± 6.32</td>
<td>48.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.65 ± 0.01</td>
<td>18.04 ± 0.34</td>
<td>157.84 ± 7.66</td>
<td>53.23</td>
</tr>
<tr>
<td>Atropine</td>
<td>Parasympathetic</td>
<td>2.80 ± 0.06</td>
<td>18.10 ± 0.40</td>
<td>148.80 ± 5.46</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.87 ± 0.02</td>
<td>14.66 ± 0.38</td>
<td>149.54 ± 6.13</td>
<td>47.37</td>
</tr>
<tr>
<td>Dibenzyline</td>
<td>Alpha-Sympathetic</td>
<td>3.00 ± 0.06</td>
<td>17.59 ± 0.83</td>
<td>134.63 ± 5.86</td>
<td>45.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.62 ± 0.01</td>
<td>15.79 ± 0.17</td>
<td>135.53 ± 4.58</td>
<td>62.93</td>
</tr>
<tr>
<td>Propranalol</td>
<td>Beta-Sympathetic</td>
<td>2.71 ± 0.03</td>
<td>18.40 ± 0.41</td>
<td>164.44 ± 17.28</td>
<td>50.69</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.31 ± 0.01</td>
<td>17.78 ± 0.29</td>
<td>117.91 ± 5.64</td>
<td>58.51</td>
</tr>
</tbody>
</table>

* ± S.E. on the least mean squares slope.
exchange with bone and urine formation.

The effect of the various neural blocking drugs on 45Ca incorporation into the egg shell at 30 min and two hours is shown in Table XIII. After 30 min approximately 30% of the tracer dose had been incorporated into the egg shell, regardless of whether the drugs were present or not. After 120 min approximately 50% of the tracer dose appeared in the egg shell and the drugs appeared to have no effect on incorporation at this time period either. Thus the data implies that none of the drugs had any marked effect on either the rate of incorporation or the absolute amount of calcium incorporated into the egg shell.

The kinetic parameters of the tracer disappearance curve, during active shell calcification, in the presence and absence of the neural blocking drugs are shown in Table XIV and summarized in Table XV. The half-life of the slowest component was approximately 150 min, that of the intermediate component approximately 18 min, and that of the fastest approximately 3 min. The presence of the neural blocking drugs had no significant effect on any of these components. Thus the results were unable to demonstrate any effects of the neural blockade on any aspect of calcium metabolism in the laying hen.

The lack of effect of a neural blocker on a system is not definitive proof that the branch of the nervous system blocked by that particular drug is not involved. The blocking doses selected for these experiments gave 70-80% blockade, since the large doses required to give 100% blockade would probably
TABLE XV

A Summary of the Mean Half Lives of the Three Exponential Components Calculated from the Arterial Plasma 45Ca Disappearance Curve in Hens Under Various Conditions

<table>
<thead>
<tr>
<th>Egg In Shell Gland</th>
<th>Physiological State of Hen</th>
<th>Drug</th>
<th>Type of Blockade</th>
<th>Fast (k₁)</th>
<th>Intermediate (k₂)</th>
<th>Slow (k₃)</th>
<th>Number of Hens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>Active Lay</td>
<td></td>
<td></td>
<td>3.26 ± 0.33*</td>
<td>18.27 ± 0.51</td>
<td>149.45 ± 12.57</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>ATROPINE</td>
<td>Parasympathetic</td>
<td>2.84 ± 0.03</td>
<td>16.38 ± 1.72</td>
<td>149.17 ± 0.37</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DIBENZYLNE</td>
<td>Alphasympathetic</td>
<td>2.81 ± 0.19</td>
<td>16.69 ± 0.90</td>
<td>135.08 ± 0.45</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PROPRANALOL</td>
<td>Betasympathetic</td>
<td>3.01 ± 0.30</td>
<td>18.09 ± 0.31</td>
<td>141.17 ± 23.26</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>Active Lay</td>
<td></td>
<td></td>
<td>2.61 ± 0.84</td>
<td>17.72 ± 2.54</td>
<td>258.97 ± 45.87</td>
<td>4</td>
</tr>
<tr>
<td>No</td>
<td>Out of Lay</td>
<td></td>
<td></td>
<td>3.84 ± 0.36</td>
<td>22.55 ± 1.89</td>
<td>303.07 ± 0.93</td>
<td>2</td>
</tr>
</tbody>
</table>

* MEAN ± S.E. ON THE MEAN

ALTHOUGH DIBENZYLNE IS AN IRREVERSIBLE BLOCKER BOTH ATROPINE AND PROPRANALOL ARE COMPETITIVE BLOCKERS, COMPETITIVE BLOCKADE MAKES DEFINITION OF BLOCKING DOSES DIFFICULT, BECAUSE AN INCREASE IN THE AGONIST CONCENTRATION WILL DISPLACE THE BLOCKER FROM RECEPTOR SITES AND A RESPONSE CAN ENSUE. DOLLEY ET AL (1969) CITE SEVERAL STUDIES WITH PROPRANALOL WHICH SUPPORT THIS CONCEPT. SINCE THE ABSOLUTE CONCENTRATIONS OF AGONIST NEAR THE RECEPTOR SITES ARE UNKNOWN, AND MAY LOCALLY BE VERY HIGH, THE POSSIBILITY OF DISPLACEMENT OF COMPETITIVE BLOCKERS CANNOT BE RULED OUT. Thus, while the pharmacological studies could have demonstrated a neural effect if one had been present, the lack of response does not necessarily prove that the nervous system is not involved.


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APPENDIX: A DESCRIPTION OF HOW A UNIFORM POPULATION OF HENS WAS OBTAINED WHOSE EGGS WERE IN THE LINEAR PORTION OF EGG SHELL CALCIFICATION

Studies on egg shell calcification required a uniform population of birds in the same stage of the calcification process. Burmester et al. (1939) and Bradfield (1951) have shown that egg shell calcification starts approximately five hours after the egg enters the shell gland and proceeds at a constant rate until just before the egg is laid some 15 hours later. Therefore birds taken somewhere in the middle of this 15 hour period would comprise a good experimental population.

Under ordinary photoperiods, i.e. lights 12 or 14 hours out of the 24, the domestic hen lays her eggs on two or more successive days, fails to lay on one day, and then approximately repeats the pattern. The eggs laid on consecutive days constitute a sequence or clutch. The first egg of the sequence is laid during the early or midmorning hours, subsequent eggs at later hours on successive days until a sequence is completed with lay of the terminal egg during early to late afternoon hours, Fraps (1955). Since egg shell calcification takes place in the last 15 hours before lay, or oviposition, most of this occurs at night.

It appeared advantageous to change the laying pattern of the experimental birds so that experiments could be conducted during a normal working day. Numerous workers have shown that light plays a large role in the control of ovulation in hens, see Fraps (1970) for references. Warren & Scott (1936)
Fig. 18. Diagram of the circuit used to monitor the time of oviposition in the experimental hens.

Once the egg was laid it rolled into the trough outside the cage and stopped light from the lamp from hitting the LDR, thus shutting off the clock at the time of lay.

Explanation of Symbols.

Lamp – No. 222 Prefocussed Lamp.
LDR – Phillips 3873/0302 Light Dependent Resistor.
E.F. – Emitter Follower NPN Transistor MPS 854.
Clock – Ingraham 115V, 2W, 60C Wall Electric Clock.
T1 – Hammond Transformer 262E6.
T2 – Hammond Transformer 166G25.
D1-D4 – Motorola Rectifier Diodes IN4001.
Z1-Z2 – Zener Diodes Motorola MZ 1000-17.
SUCCEEDED IN COMPLETELY REVERSING THE LAYING CYCLE OF A GROUP OF HENS BY REVERSING THEIR LIGHT SCHEDULE. THE BIRDS TOOK ABOUT 60 HOURS TO ADAPT TO A SUDDEN CHANGE IN THEIR LIGHTING REGIME, FROM LIGHT DURING THE DAY TO LIGHT DURING THE NIGHT. AFTER THIS THE BIRDS ONLY LAID DURING THEIR NEW LIGHT HOURS, I.E. AT NIGHT. THE CLUTCHES OF EGGS LAID SHOWED THE SAME TIMING PATTERNS AS THEY HAD DURING NORMAL DAYLIGHT HOURS.

HENS USED IN THE EXPERIMENTS REPORTED HERE HAD THEIR LAYING CYCLES REVERSED IN A SIMILAR MANNER. THEY WERE SUBJECTED TO TWO SUCCESSIVE 12 HOUR DARK PERIODS SEPARATED BY A 30 MINUTE LIGHT PERIOD TO ALLOW FEEDING. UNDER THEIR NEW LIGHT SCHEDULE THE LIGHTS WERE ON FROM 7:30PM TO 9:30AM.


HENS WITH CLUTCH LENGTHS FROM 2-5 EGGS WERE USED IN THE CALCIFICATION EXPERIMENTS. THEY WERE ANESTHETIZED AT 11:00AM-12:00NOON ON THE DAY FOLLOWING THE OVIPOSITION OF THE FIRST EGG IN THE CLUTCH AND THE 45Ca WAS INJECTED BETWEEN 1-2PM. THEREFORE THE EGG SHELL CALCIFICATION STUDIES WERE CONDUCTED ON THE SECOND EGG IN THE CLUTCH 7-12 HOURS BEFORE IT WOULD HAVE BEEN SPONTANEously LAID, I.E. IN THE MIDDLE OF THE
<table>
<thead>
<tr>
<th>Number of Eggs in Cycle</th>
<th>Egg Number (Time of Lay)</th>
<th>Total Number of Cycles</th>
<th>Number of Hens</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10PM-4AM</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>9PM-1AM</td>
<td>2AM-5AM</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>9PM-11PM</td>
<td>11PM-3AM</td>
<td>18</td>
</tr>
<tr>
<td>4</td>
<td>7PM-10PM</td>
<td>10PM-1AM</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>7PM-9PM</td>
<td>9PM-1AM</td>
<td>4AM-7AM</td>
</tr>
</tbody>
</table>
LINEAR CALCIFICATION PERIOD.