THE ACCUMULATION OF MINERALS IN THE AVIAN OVIDUCT.

by

Elizabeth M. Leonard, B.Sc.

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CONTENTS

FIGURES INDEX

TABLES INDEX

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PREFACE

During preliminary reading, it was noticeable that there was no satisfactory systematic account of the oviduct, from the point of view of a production line for the manufacture of the egg white, membranes, and shell. It is as such a systematic account that this thesis is arranged. The structural knowledge of the oviduct was developed, and this was followed by investigations into the composition and properties of the end products, and the possible mechanisms involved in their production.

This leads to a slightly unorthodox presentation, with information gained from the literature being integrated with the experimental results and observations, rather than forming a separate section. It is hoped that the advantages gained by this approach outweigh the disadvantages of abandoning the more usual manner of presentation.

SECTION 1

INTRODUCTION

One of the prime characteristics of birds is that they lay heavily shelled 'cleidoic' eggs - that is to say, eggs totally enclosed in a shell or membrane; they are in fact the only group of vertebrates which reproduce solely in this way, although some reptiles do. Such a reproductive process would seem admirably suited for flying animals, in that the extent and duration of weight gain during reproduction are minimal. (Simkiss,1967). However the formation of a calcified cleidoic egg necessarily modifies greatly the animal's mineral metabolism; and indeed it has been further suggested (Worden, 1964) that "the specialised features of the avian nitrogen metabolism will probably be ultimately referable to the characteristic excretion product, uric acid, which is in turn related to the demands of embryonic excretion within the cleidoic egg". In effect, the old question of which came first has not only not been answered, but another dimension has been added to it. Besides the peculiarities of nitrogen metabolism, the bird's metabolism is also influenced by the demands of the rapid assembly of the components of the egg, particularly where there is a continuous production of eggs.

The egg and its production have always been the object of wonder and curiosity, but rather surprisingly it is only comparatively recently that serious investigations have been undertaken. In 1863, Higginson said: "I think if required on pain of death to name instantly the most perfect thing in the universe, I should risk my life on the bird's egg." In 1967, more than a hundred years later, Simkiss said: "Avian reproduction is the most specialised system that occurs in the tetrapod." (Simkiss, 1967). Romanoff, in 1949, said: "The avian egg is the product of a remarkable physiological factory.", and it is that part of this factory which produces the characteristic outer wrappings of the egg, the albumen, the membranes, and the calcified

shell (most surprising of tertiary membranes), that is, the oviduct, which is the subject of the present study.

The early and mid-19th century naturalists were interested in the gross and external characteristics of eggs. This interest is illustrated in the many museum collections and catalogues of the time. From it grew examinations of the internal structure and histology of the egg (Owen, 1866; von Nathusius, 1867-1896; Foster and Balfour, 1874; Landois, 1865, 1878, 1892) followed by anatomical examination and measurement of the oviduct. (Sacchi, 1887; Curtis, 1910).

The studies were often motivated more by curiosity than by the stricter precepts of scientific verity, and some of their observations were somewhat fanciful. For example, Yarrell (1827) thought that transaction of the oviduct caused ovarian atrophy and a change to male type secondary characteristics if not genuine sex reversal; this view was also held by Tegetmeier (1867), Sutton (1885), and Brandt (1889) - see Pearl and Curtis, (1914.) However, many of the fundamental facts of egg production were accurately described. Coste (1847) had observed the infundibulum 'embracing' the ovarian follicle; Tarchanoff (1884) had stimulated the formation of teriary membranes (white, chalazae, shell membranes, and shell) around foreign objects - as did Weidenfeld in 1905 and Curtis in 1914. Pearl and Surface (1909) ingeniously arranged that the 'foreign object' was the bird's own faeces. Cushny, (1902) made a study of oviduct secretion. Histological study of the oviduct followed (Surface, 1912; Brambell, 1925; Bradley, 1928; and the still valuable paper of Richardson, 1935); the physics of the egg were described by Moran and Hale (1936) and the rate of its formation measured by Warren and Scott (1935a, 1935b). The chemical composition of the egg and the changes which it undergoes from ovulation to oviposition were determined and re-determined (Pearl and Curtis, 1912; Asmundson, 1931; Hansen, 1933; McNally, 1933; Scott, Hughes and

Warren, 1937; Burmester, 1940); various workers removed parts of the oviduct or took tucks in the oviduct wall and recorded the effects on the egg (Pearl and Surface, 1909; Pearl and Curtis, 1912 and 1914; Asmundson and Jervis, 1933; Asmundson and Burmester, 1938); and the secretions of the oviduct were collected and analysed (Beadle, Conrad and Scott, 1938). The composition of the oviduct itself was examined (Buckner, Martin and Peter, 1925); and the formation of the chalazae was explained (Hansen, 1933; Almquist, 1936; Olsen and Byerly, 1936; Conrad and Phillips, 1938, Asmundson, 1939).

The properties of the egg shell and membranes had come in for more detailed examination (McNally, 1934; Stewart, 1935; Moran and Hale, 1936) and the importance of blood calcium levels and other aspects of mineral metabolism were recognised (Buckner et al., 1930; Common, 1933 etc.; Morgan and Chichester, 1935; Morgan and Mitchell, 1938) - the role of medullary bone as a calcium store was known (Hughes, Titus and Smits, 1927; Heller, Paul and Thompson, 1934; Kyes and Potter, 1934; Bloom et al., 1940, 1941, 1942) and the curious effects of sulphanilamide, a carbonic anhydrase inhibitor, were confirming the role of the enzyme in shell calcification (Benesch, Barron and Mawson, 1944; Scott, Jungherr and Matterson, 1944). The dynamics of the calcification process were described by Burmester, Scott and Card in 1939.

By the early 1940's many important aspects of egg formation and oviduct function were known, but the growing economic importance of egg production had yet to be felt as a stimulus to research. It was not until the 1950's that the pace of research began to quicken. The shellgland in particular was further studied histochemically by McCallion (1953), and then using electron microscopy (Johnston, Aitken and Wyburn, 1963; Breen, 1966). Knowledge of the chemical composition of the oviduct was extended by Brown and Jackson, (1960), Schraer and Schraer

(1965), and Hohman and Schraer, (1966). The mechanical properties of the shellgland were investigated by Hoover, Smith and Abott (1962), and its anatomy, particularly the blood supply, by Hodges (1966). The effects of various stimuli (both hormonal and physical) on the oviduct have been studied (Zarrow et al., 1960; Wentworth, 1960; Gilbert, Lake and Wood-Gush, 1966 - this surveys much of the earlier work on the effects of foreign objects in the shellgland wall - Sykes, 1967). The structure and composition of the egg, - yolk, white, membranes and shell - have been examined by many methods including histology, electron microscopy, and chemistry by Wolken, 1951; Tyler and his co-workers, Mather et al., 1962; Baker and Balch, 1962; Terepka, 1963 a & b; Balch and Tyler, 1964; Frank, Burger and Swanson, 1964; Bronsch and Diamantstein, 1965; Draper 1966 and 1967; the substances secreted by the oviduct and the manner of their formation have been studied by Hoover and Smith, 1958; Anfinson and Steinberg, 1951; Frank and Vohra, 1964; as have the metabolic activity and hydrogen ion concentration of the oviduct (Brown and Badman, 1962; Ogasawara, Van Krey and Lorenz, 1964; Winget et al., 1965).

Blood calcium levels and general metabolic questions are further discussed in many papers, e.g. Tyler, 1940; Mueller and Schraer, 1960; Taylor and Hertelendy, 1960; Hertelendy and Taylor, 1961; Hunsaker and Stuckie, 1961, Mehring and Titus, 1964; Misra and Kemeny, 1964; and Taylor, 1966 - these subjects, and related ones such as calcium metabolism in the developing egg and the role of carbonic anhydrase are comprehensively reviewed by Simkiss (1961 and 1967).

There is little alteration to be made to the qualitative descriptions of egg formation given by Romanoff and Romanoff (1949), although many details may now be added. A clearer picture is beginning to emerge from the additional components furnished by more refined techniques such as electron microscopy, more accurate chemical and biochemical determina-

tions, elaborate histochemistry, and so on.

There are still many gaps; in particular the quantitative aspects of mineral transport occurring to the egg in the oviduct have been neglected, and it is the object of the present study to describe these processes and to attempt to elucidate some of the mechanisms involved.

SECTION 2

MATERIALS AND METHODS

2.1 INTRODUCTION. THE HEN

Throughout the work described here, the biological materials used were hens of the flocks maintained at the Poultry Research Centre. These were obtainable with oviducts in all states of reproductive activity from immature or completely inactive to fully functional; the functional ones could be in any stage of the reproductive cycle. Birds of several breeds were used; Brown Leghorns of the partially inbred lines maintained at the centre (see Blyth and Sang, 1960), and birds derived from commercial types, one high intensity white egg-laying light hybrid, (Shaver), and one brown egg-laying bird of medium body weight (Thornber 404). A few results were obtained from broiler type stock and lines maintained primarily because of certain genetic abnormalities (Cuckoo Leghorn, Talpid).

Eggs were obtained from the flocks, in some experiments without regard to origin, but in most cases the identity and breed of the hen was recorded, as well as the date and in some cases the precise time of lay. Soft shelled eggs, when not obtained at operation under anaesthetic, were collected as and when they were laid in the normal course of events; in addition, soft-shelled eggs were for a time obtained from birds which had had a thread inserted in the shell-gland wall (see Sykes, 1953; Gilbert, Lake and Woodgush, 1966; Sykes, 1967).

No attempt has been made to define inter-breed differences in the various parameters measured, with the exception of the oviducal dimensions. This is in itself a major research project which will be based in part on the preliminary survey of major mechanisms undertaken in the present work.

2.2 HISTOLOGY AND HISTOCHEMISTRY

Although the oviduct has already been extensively described by many workers, it was decided to re-examine it, in view of the changes which might have occurred because of the years of intense selective breeding which have occurred since these studies were published. To this end, hens of different breeds and in different states of reproductive activity were killed, and their oviducts removed and prepared for examination in a standard way. This was accomplished as follows: the hen was given an injection of a lethal dose of pentobarbitone sodium (Abbott veterinary Nembutal) into the wing vein. Immediately death was judged to be imminent the feathers were plucked from the abdominal region, and a V-shaped incision made below the breastbone. The resulting flap of skin was pulled up and back over the breast, and the underlying layers of muscle and peritoneium were cut. The lowest two or three ribs were also severed. This enabled the entire breast to be pulled up and forward, exposing the whole of the body cavity. The oviduct was then removed by cutting around the cloacal opening and severing the ligaments joining the lower part of the duct to the wall of the body cavity adjacent to the backbone. The rest of the organ could then be gently pulled clear. It was immediately immersed in a bath of ice-cold Tyrode or Avian equivalent solution to inhibit degenerative post-mortem changes, and there the ligaments, connective tissue, and any other tissues which remained attached were dissected off, care being taken not to cut into the oviduct wall. The organ could now be extended to its full length. The oviduct was then completely everted starting from the cloacal end. After eversion it was worked onto a tapered Perspex strip of suitable size - in the case of the active duct, this was of maximum width slightly less than the diameter of the forming egg, and usually 1/16" thick. For smaller ducts, the size of the strip was chosen so as not to over-extend the organ; a range of sizes was kept

available, as it was not always possible to estimate the required size in advance. The everted specimen was then immersed in solution in a black Perspex trough of a suitable length where it could be examined with a low power dissecting microscope, or for histology and subsequent display, it was placed in a vertical Perspex cylinder, filled with Formalin-Tyrode solution. Pieces could be cut from this intact everted oviduct at leisure for embedding and sectioning, leaving the rest of the organ clearly visible for comparison with and precise location of stained sections.

This procedure has several advantages; its simplicity and rapidity mean that the oviduct, and particularly the delicate functional epithelium, is completely exposed for inspection or to the action of the fixative. The entire organ is conveniently preserved in a standard form; histological specimens may be taken from it at any time, and their location is immediately and precisely obtainable, while the rest of the organ remains available for comparative purposes. This procedure of eversion and mounting could be of value to pathologists and other workers in the field, particularly where egg production is affected, for example by diseases such as Infectious Bronchitis or "watery white" conditions.

Some oviducts were fixed with partly-formed eggs *in situ*. For this, the bird was killed with intravenous Nembutal and the abdominal cavity exposed as described above; the oviduct was loosely ligated immediately above and below the egg, dissected out, and immersed in buffered formalin without eversion. Blocks were prepared from the complete assembly of egg plus oviduct, and sections were subsequently cut and stained to show the oviducal epithelium in contact with the egg exterior. The fixation of the oviduct with an egg *in situ* was first utilised by Frobose (1928).

In general, the appearance of the everted oviduct differs little from the descriptions given in the literature. (See Section 3). However, the use of specific staining procedures has revealed some interesting new aspects. The stains used were: Periodic acid/Schiff; Alcian blue; van Gieson; and von Kossa. (Casselman, 1959; Pearse, 1960; Lewis, 1962; Baker, 1966). The first three were used in the studies of the entire length of the oviduct; the von Kossa calcium stain gave interesting results only in the oviducts which contained eggs.

The periodic acid/Schiff test consists of a preliminary oxidation followed by Schiff's reagent, a complex reduction product of basic fuschin with an affinity for aldehyde groups which may be uncovered by the oxidation of both acidic and neutral polysaccharides. The conditions of the reactions and the procedure are discussed in some detail by Casselman (1959). Under the conditions used, a positive reaction indicates the presence of acid or neutral mucopolysaccharides in the oviduct sections. Such a positive reaction was consistently observed in goblet cells of the surface epithelium from the lower part of the infundibulum, where staining occurred in occasional cells, throughout the entire length of the magnum, and in the upper isthmus region. The lower isthmus and the shellgland were negative to the test, but a band of positive-staining epithelium was present at the shellgland-vaginal junction and for some distance down the vagina. In no region of the oviduct did the tubular gland cells show a positive reaction.

While the test will be positive for alpha-glycol groups from any source, which includes a wide variety of carbohydrates, in practice the simple sugars and their derivatives are lost during fixation, and a positive reaction may be taken to indicate the presence of polysaccharides, mucopolysaccharides, or glyco- or muco-proteins.

The procedure for Alcian blue staining is also given by Casselman (1959). At an appropriate pH, this stain is specific for acidic mucopolysaccharides; comparison with autoradiographs of sections labelled with ^{35}S suggests that it is selective for sulphated mucopolysaccharides. (Casselman, 1959). Preliminary tests showed pH 2.3 to be suitable; at higher pH, staining becomes non-specific.

The von Kossa method for the demonstration of calcium requires that the calcium be present as carbonate or phosphate. Treatment of the section with silver nitrate for 10 minutes or more causes the calcium to be replaced by silver, which is reduced to the black metallic form on exposure to sunlight. (Pearse, 1960).

2.3 SURFACE AREA OF THE REGIONS OF THE OVIDUCT

One of the most immediately apparent characteristics of the avian oviduct is the extremely complex appearance of the mucosal surface. In the magnum, the folds are large and smooth in outline and spirally aligned; in the isthmus they are smaller, more closely spaced and more regularly longitudinal. In the shellgland, there are five main folds or rugae, as well as many smaller villi. In order to obtain an estimate of the functional mucosal area, sections of the different regions in different functional states were examined microscopically and tracings were made of the serosal and mucosal outlines using a camera lucida attachment. The lengths of these were then measured using a map-reading wheel. By including a scale it was possible to convert these measurements to real values; it was however considered preferable to use the ratio of mucosal to serosal length, as any shrinkage of the tissue during fixation should affect both approximately equally.

Fisher and Parsons, (1950) developed a method for the estimation of surface area in the rat small intestine, which is a similar tissue. They considered the mucosal surface as a plane studded with rectangular prisms of dimensions b x w x h. The mean area of one prism, excluding the top, is $2h(b+w)$.

If the number of prisms or villi per unit serosal area is $n_c n_l$, then the ratio

$$R_a = \frac{\text{mucosal area}}{\text{serosal area}} \left(= \frac{\text{surface area}}{\text{base area}} \right) = 2h\,(b + w)\, n_c n_l + 1$$

$$= 2hbn_c n_l + 2hwn_c n_l + 1$$

Now if we define:

$$R_c = \frac{\text{mucosal length}}{\text{serosal length}} \quad \text{for circumference, and}$$

$$R_l = \frac{\text{mucosal length}}{\text{serosal length}} \quad \text{for length}$$

then:

$$R_l = 1 + 2hn_l, \text{ and } R_c = 1 + 2hn_c.$$

And defining f_l and f_c as the fractions of mucosal length and circumference not occupied by villi,

$$f_l = 1 - wn_l, \text{ and } f_c = 1 - bn_c.$$

Hence:

$$R_a = (R_l - 1)(1 - f_c) + (R_c - 1)(1 - f_l) + 1$$

Assuming that f_c and f_l are negligible, this becomes

$$R_a = R_l + R_c - 1$$

This was the formula derived and used by Warren (1939), expressed by Fisher and Parsons in their symbols. Further, assuming that R_l is approximately equal to R_c, the formula becomes

$$R_a = 2R_l - 1.$$

The formula thus derived gives an expression independent of the shape of cross-section of the villus, and also of its area. From visual inspection of the mucosal surface of the oviduct, both intact and in section, it seems that the assumptions made, i.e. that there is no flat space between villi, and that on average the surface is equally folded in the longitudinal and circumferential directions, are reasonable.

According to Fisher and Parsons, (1950), the error introduced if the villus is completely hemispherical rather than rectangular in elevation results in an under-estimate of the final ratio R_a by 3%, and this is the most unfavourable case; the value for rounded tops on the villi will thus be intermediate between 97% and 100% of the true ratio.

Since measurements are of the ratio between the two surfaces of the tissue, and any effects of shrinkage may be assumed to affect both equally, there should be no error due to the processes of fixation and embedding. Estimates by Fisher and Parsons indicate that such shrinkage could in any case be compensated for by the application

of a correction factor; for the rat small intestine they suggested that this be x1.1 for fixed tissue, and x 1.13 for further shrinkage during paraffin embedding, the overall factor thus being 1.243. A similar degree of shrinkage of the oviduct seems likely.

Fisher and Parsons held that no significant distension of the rat small intestine occurred at distension pressures of up to 35 cm. of water, as indicated by the lack of distortion of individual cells and evagination of crypts. During the passage of the egg, the oviducal wall is undoubtedly distended. Distortion of the mucosal folds does of course occur, but no stretching of the mucosal surface or distortion of individual cells was observed. The reduction in the value of R_a in the shellgland with egg in situ would thus appear to be explained by the stretching of the serosal surface only.

The question of the validity of applying measurements made on one section, usually 15 microns thick and a few cm. long, to characterise a region was investigated using serial sections from a block of active shellgland. Six sections were taken, with a spacing of at least 90 microns between each, so that a transverse distance of between 0.5 and 1.0 mm. was covered, and the variation in the measured ratios over this distance was examined. The results, expressed in terms of the longitudinal ratio, are in Table 1. Since the area ratio $R_a = 2R_1 - 1$, the coefficient of variation will be the same for both parameters, while the standard deviation and standard error will be doubled. In this case, the corresponding results are: mean $R_1 = 12.3 \pm 0.9$, and $R_a = 23.6 \pm 1.8$, with a coefficient of variation of 18.3%. This is considerable, particularly since the area over which the measurements were made is still small in comparison to the circumference of the oviduct; however, it is not so great as to affect the significance of the differences observed between different regions and different functional states, as shown in Section 3.3.

TABLE 1

SURFACE AREA RATIOS: VARIATIONS

Section	R_L
1.	10.13
2.	11.40
3.	16.49
4.	11.98
5.	10.95
6.	12.82
Mean	12.295
S.D.	2.248
S.E.	0.918
Coefficient of variation	18.3%

2.4 CHEMICAL ANALYSES

Analyses were performed on samples of oviduct tissue, and on the different components of eggs. For the former, the birds were removed from their cages with the minimum of disturbance, and killed by slow injection of 2ml. of Nembutal into the wing vein. After rapid removal, the oviduct was usually divided into: Magnum (including the Infundibulum), Isthmus, and Shellgland; in some experiments the isthmus was divided into upper, mid and lower regions. The samples were then split longitudinally into portions for the different analytical procedures.

For the egg components, eggs were collected, weighed, and broken out. A commercial egg separating cup was used to divide yolk from white, and the white was scraped off the shell as completely as possible. Particular care was taken to avoid any visible chips of shell in the white.

Content of Sodium, Potassium, Calcium and Magnesium. The samples were immediately weighed, and then dried overnight in an oven at approximately 100°C. (Preliminary trials had determined that this period was sufficient to obtain constant weight.) The samples were cooled in a dessicator, reweighed to obtain the dry weight, and then dissolved in acid. In initial experiments, 6N HCl (B.D.H. Microanalytical or 'Aristar' grade) was used; later experience showed that 1N HCl was adequate, and for some samples in later experiments, 10% trichloracetic acid (TCA) (B.D.H. 'Analar' grade) was used. Digestion for about one hour was sufficient to ensure complete extraction of the cations with the hydrochloric acid, and usually 3 - 4 hours with the TCA. The resulting solution was filtered, and made up to 100 ml. in a volumetric flask with distilled water, any undissolved residue being discarded.

The solutions thus obtained were stored in screw-cap polythene bottles until the analysis was carried out. This was done by Atomic Absorption Spectroscapy (A.A.S.).

Atomic Absorption Spectroscopy. Although the application of atomic absorption to analysis has come into quite wide use since the principle was suggested and the first practical apparatus was devised by Walsh (1953, 1955), it is perhaps not out of place to give a brief account of its advantages.

Both Emission Flame Photometry and Atomic Absorption Photometry depend on the excitation of the atoms of the element to be measured, and the specificity of the wavelength of light emitted by the transition from an excited to the ground state. In conventional flame photometry the excitation takes place in the flame of the analysing apparatus, and the intensity of the light of the relevant wavelength gives a measure of the concentration of the element present. In atomic absorption spectroscopy however, the reverse phenomenon is utilised; light of the wavelength corresponding to the desired excitation is supplied, and its absorption by atoms of the element in the vapourised sample allows the concentration of the element to be determined. The apparatus consists basically of a source of the line spectrum of the element, in most cases a hollow cathode lamp which has a cathode coated with the desired element; a vapouriser, usually a flame burner of the type used in emission spectroscopy; a monochromator (or in some instruments, a filter) to isolate the required line; and a detector, a photo-electric cell, with amplifiers, meters, and recorders added as needed. The apparatus is more fully described by David (1960), whose review also contains a concise account of the theory, and an examination of the comparative sensitivity, precision and accuracy of the method, all of which compare favourably with other available methods. The chief advantages are: in general,

the analysis may be carried out on a smaller sample than is necessary for emission spectroscopy; since the absorption takes place in the ground state of the element, sensitivity is not dependent on the excitation potential or on the temperature or other conditions in the flame, changes in which will also have much less effect on stability, which is dependent almost solely on the stability of the light source. This will have some degree of inherent fluctuation in addition to that due to the mains supply if the latter is not stabilised. The use of the ground state also improves the accuracy, as effectively all the atoms are in this state, where interference by collision transfer of excitation energy does not occur; the use of the hollow cathode line source, subject only to Doppler broadening, and an appropriately narrow slit width, enables background and radiative interference to be reduced to a level well below that present in corresponding emission measurements. Chemical interference occurs to the same extent as in emission methods, as it is due to the formation of compounds which do not dissociate at the temperatures of the flame, for example calcium phosphate. However, similar methods can be used to overcome these interferences in both techniques. The only respect in which atomic absorption may be considered inferior to emission spectroscopy is in the dilution required; not only must this be determined by a rough preliminary run, but it is usually great, thus introducing more scope for errors, particularly contamination of samples.

Apparatus and procedure. The majority of the measurements were made on an assembly which included sodium, potassium, calcium and magnesium lamps manufactured by Atomic Spectral Lamps Pty. Ltd.; a Techtron burner, Zeiss monochromator, and Beckman pen recorder. Operating conditions are given in Table 2.

TABLE 2

CONDITIONS OF USE OF ATOMIC ABSORPTION SPECTROPHOTOMETER

Element	Wavelength (A)	Lamp current (mA)	Gas mixture	Gas pressure (lb./sq.in.)
Na	5890	6	Air/Coal gas	15
K	7665	6	Air/Coal gas	15
Mg	2852	8	Air/Acetylene	15
Ca	4227	8	Air/Acetylene	20

As the relationship between the concentration of the element and the absorption of the spectral line is logarithmic, of a form comparable to the Beer-Lambert laws, (Fuwa and Vallee, 1963), the pen recorder was usually fitted with linear-logarithmic paper. Later series of measurements were made on a Techtron AA-100 assembly, conditions being largely the same. For this instrument, combined sodium/potassium and calcium/magnesium lamps were available, thus decreasing the time spent in warming-up procedures.

Operation. The appropriate lamp is allowed to warm up for about half an hour. The burner is then lit and adjusted for temperature, height, and alignment. The correct wavelength is selected approximately on the dial of the monochromator and this is then adjusted by finding the maximum intensity peak. The gain is adjusted to give full scale deflection for distilled water, i.e. zero absorption. A series of standard solutions are then introduced to calibrate the instrument; such a calibration is repeated every six or eight samples to check for drift due to heating of the burner parts or arising electronically. The unknown solutions, diluted by trial and error to a concentration within the range of the standards, are then introduced. Occasional checks at higher dilutions are necessary to eliminate the possibility of self-absorbance errors in over-concentrated solutions. The concentrations corresponding to the sample absorbances may then be read from a graph of the standards, or calculated from the computed regression line.

Under the conditions used, no interference effects are expected with sodium, or magnesium. Preliminary experiments showed that the effects of sodium on potassium readings was negligible at the ratios present; the only interference of importance is that produced by the presence of phosphate ions in calcium estimations. This was counteracted by the addition of a suppressant agent to both standard solutions

and samples. In the early experiments, this consisted of strontium chloride; later, this was replaced by lanthanum chloride, of spectral purity, as all other supplies were found to contain significant quantities of contaminating calcium. Contamination of containers was considered to be the greatest source of error in sodium and potassium determinations. Dilution errors are most serious for magnesium estimations because the sensitivity of the method is greatest for this element. These two sources of error were eliminated as far as possible by the preparation of duplicate samples, separately diluted. If a significant discrepancy appeared between the duplicates, the analysis was repeated.

Content of Phosphate, Chloride and Lipids. The following analytical methods were used: phosphate:- Fiske and Subbarow (1925) - vide Hawk, Oser and Summerson, (1947); chloride:- Cotlove, Trantham and Bowman (1958); Lipids:- Bligh and Dyer (1959), as modified by Peters and Smith (1964).

2.5 COLLECTION OF OVIDUCAL EGGS AND FLUID SAMPLES

Where this was the sole purpose of the experiment, the presence of an egg in the isthmus or shellgland was usually determined by cloacal palpation. Eggs in the magnum of the oviduct could not usually be detected by this means, and could only be predicted by consideration of the previous laying record of the hen. The hen was anaesthetised and the abdominal cavity opened as for removal of the oviduct; when the oviducal contents only were required, and the egg was low in the oviduct, a smaller incision could be used, and in these cases the technique is that of El Jack and Lake (1967). The cloaca was cleaned, and pressure was applied to the anterior end of the shellgland by two or more fingers of the left hand. A small polythene funnel and a graduated centrifuge tube are held in the cloaca near the vaginal opening for the collection of the

fluid, and the egg is removed by a second person as it is expelled. Fluid remaining in the shellgland is then gently 'milked' into the collecting funnel.

When the egg was in the magnum or upper isthmus, and the collection of fluid was not attempted, the egg was removed by carefully cutting the oviduct immediately anterior or posterior to the swelling indicating the position of the egg, whichever was the more easily accessible. The egg was then expressed into a beaker, prior to separation and analysis as already described.

2.6 OSMOTIC PRESSURE OF SOLUTIONS AND FLUIDS

Osmotic pressures were determined using the depression of the freezing point. The apparatus used was an osmometer manufactured by Advanced Instruments Inc., which, after calibration with a standard NaCl solution, gives a direct reading in milliosmoles, on a sample of 0.2 mls. or 2.0 mls. Readings were repeated on fresh aliqots. For samples in which associative or dissociative changes did not take place, reproducibility was better than 1%; for samples such as shellgland fluid, in which such changes did take place on exposure to the atmosphere, determinations were made as rapidly as possible.

2.7 PERMEABILITY OF THE SHELL MEMBRANE

Membranes were obtained from four sources: (1) Soft-shelled eggs occurring naturally throughout the P.R.C. flock were collected and stored in sealed polythene bags under refrigeration. (2) soft-shelled eggs laid by birds which had had a thread or Michel clip inserted in the shellgland wall in order to produce premature expulsion of the egg (Sykes, 1953; Lake and Gilbert, 1962, 1964: Gilbert, Lake and Woodgush, 1966; Sykes, 1967) were also collected and stored. (3) Normal infertile fresh eggs were decalcified in a solution of ethylene diamine tetra-acetate (EDTA). (4) Fertile unincubated

eggs were decalcified in EDTA solution. The decalcifying solution was made up from the disodium or tetrasodium salt of EDTA , of about 15g/100ml. concentration when rapid decalcification was required; when slower, more gentle decalcification was possible, a 4 - 5 g/100 ml. solution was used. There appeared to be no difference in the behaviour of the resulting membrane, although Simons and Wiertz (1963) have suggested that production of carbon dioxide by strong decalcifying solutions may affect the inner layer. On the other hand, the possibility that the long immersion required for completion of decalcification in the weaker solutions might influence the behaviour of the membrane did not seem to be substantiated. The breed of bird laying the naturally occurring soft-shelled eggs was recorded, but no difference between breeds was found.

Eggs from all four sources were treated in the same way, as follows: the membrane was punctured and the egg contents discarded. The membrane was then washed with distilled water, dried on filter paper, and covered with damp filter paper to keep it from drying out. Pieces were then cut from the membrane, placed over the open end of small test-tubes containing 1 ml. of one of the solutions, and held there by a tightly wound rubber band. The test tube was then inverted and the mouth immersed in a large test-tube or sample tube containing 10 mls. of the other solution, so that the two solutions were separated by the piece of membrane (see Figure 1). The tubes were left in this way overnight; they were then removed, the membrane inspected to ensure that it had remained intact, and the solutions tested. This was done in several ways: the presence of protein in a solution was detected by the Biuret test or by precipitation with trichloroacetic acid (TCA). Approximate quantitative measurements were obtained by centrifuging the solution after TCA precipitation (15 minutes at 3,000 r.p.m.) in a weighed centrifuge tube, and obtaining the weight of the dried precipitate. It was not possible to isolate the precipitate by filtration, as it is gelatinous

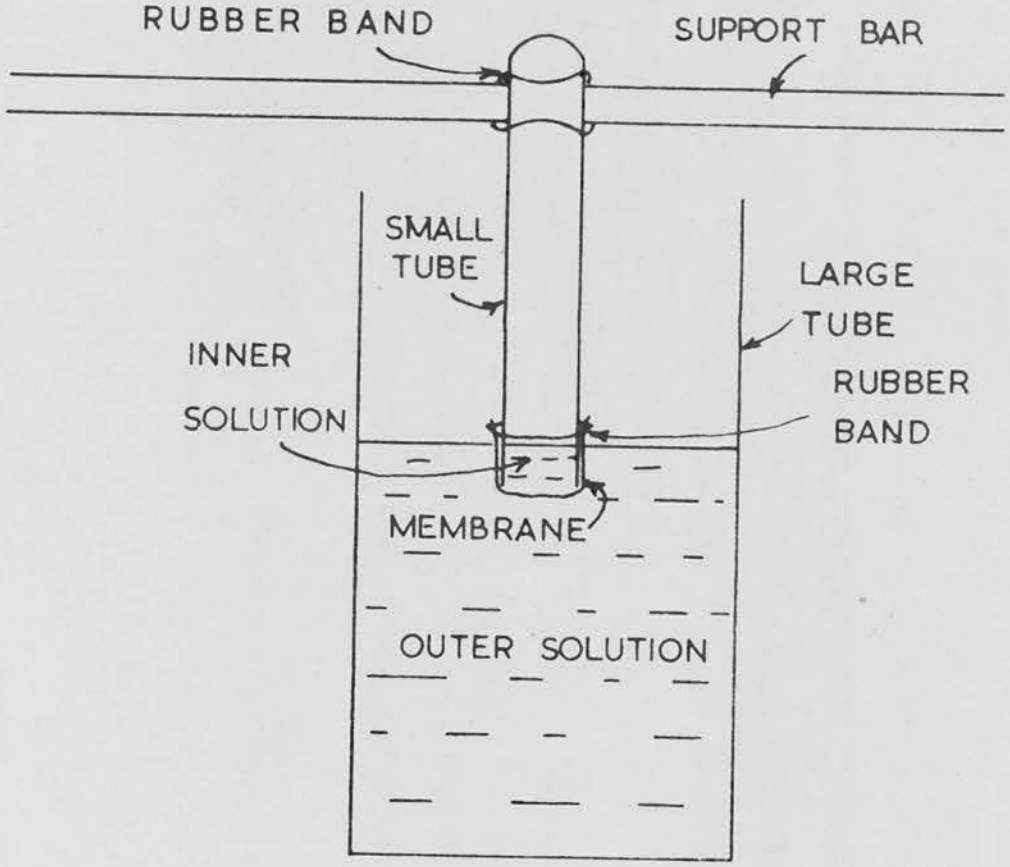


FIGURE 1:

DIAGRAM OF APPARATUS FOR TESTING PERMEABILITY OF SHELL MEMBRANE TO PROTEIN

EXPERIMENT 1

(I) Solutions in small tube
- (a) Distilled water
- (b) Saline solution (Avian equivalent D8; see Section 2.9)
- (c) Albumen solution (approximately 12g/100ml.)
- (d) Egg white

(II) Membrane
- (a) Fresh membrane
- (b) Decalcified infertile membrane
- (c) Decalcified fertile membrane

(III) Solutions in large tube
- (a) Distilled water
- (b) Saline solution (Avian equivalent)

Saline solution D8 is isosmotic with avian blood plasma - See Section 2.9.

Egg white contains approximately 12g. dry matter per 100 ml.; the albumen solution is of approximately 12g./100 ml. dried egg albumen (B.D.H.). This does not form a gel, but a non-viscous yellowish solution.

The egg white was thoroughly mixed before use, thus destroying the coherence of the thick white and forming a solution of thick and thin.

Each combination of solutions was set up in duplicate with each of the three types of membrane.

PERMEABILITY OF THE MEMBRANE: EXPERIMENT 2

(I) Solutions in small tube
- (a) Thick white, as little stirred as possible
- (b) Stirred mixed white
- (c) Albumen solution (10g./10ml.)
- (d) Saline solution, isotonic to oviducal white
- (e) Saline solution, isotonic to oviposited white
- (f) Distilled water

(II) Membrane: Fresh membrane from naturally occurring soft-shelled eggs

(III) Solutions in large tube: as (I) above with the exception of (a)

The procedure was as for Experiment 1. Samples of thick white gel (I)(a) above were obtained from a soft-shelled egg which was not fully plumped by placing the egg contents on a watchglass, and draining off the outer thin white. A slit was cut in the thick white with a scalpel, and through this the inner thin white was drained off. Care was taken to avoid puncturing the vitelline membrane. Samples of thick white were then excised with a scalpel and transferred to the small weighed tubes, so that there was the least possible disturbance of the white mass. The tube was reweighed to obtain the weight of thick white, which was usually about 1g.

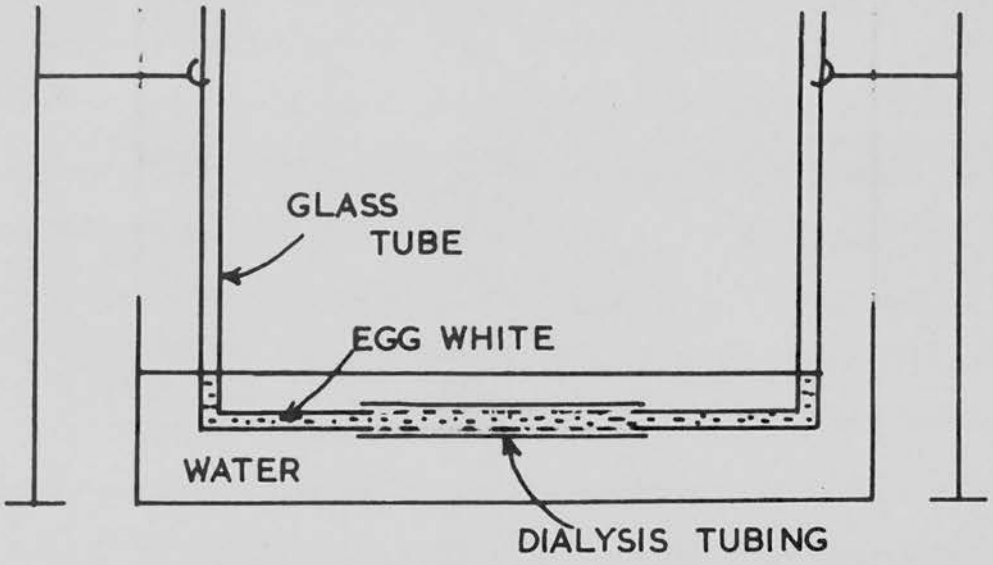
2.8 SWELLING OF EGGS IN VITRO

Naturally soft-shelled or decalcified eggs (see previous section) were placed in beakers containing 200 ml. of solution or distilled water. The solutions used were of NaCl and KCl, isotonic to: (a) plumping fluid - 8.0 g NaCl and 1.2g KCl per litre; (b) oviducal egg white - 6.8g NaCl and 0.75g KCl per litre; (c) oviposited egg white - 4.0g. NaCl and 1.8g. KCl per litre (see sections 4.5 and 4.6).

The egg was removed from the beaker at intervals of 10, 15 or 20 minutes, blotted with filter paper or tissue to remove excess moisture, and weighed. This was continued for a period of four hours, or until the egg burst; in a few cases, the procedure was carried on for a longer period, with less frequent weighings. When the experiment was over, or when the egg burst on removal from the beaker, the egg components were collected in weighed beakers and the wet and dry weights of each were determined.

The contribution of the membrane to the swelling of the egg was studied in several ways: firstly, membraneous eggs which were soaking as above were allowed to swell normally for the period of five weighings ($1\frac{1}{2}$ hours) to establish the rate of swelling. The membrane was then punctured with a seeker, a hole of $\frac{1}{2}$ to 1mm. being made, and the weighings continued for the rest of the four-hour period. Secondly, the contents of membraneous eggs were carefully transferred to a sac made from a piece of nylon stocking material, knotted at each end, and this was soaked and weighed in the same way as the soft-shelled eggs.

The behaviour of egg-white enclosed by dialysis tubing was also studied. Since the dialysis tubing does not possess the elasticity of the egg's own membrane, a different procedure was adopted: one or both ends of the section of dialysis tubing were attached to a vertical glass tube. At the beginning of the experiment, the tube was clamped so that the level of the contents was the same as the level



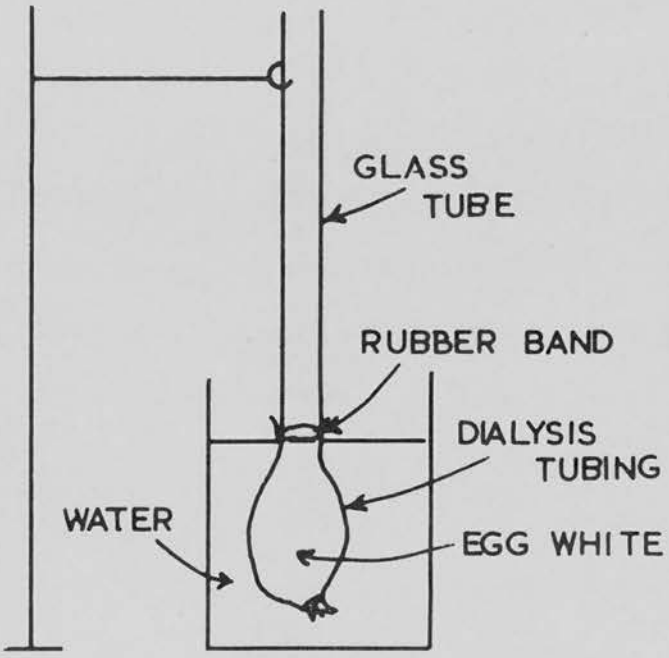


FIGURE 2:

DIAGRAMS OF APPARATUS FOR STUDYING OSMOTIC PROPERTIES OF EGG WHITE

of the external distilled water. As water passed into the tube, the level in the glass portion rose. The level attained, and the rate of rise, are dependent on the osmotic pressure of the eggwhite, although they do not provide a strict measure of it because of the length of time taken to reach equilibrium and the gravitational effect of the column of fluid.

Two procedures were used; in the first, an approximately 10 cm. length of "Visking" tubing of 6.3mm. radius when filled was held between two L-shaped pieces of glass tubing of similar diameter; egg-white (mixed white from normal oviposited eggs) was introduced by pipette. The height attained by the egg-white solution in both glass arms was recorded. As this preliminary experiment was difficult to set up, and the small quantities contained by the tubing were reflected in the small slow rises which occurred, the second procedure was devised. This consisted of a sac of "Visking" tubing of approximately 5 cm. diameter, held to a 1 cm. diameter horizontal glass tube. In this experiment, the thin white was drained from the separated white of an oviposited egg, and the remaining thick white was placed in the Visking sac, which was then fastened to the glass upright.

Hydrostatic pressure of swollen eggs. Eggs which had been soaked in water or saline for some time became turgid, and in some cases burst. When they did so, or when the membrane was cut to separate the components, the outer thin white spurted out. It was clear that considerable hydrostatic pressures must exist in such eggs, and attempts were made to measure these. This was done both with a simple water-filled manometer and with a pressure transducer calibrated by immersion in water. In both cases the reading was obtained by puncturing the swollen membraneous egg with a hypodermic needle. If a small bore needle was used, the pressure took some time to register on

the manometer or transducer output, and by the time it had reached a maximum, the contents of the egg had begun to seep out round the needle. If a larger needle was used, the pressure was transmitted almost immediately, but the resulting puncture of the membrane weakened it sufficiently for a split to occur, thus releasing the internal pressure almost equally rapidly. True values for the hydrostatic pressure developed inside the egg were thus unattainable, by this procedure.

2.9 DEVELOPMENT OF AN AVIAN EQUIVALENT SOLUTION

The derivation of a practical avian physiological solution offers difficulties, and must be arrived at by a series of compromises. A wide variety of physiological solutions have been developed for many different species; but the different body temperatures, and blood composition and pH of the bird suggested that none of these would be entirely suitable. The composition of blood plasma from hens and from other species is given in Table 3; in Table 4 are the compositions of the substances which are to be found on the mucosal side of the oviduct at different stages of egg formation, and Table 5 gives the compositions of some physiological solutions. The only solution which has been previoualy developed for avian tissues is the 'Avian Ringer' of Benzinger and Krebs (1933), which is included in the table, but which differs in some respects more than some of the other solutions from the composition of the hen's blood.

In order to simulate conditions in the living animal, the physiological solution should correspond to the natural milieu as nearly as possible in ionic composition, pH, temperature, and osmolarity. The ionic composition and osmolarity to be found on the mucosal side of the oviducal wall, that is of the oviducal fluids and the egg white, vary greatly (Table 4, and see Sections 4.5 and 4.6); recent measurement of the pH of the oviduct in anaesthetised birds (Ogasawara, Van Krey and Lorenz, 1964; Winget, Mepham and Averkin, 1065) show that this too has a wide range, 7.25 - 7.87, and varies with time in any one bird; in fact, the variation follows a cycle related to egg formation. According to Romanoff and Romanoff (1949), freshly secreted albumen is slightly acidic, which implies that the range of pH which the oviducal mucosa must be able to tolerate is even wider. The natural environment of the serosal and deep layers

of the oviduct, that is the body fluids, is much less variable. Hence, the composition of avian blood plasma was taken as the standard to be approached as nearly as possible.

For consideration of cellular function, it is the concentration of ions in water which is relevant. Unfortunately, in the literature the analyses are usually expressed in terms of plasma with no comment on the protein content, or, in the case of laying hens, the lipid content of the blood. Thus comparisons are difficult, and for this reason, and because values in the literature may refer to different breeds of bird, analytical figures obtained at the P.R.C. have been used as a basis for the synthetic solution.

The pH of avian blood is 7.45 (Spector, 1956), and its osmolarity was found by measurement of the depression of the freezing point to be 321 m-osmole/litre of plasma water. (P. E. Lake, personal communication.)

Concentrations of calcium, magnesium, and potassium chlorides and of sodium carbonate were chosen to give values of calcium, magnesium, potassium and carbonate corresponding to those of the avian blood analyses; the two most common ions, sodium and chloride, were used to adjust the osmolarity to approximately that of the plasma. The resulting solution was buffered by the addition of NaH_2PO_4; the quantity required was determined experimentally as follows. A series of solutions with a range of phosphate concentrations was made up. Each was warmed to 41°C and gassed with 95% oxygen, 5% carbon dioxide at a rate of about 40 ml./minute, and the pH was measured using a glass electrode. Readings were repeated until the pH had reached a constant value. The solution which had a pH of 7.45 under these conditions had the designation 'D8' in this experiment. The composition of this solution is given in Table 6.

The solution had been chosen to have the desired ionic composition and the desired pH at the temperature of the avian body. The sum of the ionic components, if they were completely dissociated, would suggest an osmotic pressure of 364.1 m-osmoles, which is undesirably high. However, it seems that dissociation is not complete under these conditions, as measurement of the osmotic pressure by depression of the freezing point gave a value of 327 m-osmoles, which is less than 2% above the value obtained for blood plasma.

The development of this solution was highly empirical, and no attempt has been made to describe the behaviour of the ions in it. It seems likely that some degree of recombination takes place; in particular, it is surprising that such a high concentration of bicarbonate remains in solution. However, no precipitation was observed as long as the salts were mixed in solution and in the order suggested in Table 6. Analysis of the solution during experiments, and measurement of the pH and osmolarity, gave values closely similar to those obtained for avian blood plasma. The purpose of the solution is to maintain physiological function in isolated tissues, and in this, too, some degree of success was attained.

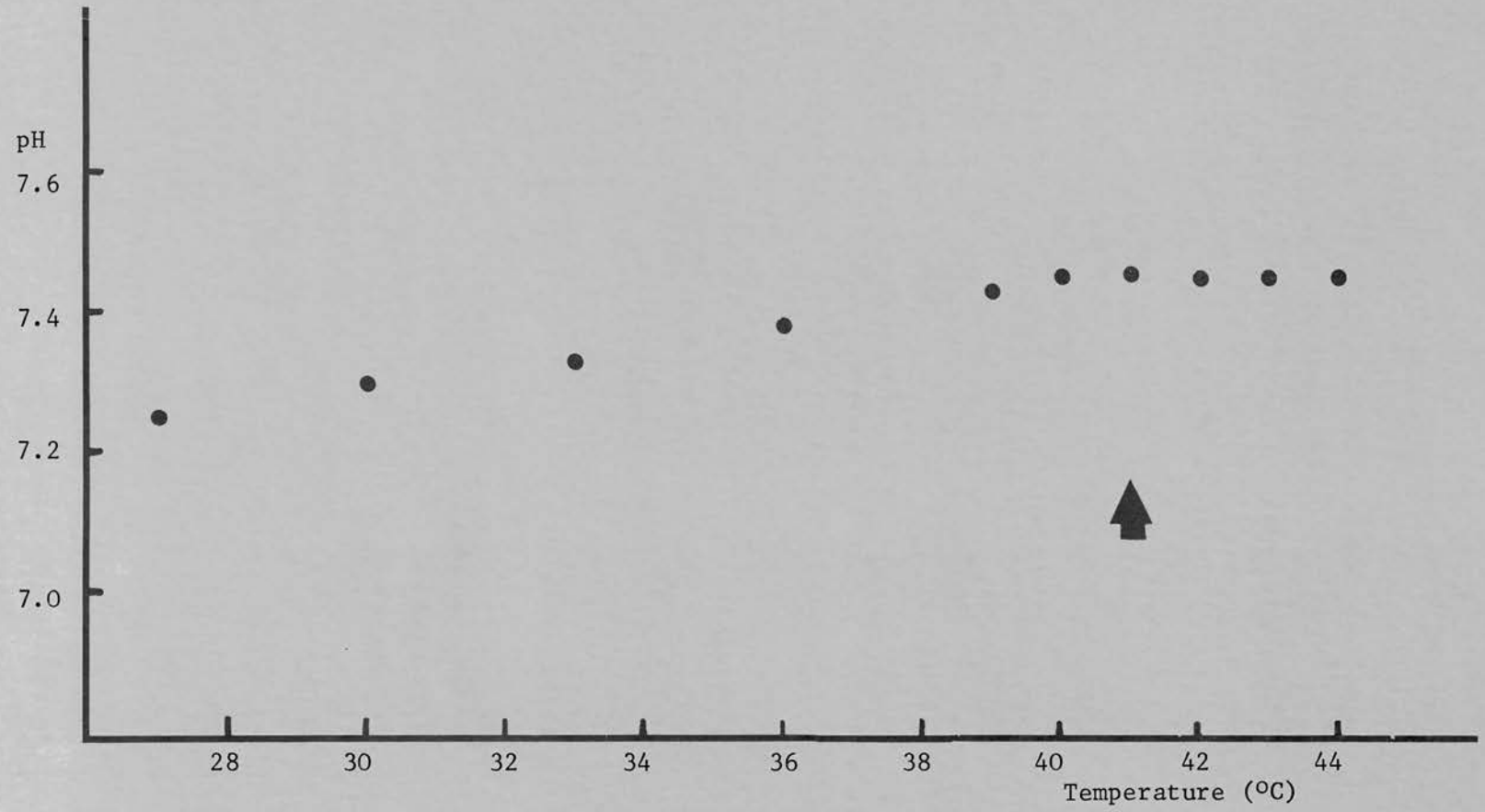


Fig.3. Variation of pH of Avian Equivalent Solution (D8'). Each point represents the mean of at least 3 measurements by glass electrode. The arrow indicates the body temperature of the hen (41°C), at which the solution has a pH of 7.45, the same as avian blood.

TABLE 3

IONIC COMPOSITION OF BLOOD PLASMA FROM GALLUS DOMESTICUS AND OTHER SPECIES

SPECIES	Na	K	Mg	Ca	Cl	CO_2	SO_4	PO_4	m-moles litre of:
Cock (1)	171.2	6.3	2.1	6.7	122.2	29.4	-	-	plasma water
Non-laying hen (2)	-	-	0.82	2.5	-	-	-	-	plasma water
Laying hen (2)	-	-	1.35	7.5 2+ (1.8Ca)	-	-	-	-	plasma water
Laying hen (3)	158	5.7	2.0 assumed	6.1	117.8	10.5	-	2.1	serum
Chicken (4)	-	6.0	1.15	-	-	-	-	-	plasma
Chicken (5)	154	6.0	-	5.0	122	-	-	-	plasma
Human (5)	140-155	3.6	1.9	4.3-5.2	93-110	27	1.0-1.5	2.4	plasma
Rabbit (5)	158	4.1	1.5	3.5	105	-	-	0.6	plasma
Turtle (Terrapene Carolina) (5)	140	4.6	1.7	2.7	122	-	-	0.3	plasma
Frog (Rana Catesbiana) (5)	105	4.8	3.8	1.6	70	-	-	0.6	plasma
Turtle (Testudo Graeco) (5)	-	7.8	-	-	-	-	-	-	plasma

(1) M. H. El Jack, personal communication

(2) J. Filshie, personal communication

(3) Morgan and Chichester, (1935)

(4) Biological Handbooks: Blood and other body fluids. Federation of American Societies for Experimental Biology, (1961)

(5) Spector (1956)

TABLE 4

IONIC COMPOSITION AND OSMOTIC PRESSURE OF OVIDUCAL FLUIDS AND ALBUMEN

	Na	K	Mg	Ca	Cl	CO_2	O.P. (m-osmoles)
	(m-moles/litre of water)						
Plumping fluid (El Jack & Lake, (1967)	139.10	15.9	1.37	14.1	79.9	82.5	
Oviposition fluid (El Jack & Lake, 1967)	42.8	75.0	10.0	25.8	63.4	91.3	
Plumping fluid (see Section 4.6)	138	20.3		2.95			292.5
Calcification fluid (Section 4.6)	41.3	47.7	6.1	20.3			284.3
Egg albumen: (see Section 4.6)							
Magnum	120	10	12	10			
Shellgland	100	14	3 - 7	4 - 7			263
Oviposited	60	22	4	3			

TABLE 5

IONIC COMPOSITION, OSMOTIC PRESSURE AND pH OF PHYSIOLOGICAL SOLUTIONS

(Ionic concentrations in m-moles/1., osmotic pressure in m-osmoles)

Solution	Na	K	Mg	Ca	Cl	CO_2	SO_4	PO_4	O.P.	pH
Ringer	114	1.9	0.0	1.0	115	2.4	0.0	0.8	235	8.3
Locke	156	5.6	0.0	2.2	163.8	1.2 -3.6	0.0	0.0	329	-
Tyrode	149	2.68	1.05	1.8	145	11.9	0.0	0.42	312	-
'Avian Ringer' (Benzinger & Krebs, 1933)	145.2	23.15	0.99	2.92	145.4	29.2	1.03	0.00	318	7.4
Avian equivalent solution ('D8')	171.2	6.3	0.87	1.84	153.3	29.4	0.0	1.2	327	7.45
(Avian blood)	171.2	6.3	0.87	1.84 (Ca^{2+})	122	29.4	-	0.74	321	7.45

TABLE 6

COMPOSITION OF AVIAN EQUIVALENT SOLUTION ('D8')

NaCl	8.28 g.
KCl	63 ml. of 0.1 M solution
$MgCl_2$	8.7 ml. of 0.1 M solution
$NaHCO_3$	2.47 g.
NaH_2PO_4	12 ml. of 0.1 M solution
$CaCl_2$	18.4 ml. of 0.1 M solution

per litre of solution

The NaCl was dissolved in 500 ml. of distilled water; the other salts were added in the above order. The $NaHCO_3$ was dissolved in distilled water and the $CaCl_2$ solution was diluted to at least 100 ml. before being added. The NaH_2PO_4 and $CaCl_2$ solutions were added slowly while the solution was stirred continuously. Finally, the solution was made up to 1 l. in a volumetric flask, and stored under refrigeration.

2.10 TRANSPORT OF MINERALS ACROSS THE OVIDUCT WALL IN VITRO

Adult Brown Leghorn hens were killed by injection of 2 ml. of Nembutal; the oviduct was excised as previously described and immediately placed in ice-cold avian equivalent saline. The region for study was cut off and thoroughly washed with the solution; the lower end was tied off and the upper end pulled over the flanged end of a glass tube (see Figure 4) and tied firmly in position. The sac thus formed was placed in a flask of 500 ml. capacity containing the external solution previously warmed to 41°C and the internal solution introduced through the flanged tube. The assembly was maintained at 41°C in a constant temperature bath and continuously gassed with 95% oxygen, 5% carbon dioxide by means of a fine polythene tube passed into the lumen of the sac via the supporting tube.

Samples of the internal and external solutions were withdrawn at intervals for analysis by Atomic Absorption Spectroscopy. Such solutions were centrifuged for 15 minutes at 3,000 r.p.m. to remove debris. Microscopical examination showed this to be mainly cellular debris, together with some mineral debris, possibly precipitated $CaCO_3$, such as is frequently found in the lumen of the oviduct in the living bird.

It was judged that the oviduct segment was in a condition resembling the physiological as long as strong peristaltic contractions could be observed. Slight contractions could still be observed after all night storage at 4°C following an afternoon's experimentation. Peristalsis seems to be a robust mechanism in the isolated oviduct segment. It was also noted that the application of a drop of 3M KCl solution after removal of the segment from the apparatus at the end of even the longest experiment always produced a vigorous contraction, indicating that the deeper placed smooth muscle had at least some intact function. Since the majority

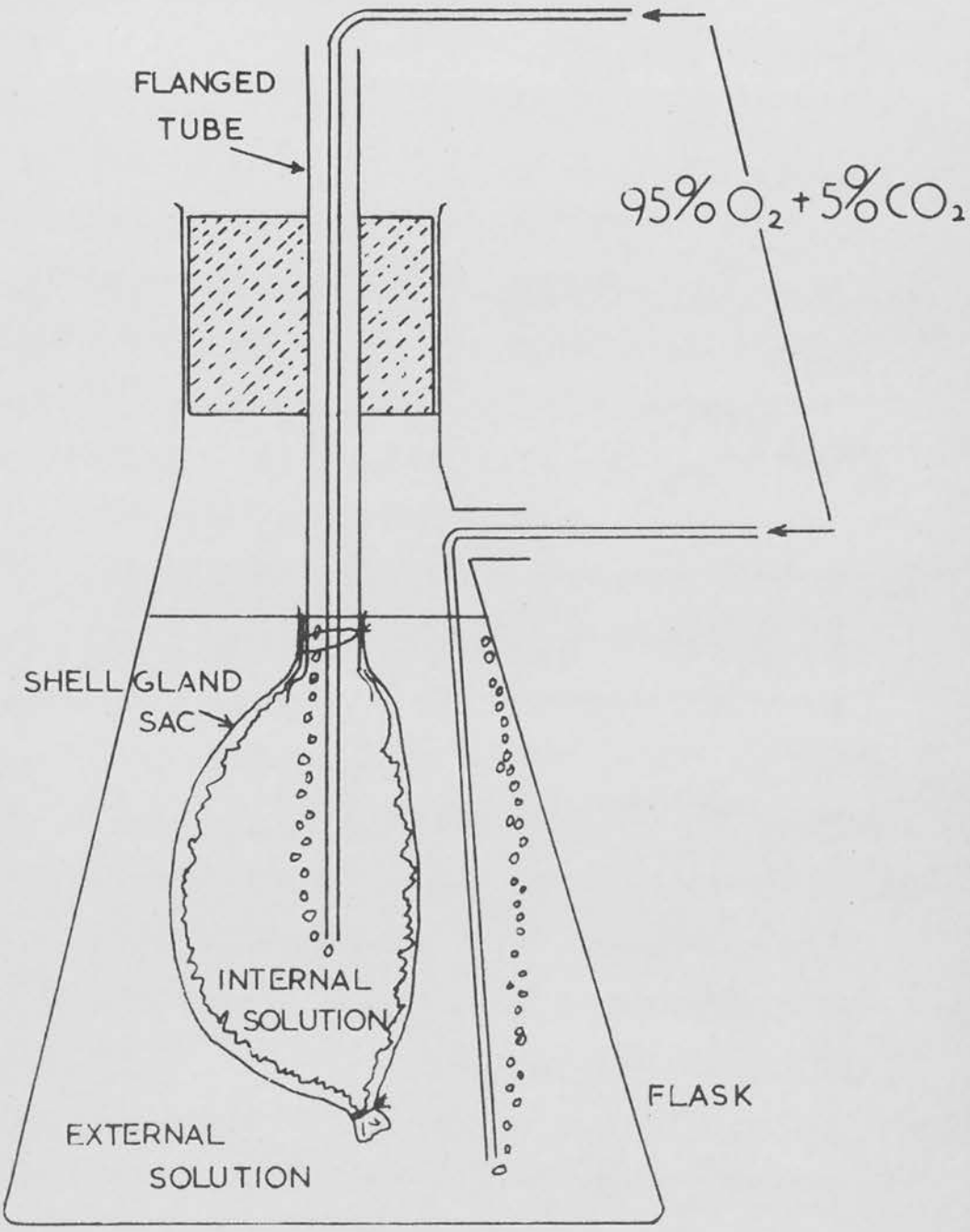


FIGURE 4:
DIAGRAM OF APPARATUS FOR MAINTENANCE
OF OVIDUCT SEGMENT IN VITRO

of the experiments lasted one hour, it is reasonable to assume that results obtained under these conditions have some relevance to oviducal epithelial function.

The oviducts were classified by observation of their functional condition at the time of death. The conditions considered were:

(a) Inactive or regressed, where the oviduct was small and the ovary contained no follicles over about 1 cm. diameter, except in some cases atretic ones.

(b) Oviducts with an egg in the shellgland. These also usually had several large follicles in the ovary.

(c) Large, well-developed oviducts, where the ovary contained large follicles, but there was no egg present in the oviduct.

2.11 POTENTIALS ACROSS THE OVIDUCT WALL IN VITRO

During the experiments on the transfer of minerals across the oviduct, (see previous section), several attempts were made to record electrical potentials across the wall. At first, two Agar/KCl bridges were used (2-3g. agar/100 ml.; 3M KCl); in later experiments, these were replaced by bridges filled with Agar/D8 solution, (2-3g./100 ml. agar; for composition of D8 solution, see Section 2.9) to avoid the necessity of applying a correction for leakage of potassium from the open ends of the bridges to analyses of fluid samples.

A KCl solution which approximated reasonably closely to 3M was obtained by adding 2,030 ml. of distilled water to the contents of a 500 g. bottle of Analar KCl. To make the bridges, 2 or 3 g. of agar powder was dissolved in 100 mls of this solution or of D8 solution. The 2g./100 ml. solution was easier to handle while the bridge was being made, but if a bridge of large internal diameter was required, or if the bridge was to be used in a vertical position, the greater rigidity of the 3g./100 ml. gel was preferred. While it was hot, but not boiling or aerated by stirring, the agar solution was drawn into a polythene or nylon cannula of the required diameter by attaching a vacuum pump to the end of the cannula. It was found advisable to include a length of translucent tubing as well as the usual trap to prevent the agar solution from reaching the pump, where contact with the cold water caused solidification of the gel and jambing of the valves. When the gel had set in the cannula, pieces of the required length were cut off, and the ends constricted slightly to keep the filling in place by heating gently and squeezing with an artery clamp.

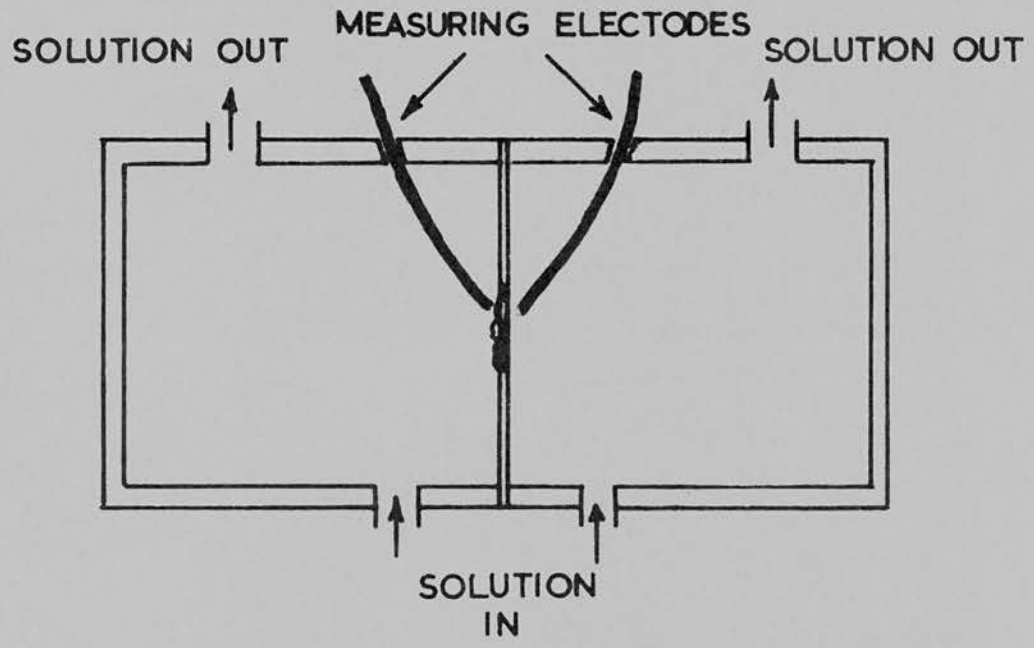


FIG. 5: DIAGRAM OF APPARATUS FOR MEASURING POTENTIAL ACROSS SECTION OF OVIDUCT WALL IN VITRO

The two bridges were attached to calomel electrodes and thence to a Vibron 33B electrometer. Any imbalance between the electrodes was backed off using a Mallory cell and a helipot; the output from the Vibron was taken to a pen recorder.

In all the experiments in which the potential was recorded, regular fluctuations of 3 - 4 millivolts were observed. These appeared to correspond to the peristaltic contractions of the oviduct sac, and were possibly caused by changing flow potentials. Apart from these, the potential did not depart significantly from zero. Attempts to measure potentials in these experiments was thus abandoned, as the adjustment of the electrodes detracted from the efficiency of the sample-taking.

Instead, smaller excised pieces of oviduct were used, in an apparatus similar to that used by Ussing for the measurement of potentials across amphibian skin. (Ussing and Zerahn, 1951). This consists of two Perspex half-cylinders between which the tissue is clamped. One or more Agar/salt bridges enter each chamber. The bridges were connected through calomel electrodes to the Vibron as before. The chambers were filled with the D8 solution, circulated by a peristaltic pump (Fison's Multifix) from reservoirs where it was continually gassed with 95% oxygen, 5% carbon dioxide, and maintained at a constant temperature. The temperature was adjusted so that a thermometer near the outflow from the chambers showed a temperature of 41°C, corresponding to the body temperature of the bird. (Sturkie, 1965.)

The piece of oviducal tissue was removed from the bird as rapidly as possible after death and transferred immediately to the apparatus. Glucose was added to the solutions in a concentration of approximately 1g./litre, and in some experiments the glucose concentration was doubled, and adenosine triphosphate (ATP) was added to give a final concentration of approximately 10^{-7} g./litre.

2.12 POTENTIALS ACROSS THE OVIDUCT WALL IN VIVO

Potentials across the oviduct *in vitro* were measurable, but rapidly disappeared even although the solutions bathing the tissue were oxygenated and energy sources (glucose, ATP) were available (see Section 6.3). It seemed likely that the failure to maintain the tissue was due to the difficulty of oxygenating such a thick tissue. Clearly, if it were possible to make potential measurements in the living bird, this problem would be avoided, although the conveniences of the isolated preparation would be lost. Accordingly, a series of such measurements was carried out.

The experimental bird was anaesthetised with intravenous Nembutal. As individual birds differ in their response to pentobarbital, the dose was administered slowly until anaesthesia was established. It was maintained throughout the experiment by administering ether whenever the bird's increased rate of respiration or reflex response to stimuli indicated the need. The bird was restrained on an operating table surrounded by a Faraday cage. The abdominal area was plucked and an incision made to expose the body cavity. The oviduct was located and the position of the egg, if there was one in it, and the apparent physiological state of the organ noted. A small puncture was made in the magnum or isthmus of the oviduct, and a salt bridge was inserted. In some cases a sleeve of polythene tubing of slightly greater diameter than the bridge was first tied into the hole with a purse-string suture, but this was found to be unnecessary and in later experiments was omitted. The bridge was slid into the oviduct until it was found by feeling through the oviducal wall that the end was in the desired position. The neutral electrode bridge was placed in the body cavity near the oviduct, and if necessary the region was moistened with warm D8 avian equivalent solution to improve contact. The incision was

covered by a pad of cotton wool moistened with warm D8 except when the bridges were being moved, and a hot-water bottle on the operating table and a powerful spotlight above it helped to maintain the bird's body temperature.

When the potential had stabilised and a reading had been obtained, the bridges were reversed and the erstwhile reference bridge placed in the same region of the oviducal lumen to obtain a duplicate measurement; frequent checks for zero drift were made by placing both bridges in the body cavity, and the ends of the bridges were trimmed occasionally to remove contaminating deposits of oviducal secretions or blood clots. Deposits of calcium were frequently found when the bridge was in the shellgland region, and occasionally when the bridge was in the isthmus some membrane-like fibres would be found on it.

For most of these experiments, a Keithley 603 electrometer was used in place of the Vibron, as the scale factors were found more useful. The output of either electrometer was fed (that of the Keithley through a voltage divider adjusted to match the input of the recorder) to a Bausch and Lomb variable speed pen recorder, which had been pre-calibrated for both speed and potential, thus enabling the record to be analysed later.

When the experiment was completed, the bird was killed with a further injection of intravenous Nembutal. This was usually after about one hour. In one experiment, however, on a bird which had an egg in the lower half of the magnum initially, measurements were continued for six hours. By this time the egg was in the shellgland and partially plumped.

SECTION 3
RESULTS: THE OVIDUCT

3.1 INTRODUCTION

The following observations on the oviduct were made with the object of confirming the descriptions of the organ in the literature, resolving apparent contradictions wherever these occurred, and determining the range of variation which exists in the modern light hybrid as represented by the birds of the P.R.C. flock. It was hoped to produce a coherent and logical description of the oviduct. To this end, information drawn from recent literature and other sources than the experimental results of the present study have been incorporated in this section wherever seemed most appropriate to the description, rather than segregated as a separate section.

In embryological development, the oviducal fields, of thickened peritoneal epithelium, become organised during the fourth day of incubation. In both sexes the two ducts grow down alongside the mesonephric ducts until they approach the cloaca. This occurs about the ninth day. Thereafter, the right duct in the female and both ducts in the male regress; they are vestigial by the 16th and 15th days respectively. (Witschi, 1961; Scheib-Pfleger, 1955).

The development of the oviduct during the growth of the young hen is described by Kar (1947). After a stage of some 20 weeks during which the oviduct enlarges proportionately to the rest of the body (isogony), there follows a brief period of hormonally-induced hypertrophy of the oviduct, and of the other sexual apparatus, such as the comb and wattles (heterogony). In the latter period, the glandular structure of the oviducal walls develops to the mature state.

The oviduct remains closed and only superficially attached to the cloacal wall until the approach of sexual maturity, when it

develops its opening into the left medial aspect of the ventral wall of the cloaca (Greenwood, 1935). The right oviduct ordinarily remains infantile (Finlay, 1925) but may be persistent (Greenwood, 1935; Quinn, Burrows and McNally, 1939; Bryant, 1944; Bradley & Grahame, 1960), and even in rare cases functional (Chappelier, 1913). Cyst development is a frequent finding at this site; Williamson (1965) has suggested that every hen is capable of developing a large right cystic oviduct, and many do, to the detriment of their laying performance.

The normal mature oviduct in a bird weighing from 1.5 - 2 kg, and laying an egg of about 60 g, is a long intestine-like tube, which, although much folded and convoluted, occupies a large part of the left half of the abdominal cavity. In the inactive state, its length is from 10 to 20 cm., and its diameter at its narrowest region (the isthmus) is 0.5 cm. and at its widest (the shell gland) is 2.4 cm.; it weighs only four or five grams. When the duct is fully functional, these dimensions increase to a length of from 40 to 90 cm., and a corresponding diameter of 0.6 to 3 cm. and weight of about 50g. It is also extremely extensible. Figures for other types of bird are given by Romanoff and Romanoff (1949), Bradley (1960), Hoover, Smith and Abbott (1962).

The oviduct is held in position in the body cavity by two complex ligaments, which are membranous fan-shaped folds of the peritoneum. The dorsal ligament has one border attached to the duct and the other to the dorsal wall of the body extending from the fourth thoracic rib to the cloaca. The ventral ligament has its ventral border free, but the two ends are thickened and attached, and both contain muscular tissue; the anterior end is in the vicinity of the ovary, while the caudal end terminates as a muscular cord which

divides round the vagina, holding it firmly in place and maintaining its characteristic 'S-bend'. This kink is responsible for keeping the egg in the shellgland during calcification (Kar, 1947), as is evidenced by the fact that hens having a straight vagina, produced by the injection of oestrin in early incubation, laid shell-less eggs. (Greenwood and Blyth, 1938). Both ligaments support the large numbers of blood vessels which supply the oviduct. The muscles in the ligaments at each end of the oviduct are said to be continuous with the outer longitudinal muscle coat of the oviduct (Curtis, 1910; Kar, 1947).

The length of the oviduct can be divided into five regions on the basis of its appearance (vide Romanoff, 1949). On functional and histological grounds the divisions are essentially similar, but there is some disagreement on the exact area covered by each term. It is hoped that the evidence to be presented about the structure and function of the different regions will clarify this situation. The main regions are: (1) infundibulum or funnel; (2) magnum or albumen-secreting region (this has been referred to by some authors as the convoluted glandular portion; it is not however alone in either of these attributes); (3) the isthmus; (4) the shellgland or uterus; and (5) the vagina.

Throughout its length, the oviduct wall varies in thickness, but basically there are usually seven layers of tissue present. These are (1) a serosal covering, continuous with the peritoneal lining of the body cavity; (2, 3 and 4) two layers of smooth muscle, separated by a thin layer of connective tissue, the outer longitudinal, but slightly spiral (hence the occasional mis-description of it in early literature as circular) and an inner circular layer; (5) a submucosal layer of connective tissue, in which the oviduct's plentiful supply of blood vessels ramifies and (6) the secretory

mucosa, consisting of a dense layer of tubular glands, closely packed and interspersed with capillaries, which reach to just below the final layer (7) of ciliated columnar epithelium. Unlike the intestine there does not appear to be a true muscularis mucosae.

These layers are present in the main throughout the length of the duct. However, the mucosa varies in its histological appearance and histochemical reactions in different parts. These differences can be related to the different functions of the regions of the oviduct and such considerations enable a more precise definition of the various regions to be attempted.

3.2 DIMENSIONS

During the preparation of everted oviducts for histology, it was possible to measure the length of the entire organ and of the various regions in a number of specimens. These results are recorded in Table 7. Some difficulty was experienced in deciding precisely where a region terminated in the case of oviducts which had been fixed for some time, or where the junction of the regions is irregular, as happens frequently with every junction except at the magnum - isthmus. No great accuracy is thus claimed for the values, which are in any case too few to be representative; however, they serve to give some idea of the variation to be expected in the organ, in a number of breeds and different functional states.

The longest oviducts are those from high intensity white egg layers in full production (Shavers 1 - 4). These have a mean length of 75.0 cm., and one reached more than 80 cm. Next in overall length are the brown egg layers, also of modern high-density stock (Thornber 404s), which have a mean length of 66.6 cm. Two Shaver oviducts in a slightly regressed state, but by no means in full moult, follow at 52.15 cm. The two remaining breeds represented are the Poultry Research Centre's Brown Leghorns, partially inbred and of a line selected for high laying intensity, but not reaching the production levels of commercially derived birds; both of these were in pause, and the oviducts are the smallest in this group - and two birds from a line of Cuckoo Leghorns which were remarkable for their low laying rate, something of the order of one egg per two weeks being not uncommon. Although both these birds were considered to be in lay, the mean oviduct length is only about 7 cm. greater than that of the Shavers out of lay. Photographs of three of the oviducts are shown in Figure 7. These comprise one of the active oviducts from each of the two commercial breeds, and one from a Cuckoo Leghorn.

The photograph also demonstrates the other interesting aspect of the measurements, which is the differences in the length of the individual regions in oviducts which produce eggs of similar size and composition. Differences in the two terminal regions, the infundibulum and the vagina, are probably not of any relevance to the formation of the egg. The length of the vagina in particular is very prone to be changed by the precise method of dissection, and the extent to which the ligaments have been removed. Complete removal of the ligament and some of the serosal layer of this part of the oviduct allows the vagina to extend to a length which is possibly longer than the functional length *in vivo*. Removal of most of this tissue is necessary before the vaginal segment can be straightened for the introduction of the stretcher. The main difference between brown and white egg producers in the specimens examined appears to be a relative shortening of the lower isthmus and shellgland regions. No distinction was attempted between the upper and mid isthmus regions in these measurements, as the junction can only be satisfactorily delineated by a histochemical examination of the functioning oviduct. Table8 shows that in the brown egg producer the magnum percentage is greater, yet the absolute lengths are almost identical. The lower regions contribute correspondingly less.

The ability of the oviduct to grow in the 14 or so days before lay, or to regress in a pause period, is striking. Most of the decrease in the resting oviduct length and mass arises from shrinkage of the magnum. The shellgland may be reduced to half its length, but, in terms of tissue mass disappearance, the relative loss is far greater in the magnum. There are other changes, particularly the lessening in depth and complexity of the cells of the mucosa, which demonstrate still further the remarkable capacity of the most active parts of the oviduct to regress.

TABLE 7

OVIDUCT DIMENSIONS

Some measurements of the lengths (cm.) of segments in everted oviducts

Type of bird and state	Infundibulum	Magnum	Upper and mid isthmus	Lower isthmus	Shell-gland	Vagina	Total
Shaver in lay	15.2	32.8	9.2	4.1	6.2	6.1	73.6
"	15.8	32.8	8.3	5.6	7.2	5.6	75.3
"	11.2	32.5	6.2	6.3	7.4	7.4	71.0
"	13.6	37.6	8.2	5.7	8.4	6.7	80.2
Mean	14.0	33.9	8.0	5.4	7.3	6.5	75.0
Shaver out of lay	12.8	24.9	4.5	2.9	4.0	3.3	52.4
"	12.4	25.2	4.2	3.2	3.9	3.0	51.9
Mean	12.6	25.1	4.4	3.0	4.0	3.2	52.1
Thornber 404 in lay	8.9	29.3	10.6	2.9	6.7	6.6	65.0
"	8.3	35.7	11.3	3.5	5.1	4.1	68.0
"	7.9	35.6	10.4	3.3	5.3	4.4	66.9
Mean	8.4	33.5	10.8	3.2	5.7	5.0	66.6
Cuckoo Leghorn in lay	7.5	25.8	9.1	4.0	7.3	7.5	61.2
"	7.0	26.0	8.7	3.9	5.2	6.6	57.4
Mean	7.3	25.9	8.9	4.0	6.3	7.1	59.3
Brown Leghorn out of lay	5.7	12.6	5.0	2.3	5.2	2.1	32.9
"	5.1	13.2	4.1	2.4	4.2	2.8	31.8
Mean	5.4	12.9	4.6	2.4	4.7	2.5	32.4

TABLE 8

OVIDUCT PROPORTIONS

The percentage represented by the regions

Type of bird and state	Infundibulum	Magnum	Upper and mid isthmus	Lower isthmus	Shell-gland	Vaginina
Shaver in lay	21	45	12	6	8	8
"	21	44	11	7	10	7
"	16	46	9	9	10	10
"	17	47	10	7	11	8
Mean	19	45.5	10.5	7	10	8
Shaver out of lay	24	47	9	6	8	6
"	24	49	8	6	7	6
Mean	24	48	8.5	6	7.5	6
Thornber 404 in lay	14	45	16	5	10	10
"	12	52	17	5	8	6
"	12	53	15	5	8	7
Mean	13	50	16	5	9	8
Cuckoo Leghorn in lay	12	42	15	7	12	12
"	12	45	15	7	9	12
Mean	12	43.5	15	7	10.5	12
Brown Leghorn out of lay	17	38	15	7	16	7
"	16	41	13	8	13	9
Mean	16.5	39.5	14	7.5	14.5	8

3.3 SURFACE AREA OF THE OVIDUCT

It is clear from the appearance of the ridged mucosal surface of the oviduct that the area of this secretory surface varies greatly in different regions, whereas the diameter of the empty duct is comparatively constant. On this area depends the number of cells of a given size, and hence probably the secretory capacity, of the columnar epithelium. The relationship of surface area to secretory capacity is of course only approximate because it tells little about the underlying tubular glands. However, it seems to be as likely to give an indication of this capacity as is cell height, which was a parameter previously used (Cole, 1938). It is of some use in the consideration of transport of substances across the oviducal wall, since all material even from the duct of a tubular gland must cross this sheet of cells, either through a duct or through the cells themselves, as might happen with some ionic transport. Estimates of the total area were made by the method of Fisher and Parsons - see Section 2.3. They are most usefully expressed as the ratio of the mucosal to serosal surface areas. They vary markedly with the region and functional state of the organ - see Table 9. The ratios for the magnum ranged from 2.0 for a non-functional duct, to 4.1 for the fully functional state. For the isthmus they were similar, 2.6 and 3.3 respectively, rising to 7.1 in the lower isthmus of the bird in full lay. Both regions demonstrated a capacity for hypertrophy, presumably due to accumulated, undischarged secretions, in birds which had (a) recently stopped ovulating; (b) been ovulating, but the yolk was consistently lost in the body cavity rather than passing down the oviduct ('internal laying'); or (c) had the oviduct surgically interfered with by the insertion of a thread in the shellgland wall for the purpose of inducing the production of soft-shelled eggs. In these cases the values were 5.4 - 6.2 for the magnum, and 9.2 - 11.3 for the isthmus.

The shellgland, as might be expected from its appearance, had a much greater surface area than either, both relatively and absolutely. The ratios ranged from 3.2 for the most immature oviduct studied, increasing with increasing organ activity (as judged by length and histological appearance, and the state of the bird's ovary) to almost 40 for a bird in full lay. In addition, the area of the serosal surface is greater in the 'pouch' of the shellgland for a given longitudinal distance, than in the other regions.

When the area was fixed and sectioned with the forming egg in situ, the ratio was not so high, since the villi are compacted by the egg. The higher value in the empty pouch may however give a better idea of the area potentially available for the processes of shell production and transport of ions and water into the egg. The ratio is of course also affected by the stretching of the serosal surface due to the distension of the oviduct by the egg.

Suitable sections of vagina were not available for measurement; in the non-functional oviduct there is negligible folding of the vaginal surface, and the ratio is probably close to unity. In the secreting state, the appearance of the outline is similar to that of the upper part of the isthmus, where a ratio of 2.5 - 3.0 is found.

The differences in the different regions seem to reflect the kind and amount of underlying secretory activity. That they reflect the proportional activities also may be deduced from the experiments of Asmundson and colleagues (Asmundson and Jervis, 1933; Asmundson and Burmester, 1936, 1938) in which it was demonstrated that the removal of different parts of the oviduct altered the composition of the egg in a way determined by the location of the part removed.

The shellgland in a laying hen has a large surface area whether or not an egg actually passes down the oviduct. It does not become engorged with secretions as do the magnum and the isthmus in anticipation of a descending yolk. This engorgement persists and indeed

TABLE 9

Ratios of mucosal to serosal surface areas of the regions of the oviduct in different functional states

Functional state	Magnum	Isthmus: Upper	Isthmus: Lower	Shellgland
Functionally immature	2.0			3.2
Regressed	2.4			3.4
Moult				8.5
Early redevelopment	3.1			5.9
Partial regression		2.6		10.5
Early regression		9.2		16.8
Early regression				15.4
Internal layer	5.4		11.3	17.2
Full lay	4.1	3.3	7.1	39.9
Full lay, clip in shellgland	6.2			
Egg in isthmus			10.0	
Egg in shellgland				8.5
Egg in shellgland				14.1

FIGURE 6:
DIAGRAMATIC REPRESENTATION OF CROSS-SECTION OF AN EGG IN THE SHELL-GLAND DURING SHELL FORMATION

induces an hypertrophy of the epithelium if the bird becomes a persistent internal layer. In fact, Burmester and Card (1941) showed that birds with ligated oviducts could on occasion produce yolkless eggs.

3.4 HISTOLOGY: THE REGIONS OF THE OVIDUCT

Infundibulum. The proximal end of the oviduct is thin-walled and funnel-shaped; its opening, the ostium tubum abdominale, has a delicate fimbriated edge. Near the time of ovulation, the peristaltic action of the muscle of this region becomes vigorous, and the mouth of the funnel, which is normally slit-like, embraces and engulfs the ovum as it is released. This activity is pronounced only at the time of ovulation, and is presumably under hormonal or nervous control.

The diameter of the funnel mouth is 8 or 9 cm.; the length of the region is about the same. Some authors (Bradley, 1928; Giersberg, 1922) consider the infundibulum to be only the first 2 to 3 cm., that is the thin-walled, tapered part, and differentiate the lower part as the "tube" or neck of the infundibulum. It is to this part that Richardson gives the name of chalaziferous region.

The upper part of the infundibulum has comparatively little muscle, and the two layers are not so clearly defined as elsewhere. The connective tissue layer which surmounts the muscle is well-marked; it is covered by a single-layer of epithelial cells, there being no tubular glands at this point. The epithelial cells are columnar and ciliated, and are similar to the ciliated cells found throughout the length of the duct. The cells seem to be about 30 microns in height, and stain only faintly with the procedures used in this study. The nuclei of the cells are prominent and oval in shape, and usually situated in the basal part of the cell.

As the lumen of the oviduct narrows and the neck of the infundibulum is approached, the muscle layers become more clearly separated, and the surface of the duct becomes more deeply folded. Here, the other two types of cell begin to make their appearance. First, occasional goblet cells are found in the epithelium; these are mostly near the top of the ridges. Then, in the grooves between ridges,

appear small collections of gland cells with no intervening ciliated cells. These cells tend to be less markedly columnar than the others, and stain more densely. Giersberg (1922) suggested that the glandular grooves secreted a lubricating material, not unlike blood plasma in composition, which could facilitate the descent of the yolk. The apical cytoplasm of the goblet cells contains granules which stain intensely with PAS, but not with Alcian Blue. The cells have a characteristic waisted shape, reminiscent of a thistle or a goblet. The cells of the glandular grooves have a prominent granular basophilic cytoplasm in H & E stained section. The position of the nucleus is irregular but frequently basal. The nucleus of the tubular, unicellular glands is almost invariably basal.

In the lower, cylindrical part of the infundibulum, the ridges are higher and the PAS-positive goblet cells alternate regularly with the ciliated epithelial cells. Occasional glandular grooves are still found, but these have mostly given way to fully invaginated tubular glands of the same type of cell. These are fairly simple and short, and undistorted by crowding. The cytoplasm of the cells is granular, and some are faintly PAS-positive, as are the deposits of secretion which may be found in the lumen of a tubular gland in the active duct, particularly if the tissue is fixed shortly prior to the passage of the yolk. It is this region of the duct which Richardson named the chalaziferous region, and which is considered by most workers to be responsible for the secretion of the innermost rather opaque layer of albumen, the chalaziferous layer, extensions of which at the poles of the egg form the fibrous chalazae, anchor-chains of the yolk. The name is derived from the Greek word for a hailstone.

Magnum. Inspection of the everted oviduct (Figure7) reveals that the thin-walled infundibulum is gradually replaced by the thick glandular mucosa and the smooth, large folds and grooves typical of

Fig.8. Magnum of mature, active oviduct (Shaver). Stained with Alcian blue, counterstained H & E. The secretion-swollen goblet cells of the surface epithelium give the appearance of a continuous blue border. x 160.

the albumen-secreting part of the oviduct. The region, which extends for 25 - 40 cm. in the laying bird, approximately 60% of the total oviduct length, is characterised by much evidence of secretory activity, both in the epithelium and in the tubular gland layer. The distinct layers of muscle and connective tissue are now thicker; the sub-mucous layer is vascular, and there are occasional islands of lymphoid tissue. The tubular glands in this area are completely invaginated, and it is only rarely that glandular grooves or patches are found, although examination of immature ducts at various stages of development makes it quite clear that this is the mode of their formation, and in the magnum of these oviducts such patches and grooves are frequent. The cytoplasm of the tubular gland cells is filled with acidophilic granules and secretory globules; the glands are coiled and branched and packed together so as to make the task of tracing the course of one gland almost impossible (Surface, 1912). Even so, the ducts which open into the lumen are surprisingly few for the rate of secretion which occurs (Bradley, 1928). The epithelial cells increase in height along the length of the magnum. In formalin-fixed paraffin sections they range from as little as 10 microns in the upper magnum to 25 or 30 microns in the lower magnum. The goblet cells stain strongly with both PAS, and, from the bottom of the infundibulum downwards, Alcian blue, indicating the presence of both acidic and neutral mucopolysaccharides.

Cole (1938) and Conrad and Scott (1942) related measurements of cell heights to the albumen-secreting activity of the magnum. Conrad and Scott found that the height of the cells at a point 5 cm. above the isthmus did not vary with the time of the egg-laying cycle. Cole found a significant difference between the heights of cells taken from the equivalent regions of oviducts which consistently

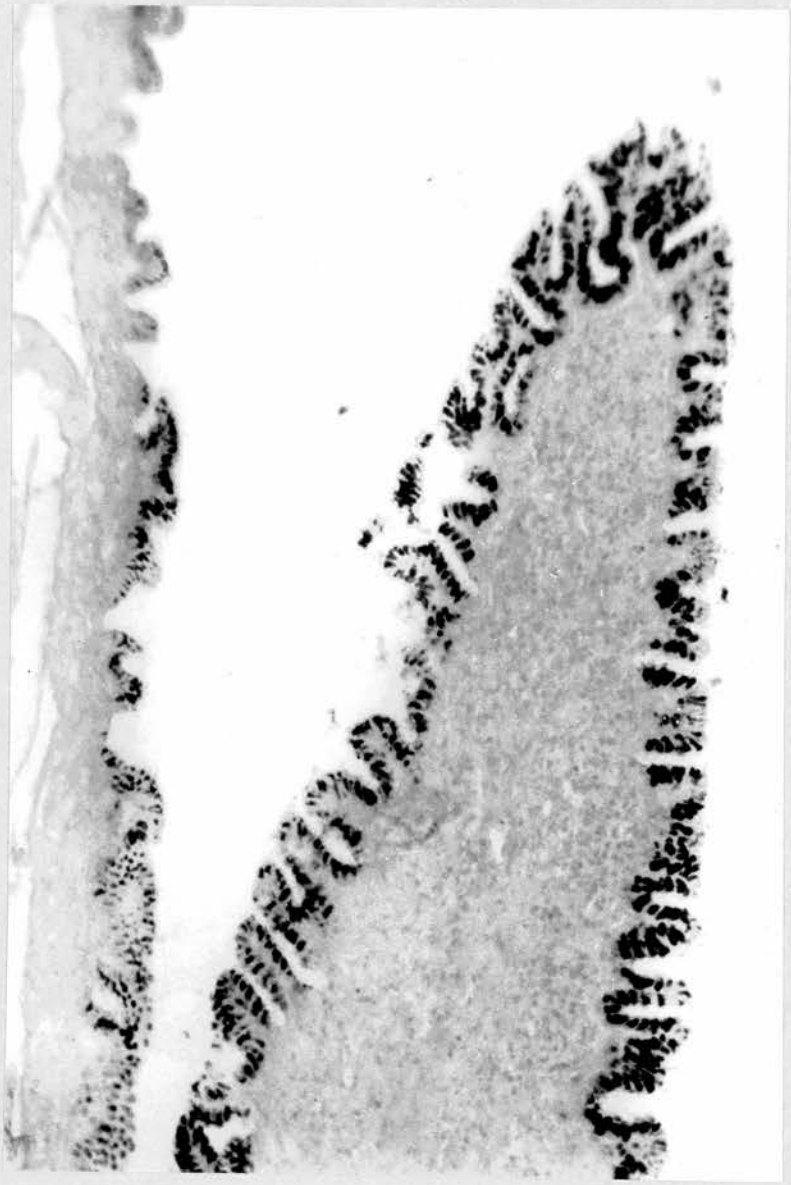


Fig.9. Magnum of immature oviduct. Alcian blue, counterstained with eosin. Many of the goblet cells of the surface epithelium show blue staining; this staining disappears at the bottom of the invaginating glandular grooves. x 160.

produced eggs with good or poor 'score' for thick white condition, the good egg producers having the taller cells. He also found that sections of coagulated 'good' whites contained a higher density of fibres showing an affinity for the same mucopolysaccharide stains as the goblet cells. He used mucicarmine and thionine blue, which react to a very similar range of materials to the PAS method and Alcian blue respectively.

Sturkie (1965) states that 'most observers believe that the goblet cells of the epithelium secrete mucin, which forms the thick albumen, while the tubular glands produce the thin albumen'. The secretions of the two types of cell are in fact probably closely intermingled, the more liquid tubular gland products being held by a meshwork of fibres or sheets of gel-like but non-thixotropic secretions mainly originating from the goblet cells. The inner thin white is assumed to be formed by the twisting of threads of the mucin into the chalazae, leaving a surplus of the liquid components; the formation of the outer thin white seems likely to be attributable to the addition of a similarly fluid component during the process of plumping.

Using the cell height as an index of secretory activity, the results of Cole (1938) are in accordance with the observations of Asmundson et al on the secretory activity of the parts of the oviduct. (Asmundson and Jervis, 1933; Asmundson and Burmester, 1936, 1938).

Although their height does not change, the precise appearance of the cells of the magnum depends on the stage of the egg-laying cycle at which it is examined, corresponding to the discharge of secretory products during the formation of the egg. This is particularly clear when an oviduct is examined with an egg in transit. Below the descending yolk, the tissue is distended and the glands crowded.

The goblet cells of the epithelium become so swollen that they almost entirely obscure the ciliated cells; at low magnification, the oviduct walls seem to be edged with a continuous band of bright blue (in Alcian Blue stained sections) or magenta (in the PAS ones). At higher power, the intervening counter-stained ciliated cells are distinguishable and the "foamy" appearance of the goblet cells is resolved into large globules. These tend to be larger and to stain more deeply near the luminal edge of the cell, which often bulges outwards, but are present throughout the cytoplasm, and even seem to compress the nucleus. In the immediate region of the egg, the bulging edge of the cell is sometimes found to be ruptured (although this has been observed in parts of oviducts not adjacent to forming eggs, and may be an artefact), and the discharge of the secretory globules may be easily envisaged. In fixed sections, the discharged contents of such a cell may be seen as a slightly thready mass protruding from the epithelium. The tubular glands are also swollen with secretory granules in the cells and masses of secretory substance in the lumen of the gland. This is most noticeable in glands sectioned near to the surface of the oviduct; glands sectioned near the connective tissue layer are rarely so swollen, and do not seem to be strictly in phase with the egg-laying cycle. Bradley and Grahame (1960) state that the tubular glands secrete their products during the whole period of egg formation, while the goblet cells discharge their contents on passage of the yolk. In any case the substances collected in the lumen of the gland at this level may be assumed to have a long passage before their release into the oviduct. It was also noticed that secretory masses in sections of tubular glands near the base of the mucosa were occasionally slightly PAS-positive; this was very rarely so near the epithelium. The appearance of the tubular glands in the immediate region of the egg is similar, but the

slight indentations in the surface of the oviduct which mark the openings of the tubular glands are more clearly visible. In general, there seem to be more of these openings between the ridges than on their upper surfaces. In some cases, considerable accumulations of secretion are found in a hollow where a number of glands have their termination. After the passage of the egg, the lumen near the opening of a tubular gland is usually empty and patent, the cells are undistorted and lightly stained, and the granules which they contain are smaller than in the undischarged gland. These granules are always smaller than those found in the epithelial cells, and more discrete. They do not stain with PAS or Alcian Blue, but are strongly acidophilic; during the preparation for the passage of the egg, the granules grow in size and density, and the cells swell slightly, while the nuclei seem to shrink. The lumen of the gland becomes invisible, or filled with a lightly-stained secretion, which often appears to have shrunk slightly during fixation, and which has no observable structure.

Isthmus. In the lower reaches of the magnum, the cells of the epithelium are particularly tall; the goblet cells are distended by large globules of secretory products which stain intensely with PAS and Alcian blue. At that portion of the oviduct where the magnum region appears to end in a clear ring of translucent tissue easily visible to the unaided eye this stage of affairs ceases dramatically (Figure 10, Figure 11). Here the smooth curving folds of the magnum above and the somewhat smaller ridges of the isthmus below can be distinguished. The tissue of this intermediate ring is less easily extensible than the rest of the tube, and the wall, though thin, is strong. This is seen during the eversion procedure, when the ring appears as a constriction, noticeably narrower than any other part of the duct, through which the stretcher may have to be forced. In

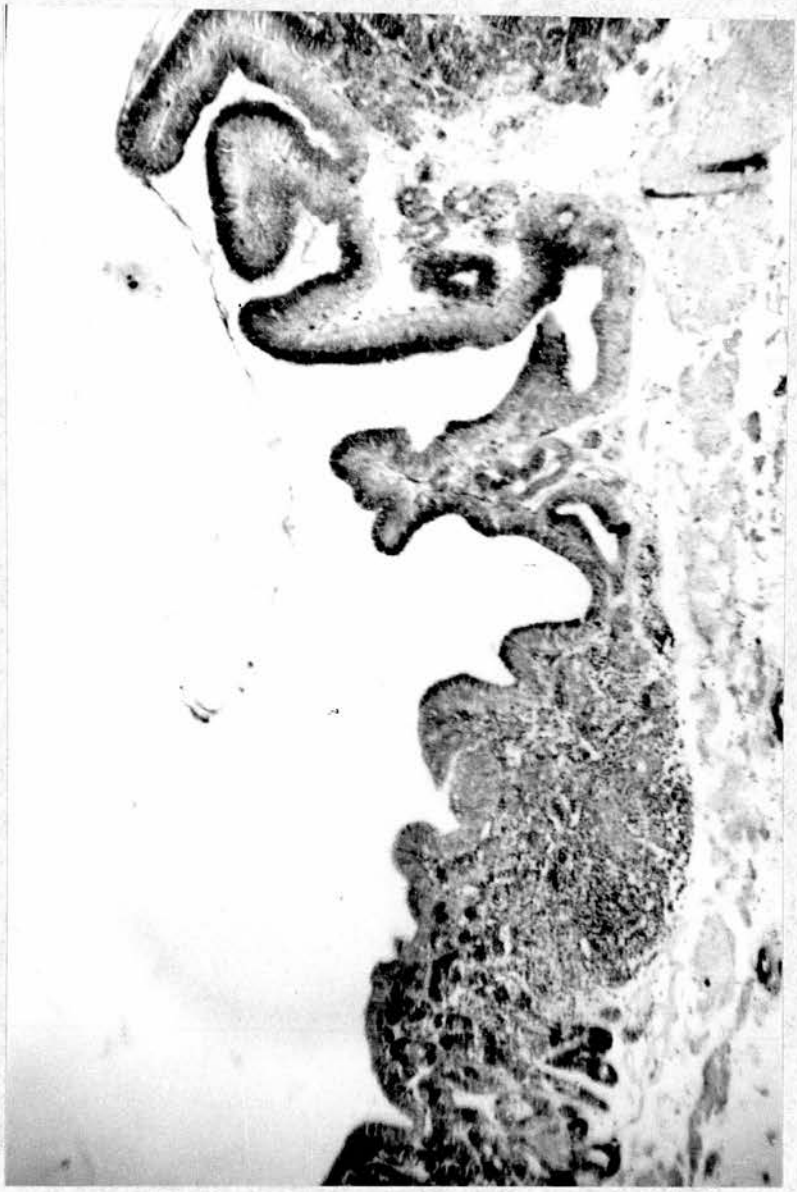


Fig.10. The mucous-secreting region at the lower end of the magnum and the junction of magnum and isthmus, stained by the PAS method, counterstained with light green and haemalum. Oviduct from a Thornber 404 hen. The goblet cells of the mucous-secreting region are swollen with PAS-positive granules. These cease abruptly at the junction ring. x 80.

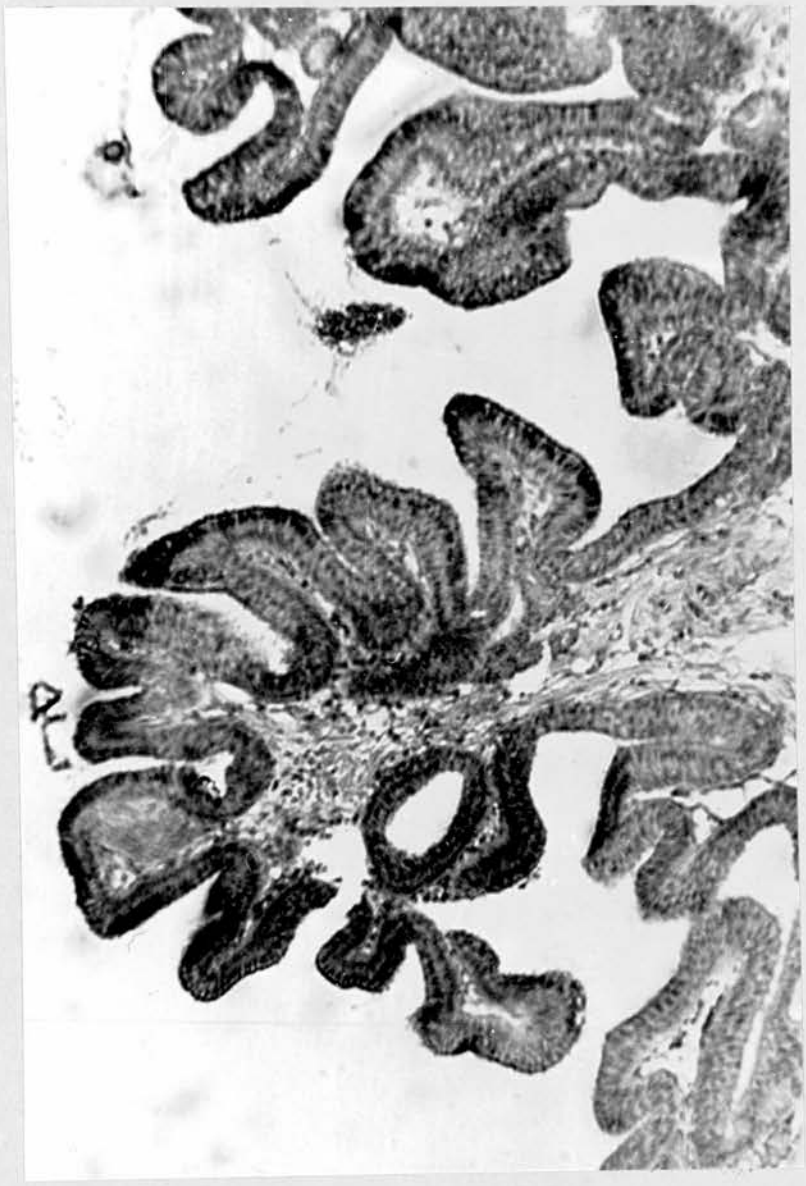


Fig.11. Mucous-secreting region and junction of magnum and isthmus from a Shaver's oviduct, stained with alcian blue and counter-stained with H & E. As in Fig.10, the large goblet cells filled with globules of substances staining as mucopolysaccharides are found only above the junction, which is very sharp. x 80.

section, the muscle layers of the oviduct wall in this region, which extends for a millimetre or so, appear unchanged, but the tubular gland layer is found to be absent. Its place is taken by a thickening of the submucosal layer of connective tissue to form a ridge, of about the same height as those in the isthmus but with a more crinkled surface epithelium. Within the same distance, the staining characteristics of the epithelium change abruptly, usually within a dozen or so cells. The vivid blue of the Alcian blue positive goblet cells, here at their tallest, disappears almost entirely. Only the faintest traces are present, and since they extend for only a few millimetres, these may be due to contamination by secretions from the magnum. Similarly, the strong PAS-positive reaction vanished; below this magnum-isthmus junction the goblet cells retain a degree of weaker staining of the rather vacuolar-looking cytoplasm, particularly at the free edge of the cell, but in contrast with the intensity of their counterparts above the junction they present a washed-out appearance.

This appearance of the epithelium continues for the whole of the upper isthmus region. The general outline of the oviducal wall has changed from the broad smooth curves of the magnum to the lower, narrower, slightly spiral ridges of the isthmus (Figure 7). The tubular glands, which rapidly become re-established, are similar in appearance to those of the magnum, but their secretions, where these are visible in the lumen, tend to form long threads rather than the amorphous masses which are found in the magnum. Some workers have held that the secretions are in the form of granules, which later coalesce into threads, from which it is generally agreed that the shell membranes are formed; the present study throws no light on this possibility. Romanoff states that the surface of the isthmus

Fig.12. Junction of the mid-isthmus region,which like the upper isthmus has acidophilic granules in the tubular gland cell cytoplasm, with the lower isthmus region,which does not. The transition is more gradual than that of magnum to upper isthmus, but is quite clear. Epithelial goblet cells show some staining with PAS. Stained by PAS method,counterstained light green and haemalum. x 160.

is darker than that of the rest of the oviduct; this does not appear to be true of all specimens, and may be a variable of the state of activity and thus of vascular engorgement. Alternatively, he may have been referring to the lower isthmus, which has a dark reddish colour similar to that of the shellgland. It is not clear from Richardson's paper whether this is the region to which he applied the name of mucous-secreting region, and described as being the lowest part of the magnum; it is so labelled on his photograph, but the text leaves the matter in doubt, particularly as he does not mention the magnum-isthmus junction ring, although it is visible in the illustration and had been remarked on by earlier workers (Surface, 1912).

After a length of about 6 to 7 cm., the ridges of the surface become rather higher. This is accompanied by a marked increase in the opacity of the tissue, whether fixed or untreated. The transition is fairly gradual, and there is no striking change in the histology of the surface epithelium, which continues to consist of an alternation of lightly stained ciliated cells and tall goblets in which the secretory granules take a light PAS-positive stain near the apical cell surface, or of the tubular gland layer, the cells of which still contain acidophilic granules.

In most of the breeds from which oviducts were examined, the mid-isthmus region is rather longer than the upper isthmus, usually about 9 cm. The transition to the lower isthmus is also fairly gradual; the tissue takes on a reddish tinge possible indicative of increased vascularity, the diameter of the oviduct lumen begins to increase preparatory to the expansion into the pouch of the shellgland, and the surface of the ridges becomes more elaborate, with increased secondary folding. Microscopically, the increased surface area of the epithelium is striking; the goblet cells, some of which had

continued to contain PAS-positive material at least in the outer part, now almost all show this reaction, though they are still Alcian blue-negative, and the intensity of the staining is much less than in the magnum. The most obvious difference, however, is in the cytoplasm of the tubular gland cells, where the acidophilic granules disappear abruptly; the appearance of the mucosa is pale and vacuolated in H & E stained sections and no part of the cells seems to bind any of the stains used to a noticeable extent.

Shellgland. The lower isthmus merges into the shell gland pouch, where the egg is held during plumping and shell deposition. The term "uterus" is frequently used for this region, on the analogy of the mammalian uterus where the foetus is held during embryonic development. The comparison is of limited usefulness due to the almost complete difference in function; most authors over the last forty years - or more - have agreed on the unsuitability of the term, while continuing to use it; there seems to be no need to use it again here. The tissue of the shellgland is a very deep pink colour probably due to the ample blood supply, particularly during the period of active shell formation, when it has been estimated that the amount of blood in the region increases by an average of 42 per cent. (Hodges, 1966). The connective tissue and outer muscle layers are possibly slightly thinner than elsewhere in the oviduct, although there are more capillaries in all layers. The inner muscle layer is not essentially thicker, but the fibres continue into the cores of the large spiral ridges, or rugae, usually five in number, which are one of the most immediately obvious features of the region. Secondary and tertiary folding on and between these ridges increases the surface area of the epithelium considerably, and the epithelial cells, which are taller than in any previously described region (their height has been steadily increasing with successive regions),

now fail to stain with PAS as well as Alcian blue, and the cytoplasm does not stain with eosin or other acidophilic stains.

Hoover, Smith and Abbott (1962), studying the mechanical properties of the shellgland, concluded that the volume which could be contained in the pouch is limited by the size of the body cavity rather than the mechanical properties of the tissue. When the body cavity had been surgically opened, a distension of 300 mls., i.e. x5 times the volume of an average egg, was easily obtainable; the increase in pressure and decrease in wall thickness were less than predicted from geometric calculations. Clearly, the organ is well able to contain an egg for long periods; with distensions comparable to normal eggs, the authors concluded that the pressure changes were small, although functionally significant, and that after the initial distension the muscle and glandular layers did not change appreciably in thickness.

The mucosal layer is thick and the tubular glands are tightly massed together. They are both more complicated individually and greater in number and density than in any other region of the oviduct. Some traces of secretory accumulations appeared in occasional sections, but it is not certain that these are not a result of artefact. The cytoplasm of the tubular gland cells did not take up any of the stains used, throughout the shellgland and in the lower isthmus and vaginal glands. This is in contrast with the description by Bradley and Grahame (1960) of basophilic granules, and by Fujii (1963) of acidophilic granules.

Recessus Uteri and Transitional Region. Towards the caudal end of the shellgland, both the folding of the surface and the complexity of the tubular gland layer decrease. The boundary is marked by a thick sphincter-like ring of circular muscle. Immediately distal

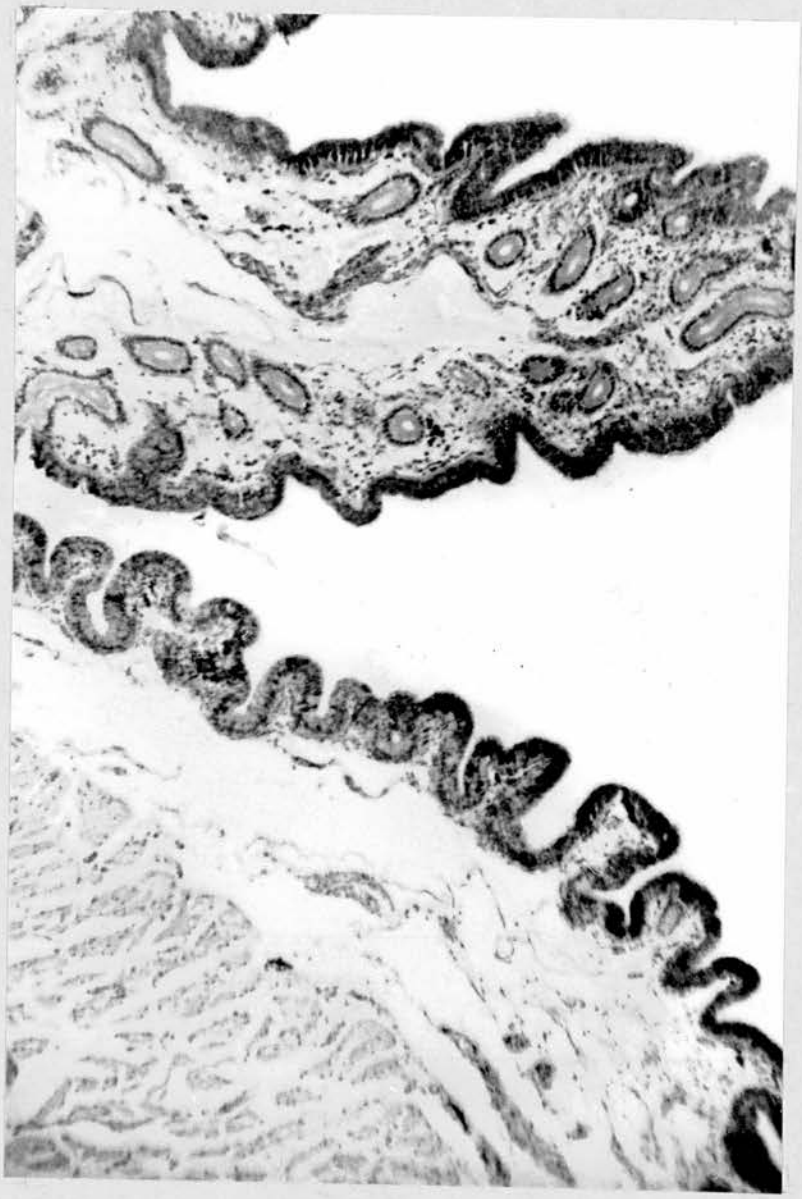


Fig.13. Transitional region between the shellgland and the vagina, showing tubular glands of the sperm-host type with patent lumen, and alcian blue staining reappearing in occasional goblet cells in the vaginal surface epithelium. Oviduct from a Thornber 404, stained with alcian blue, counterstained H & E. x 80.

to this is the region, about 0.5 to 1.0cm. long, which Fujii (1963) has named the Recessus Uteri. It is characterised by the colour of the epithelium, which is greyish-white, except just after the passage of the egg, when it appears faint pink. The ciliated cells of this region were found to contain lipid granules, the substance of which was identified as cholesterin ester. Immediately caudal to the sphincter, in the first kink of the 'S-bend', is Fujii's Vaginal Gland region, which corresponds to the Transitional Region of Gilbert, Reynolds, and Lorenz (1968). Here, the tubular glands are simple, large, usually blind-ended ducts. The cytoplasm surrounding and apical to the nucleus of the cells (which are columnar, and about 25 microns tall) contains lipid droplets. The patent lumen (according to Fujii about 14 microns in diameter), in the basal part of which are many microvilli, acts as a reservoir for sperm. These are the 'sperm host glands', 'sperm nests', or 'vaginal glands' which are responsible for the prolonged fertility of the hen. Such a function has also been attributed to similar groups of cells in the infundibulum (vide Lake, 1968).

Vagina. The vagina is usually about 12 cm. long, but it is bent into an S-shape and embedded in the muscle and connective tissue of the ventral ligament and the wall of the cloaca, into which it opens. The predominance of the tubular gland layer gives way to a region in which the thickness of the connective tissue and muscle layers is considerably increased - the muscle layers in particular are massive in comparison to all other regions - and the columnar cells of the epithelium are yet taller. The goblet cells react positively once again with both PAS and Alcian blue, although not so strongly as those in the magnum. The density of colour and distribution of

the stained globules is mainly in the apical part of the cells, as was seen in the upper isthmus region. After the packed complexity of the shell gland tubular gland layer, the appearance here is akin to the simplicity of the infundibulum. In the vagina proper, no tubular glands are found, although the surface is still folded and there is a thin layer of undifferentiated epithelial cells below the ciliated and goblet cell layer. Thus the statements of Richardson (1935) and Bradley (1960) that no tubular glands are found in the vagina, and the contradictory finding of Cole (1938), that such glands were present, may be explained, either on the basis of different positioning of the boundary between shellgland and vagina, so that the transitional region with the sperm glands was included by the former authors with the shellgland, and by the latter with the vagina, or as a misinterpretation of the degree of organisation of the sub-surface layer.

3.5 HISTOLOGY OF THE OVIDUCT WITH AN EGG IN SITU: THE FUNCTION OF THE REGIONS

The differentiation of function of the regions has been broadly understood since the work of Asmundson and Burmester (1936, 1938). They reached their conclusions by removing parts of the oviduct and observing the effect on eggs laid after operation. With the redefinition of the regions as above, and the improvements in histological technique, it is possible to be more precise.

Infundibulum. The mature follicle is released from the ovary. This usually happens about half an hour after the previous egg is laid, although the precise timing varies between breeds and individual hens. Coincidentally, the muscles of the infundibular region are at their most active, and the yolk is rapidly engulfed by the funnel. The yolk has been growing by the addition of material through the vitelline membrane, and after the egg has been laid a degree of exchange of materials between white and yolk will take place through this membrane, but during the passage of the oviduct no change can be detected, with the exception of a water gain of about 1%, of doubtful significance. (Draper, 1966). Thus, the oviduct plays no part in the formation of the yolk.

In the neck of the infundibulum, the yolk receives the first layer of its wrappings. This is a rather fibrous mucous secretion, translucent and whitish in the completed egg. It may however be somewhat thinner when originally secreted, as it is continuous with the chalazae of the egg, from which it takes its name of chalaziferous layer. The chalazae are believed to be formed mechanically from the white by condensation of ovomucin fibres caused by the rotation of the egg by the spiral ridges of the oviduct wall and by the muscular contractions of the shellgland. (Hansen, 1933; Almquist, 1936; Conrad and Phillips, 1938; Burmester and Card,

1939; Scott and Huang, 1941; Sharma, 1950; Baker and Stadelman, 1958.) The rotation in the magnum and isthmus are sufficient to cause only a slight separation of the mucin from the rest of the albumen; the chalazae as such are only observed in uterine eggs. They are fully condensed after about five hours in the shellgland, but continue to twist until the egg is laid, and will do so thereafter if the egg is rotated, to the extent of rupturing the yolk if the chalazae are overtwisted, e.g. by too frequent turning during incubation. (Olsen and Byerly, 1936). The earlier formation and greater size of the chalaza at the narrow end of the egg is attributed to the fact that this end is caudal in the oviduct during egg formation. It is not clear whether the secretion of the chalaziferous region is different from that of the magnum; it may be, as the surface epithelial cells show little staining with Alcian blue, and the tubular glands are fewer proportionately than in the lower region. This would suggest that the secretion should have a greater density of fibrous mucin than the majority of the albumen, and that the mucin should consist mainly of neutral mucopolysaccharides.

Magnum. Throughout the length of the magnum, albumen is added to the egg in a series of concentric layers. Histological staining shows that these are determined by fibrous sheets of a substance which stains as an acidic polysaccharide (Cole, 1938), in the same way as the contents of the goblet cells of the surface epithelium. These sheets appear to be formed of a loose network, and there are numerous anastomoses between them. Scott and Huang (1941) observed these layers in eggs fixed in the magnum by boiling; they may also be observed, though less clearly due to fixation shrinkage and the long coagulation time of albumen in formalin, in the sections of magnum-plus-egg obtained as described in Section 2.2. The non-staining portions of the albumen appear similar to secretions observed in the

crypts between grooves, that is to say near the orifices of the tubular glands. Cole (1938) remarked the apparent formation of fibres by the goblet cells. Although such effects were observed in several sections during the present study, it was not considered certain that they might not be due to artefact, as the albumen had a tendency to coagulate into shrunken layers with vacuoles between, which could have presented the same appearance. Chemical studies of the protein fractions of egg-white show that approximately 1.5% of the solids is ovomucin, which is insoluble, fibrous, and contains a higher concentration of the mucopolysaccharide sialic acid than other egg-white proteins. (Parkinson, 1966). Robinson & Monsey (1966) suggest that it may be regarded as a glycoprotein cross-linked by disulphide bonds and possibly also by non-covalent links such as hydrogen bonds. It may be assumed, than, that this substance plays some part in the structuring of the white; it has been suggested that the stability is due to an ovomucin-lysozyme cross-linked network (Brooks and Hale, 1959, 1961.)

The composition of the thick white does not appear to vary greatly throughout the layers, with the exception of this insoluble and indiffusible fraction. The correlation of white quality, which is to say rigidity, or fraction of ovomucin present, with goblet cell size, particularly in the more active mucous-secreting region, the lowest part of the magnum, has already been mentioned. It seems that this may also involve closer packing of the ovomucin layers, which could be of significance in the phenomenon of plumping.

Isthmus. This region has generally been supposed to have the function of adding the inner and outer shell membranes to the egg. Samples taken from the oviduct above the magnum-isthmus junction are uncovered, while those obtained caudad of this point are usually

fully enclosed in both membranes. On several occasions a half-membraned egg has been reported. (Conrad and Phillips, 1938). The earliest of these was the account of Coste (1847). According to Surface (1912), this egg was in the upper part of the isthmus. Unfortunately, no such egg was found during this study; however, eggs with one end or the other very close to the junction ring were obtained, and in all cases were either completely with or completely without both membranes. It is impossible to be certain that the position in which an egg is found on post mortem examination of the oviduct corresponds exactly to its stage of formation. However, the accounts of such eggs as Coste's, coupled with the above observations, suggest that the membranes are formed by quite a small area of the upper isthmus. Membraneous eggs have been reported in upper regions of the oviduct. (For example, Robinson, King and Bowen, 1968.) It is possible that these are a result of reverse peristalsis, which is known to occur if the hen is unduly alarmed, or during the struggles frequently caused if the bird is killed by cervical dislocation. These can be so severe that rupture of the oviduct has been observed. The formation of fibres similar to those constituting the membranes has been observed during experiments on isolated portions of the oviduct (see Section 6.2), where the segment under study extended from just above the magnum-isthmus junction to approximately the middle of the lower isthmus.

If, then, the function of membrane formation is attributed to the upper isthmus, there remain the questions: is this formation of membrane fibres confined to the surface epithelium, or the tubular glands of the region, or a combination of both? - and, what is the function of the mid and lower isthmus regions?

It is reported by Wolken (1951) and Masshoff and Stolpmann (1961) that the shell membranes are a network of branched fibres about 1 micron in diameter; the X-ray diffraction and infra-red absorption spectra are compatible with the view that the main constituent is keratin, although this evidence has been criticised as unconvincing. (Terapka, 1963a). Closer examination however reveals that there are two components, a core and a mantle, with different characteristics. The core is of keratin, and the mantle, which is less electron-dense and about 0.2 - 0.5 microns thick (Simons and Wiertz, 1963) is continuous, and similar to the organic material of the mammillae and shell matrix. It is probably a mucopolysaccharide. Richardson (1935) observed and photographed a rod-like mass of apparently about ½ μm diameter in the duct of a tubular gland in the isthmus region, clearly in the granular part (Richardson, 1935, plate 16, figures 15 and 16). The mantle of the membrane fibres is presumably responsible for the histological staining which they show; both in the present study and in the work of Robinson, King and Bowen, (1966) and Robinson and King, (1968), the membrane fibres and the goblet cells of the surface epithelium of the upper isthmus stain with the PAS method. It is thus suggested that the membranes are formed on the stimulus of the presence of the egg (or any foreign body) by the extrusion of material by the tubular glands of the upper isthmus, this being in the form of granules of ovokeratin which coalesce into threads in the glandular lumen, and are coated with a mucopolysaccharide which probably contains disulphide bonds; it is feasible that these bonds may be responsible for the cross-linking of the membrane fibres to each other, as well as to the mammillary cores, as Robinson and King (1968) suggest.

All samples of eggs fixed in the isthmus had a full complement of membranes. In addition, most of them had some degree of forma-

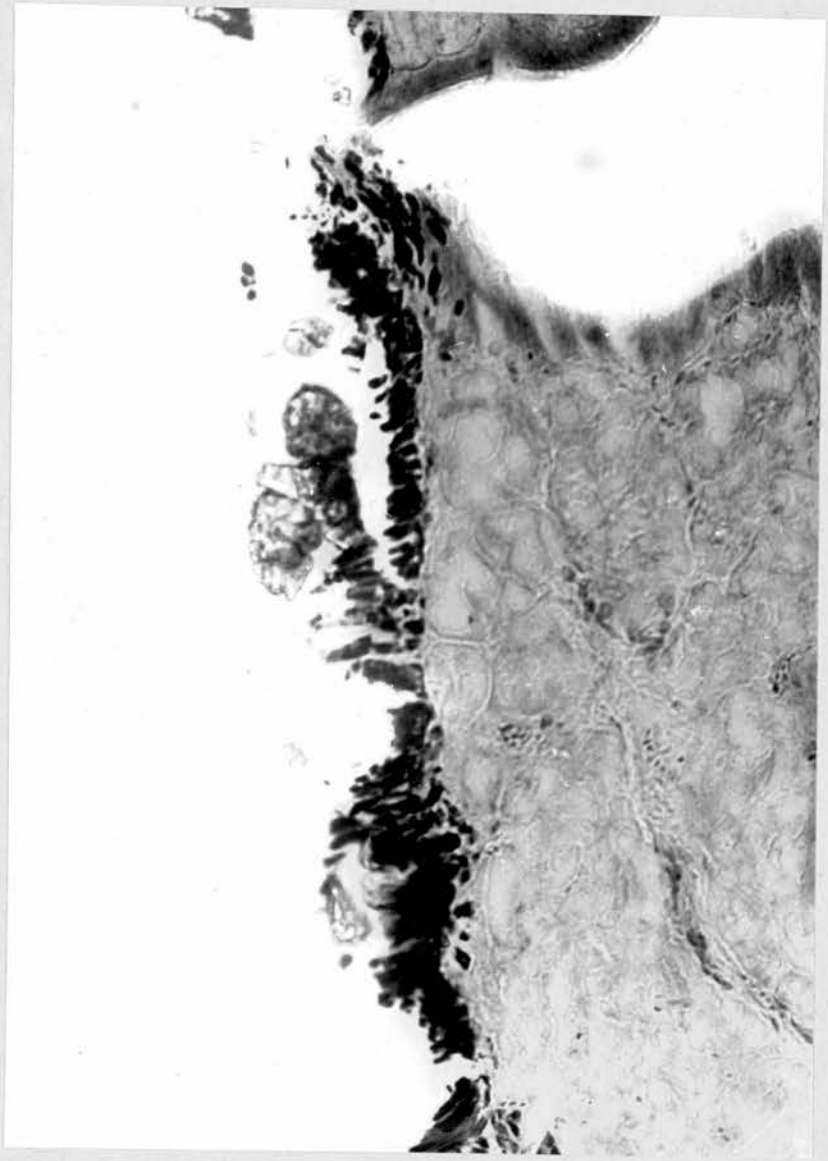


Fig.14. Lower isthmus,fixed in contact with a membrane in the early stages of calcification, showing 'cup and ball' formation of epithelial cells around mammillary cores,and material staining black by the von Kossa method (calcium) in these cells.
Counter-stained with neutral red. x 160.

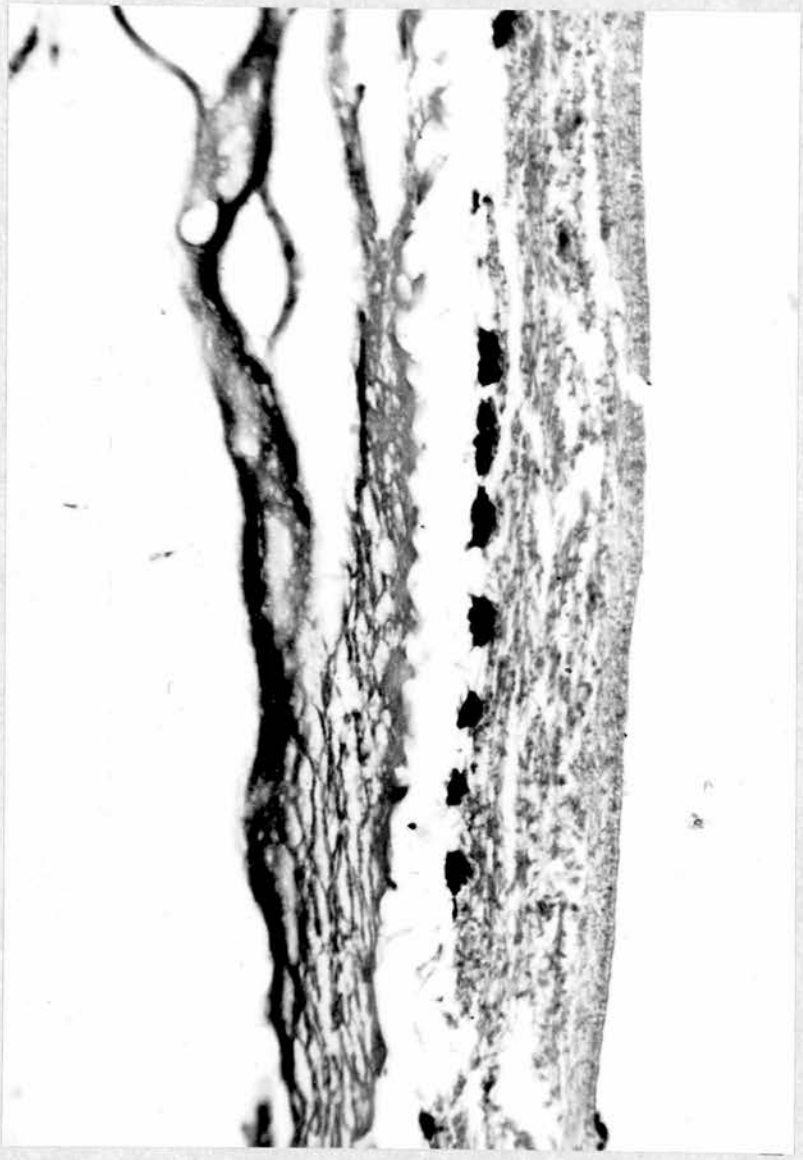


Fig.15. Radial section of a decalcified eggshell, showing the mammillary cores between the membranes and the shell matrix. The size and spacing of these cores are comparable to those in fig.14. x 160.

tion of the mammillary layer. The earliest cases of this, where the egg was in the granular part of the isthmus (i.e. upper or mid-isthmus regions) showed blobs of eosinophilic, PAS-positive material at intervals of about 60 microns along the membrane surface, each having a diameter of some 15 microns. In more fully developed cases, still usually in the upper parts of the isthmus, this material (which appears similar to that of the membrane fibre mantles) has itself acquired a mantle of haematoxylon-staining material; yet later, in samples which were most frequently found in the lower isthmus, the circular-section cores have begun to accumulate small, highly refractive crystals which stain black with the von Kossa method. (Figure 14). Examination of similarly fixed and stained samples of membranes, soft shells, and decalcified shells of oviposited eggs shows that these blobs compare in size, shape, staining properties and spacing with the mammillary cores, which are a spherical central core of about 10 microns diameter, which stains with eosin, and basic fuchsin in the PAS method; a slightly flattened mantle which stains with haematoxylon, and which gives a size of 20 by 25 microns; and a spacing most frequently of around 60 microns centre to centre, or a multiple thereof. It is also noticeable in near-tangential sections of shells and membranes that the original accumulation of mammillary core material tends to occur at a point where a number of thick membrane fibres appear to cross or join. (Figure 16). This is interesting in view of the suggestion of Robinson and King (1968a) that the core represents a specialised form of membrane fibre material, with disulphide bonds occurring predominately within the molecular chain rather than between chains. It has been reported before that membrane fibres pass into the cores (Tyler and Simkiss, 1959; Simons and Wiertz, 1963; Simkiss, 1961) - Simkiss also suggested that the disulphide links were important,

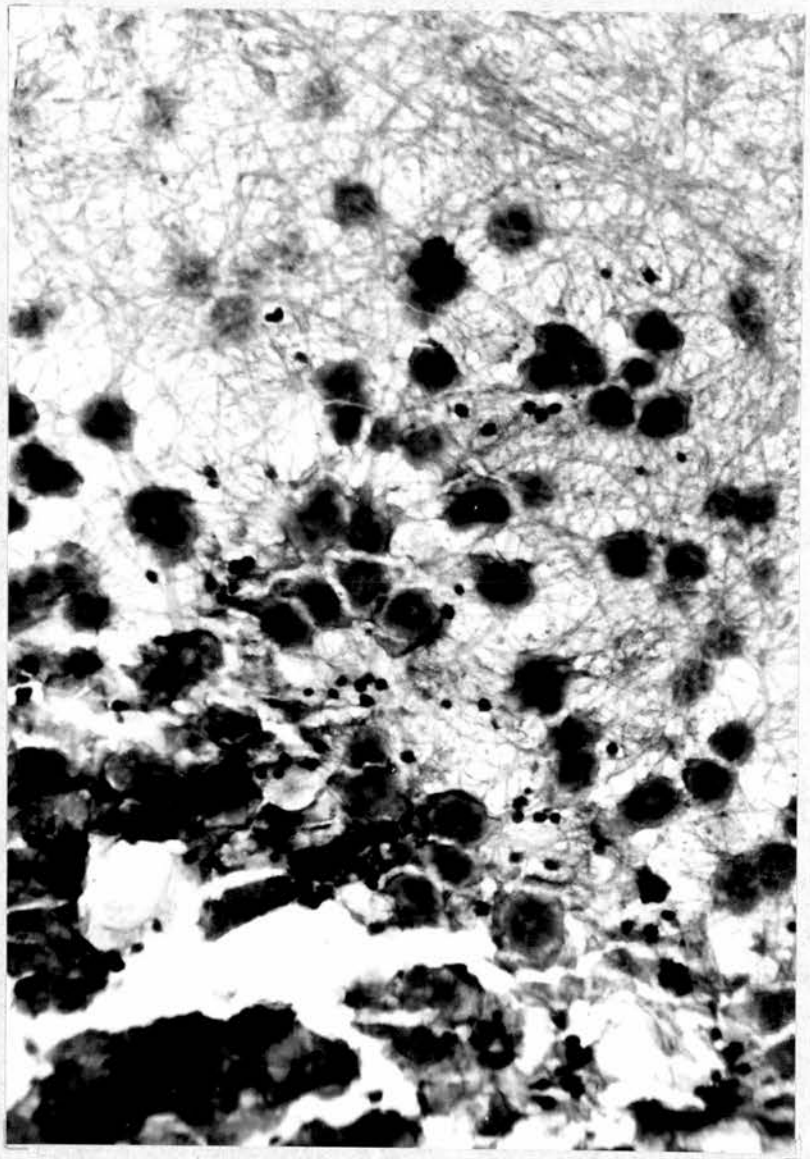


Fig.16a. Near-tangential section of decalcified eggshell, showing the mammillary cores at membrane fibre intersections. Stained by the van Gieson method. x 160.

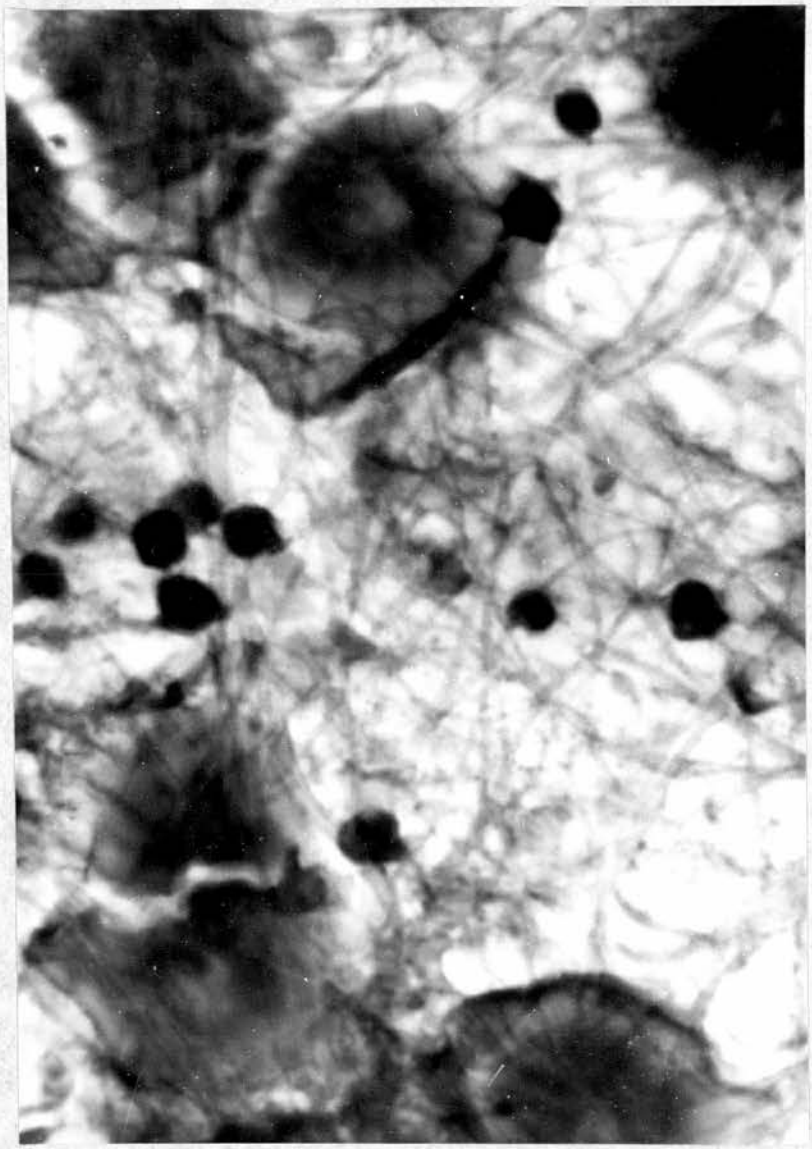


Fig.16b. Higher magnification of the same decalcified shell as fig.16a, showing the membrane fibres entering the mammillary cores. Stained by the van Gieson method. x 640.

but it is not clear whether the fibres are brought together by the core, or the core forms at a region of high fibre density. It may be that the fibres are secreted in a more regular pattern than has been assumed, and that this pattern induces the grouping of epithelial cells for core formation.

The appearance of the epithelial surface in contact with the membranes during this period of mammillary formation and crystalline seeding may also be relevant. (See figures 14 and 17). The ridges of the oviduct wall are flattened by the egg, and the membrane surface is closely in contact with the epithelial cells. These arrange themselves in groups to present the appearance of a series of 'peaks' and 'cups'. In each 'cup' a core forms. The goblet cells round the edge of the cup appear elongated, and their contents stain deeply with fuchsin and by the von Kossa method, and are rather basophilic. The shorter cells in the centre of the cup are particularly fragile, although all the epithelial cells of the isthmus in contact with the membrane show a marked tendency to rupture. It may be that this represents a genuine shedding and regeneration process, which has been suggested in the formation of the shell; indeed, early observers (Merkel and Hemsbach, 1851; Landois, 1865; quoted by Surface, 1912) believed the membrane to be formed by a transformation of the tissue of the isthmus - a view which seemed to be supported by certain similarities of behaviour in alkaline and acid solution between the membrane fibres and fibres of smooth muscle. Landois also believed the mammillae to be similarly formed from the glands of the shellgland. The history and refution of these ideas is recounted by Surface (1912). It is quite feasible that the epithelium is shed and regenerated, in the same way as that of the intestine; what is clear is that the cells are both closely attached to the membrane and liable to rupture.

The epithelial cells of the mid and lower isthmus fixed in contact with a membrane on which the mammillary cores are beginning to calcify stain by von Kossa's technique. The cells of the upper isthmus do not, nor do cells not in contact with the membrane, with however, some exceptions. Surprisingly, those parts of the surface from which the egg had been detached before treatment likewise do not develop silver granules in the cytoplasm when the von Kossa method is applied. This method is generally accepted as an indication of the presence of calcium; but it in fact shows inorganic phosphate or carbonate (which commonly occur in biological tissues only as calcium salts), and the staining could conceivably be produced by any substance, organic or inorganic, with a comparable affinity for the silver of the silver nitrate solution used. (Casselman, 1959; Pearse, 1960; Section 2.2; Section 7). It is known that a slight accumulation of calcium does take place in the isthmus region, and that this is discharged on passage of the egg, contrary to earlier reports that none is accumulated (Schraer and Schraer, 1965; Section 3.6); the fact that previous workers have been unable to demonstrate its presence histologically, either by the von Kossa technique or by any other (Richardson, 1935; Godfrey, 1948; McCallion, 1953.) is explained by the occurrence of a positive reaction only when the forming egg is present during the entire process of fixation and staining.

The cells which do show staining by the von Kossa method are those forming the 'cup and peak' shapes described. The entire cytoplasm is filled with small black granules, which appear to be quite clearly resolved under oil immersion, although their diameter was estimated at 0.1 to 0.2 mu, which is about the theoretical resolution of the microscope and lenses used. Almost all goblet cells in contact With the calcifying membrane had this appearance, and also

a few isolated goblet cells in the rest of the epithelium. These were usually located on the opposite side of a villus which was touching the membrane.

Thus it seems that the mid-isthmus region may be predominately instrumental in the secretion of the mammillary cores, although some of the organic material could be produced by the upper isthmus. Staining indicative of calcium deposition has been observed in the mid and lower isthmus, but not the upper region, and appears at the same time as the first signs of calcification on the membrane. This is also the stage of shell formation at which Robinson and King (1963) reported in the mammillae the presence of carbonic anhydrase, which has long been suspected to be implicated in shell calcification (Gutowska and Mitchell, 1945; Common, 1941; Mueller, 1962).

Other functions may be performed in the isthmus region. Pearl and Curtis (1912), and McNally (1934) held that some albumen is added to the egg, but other workers (Conrad and Phillips, 1938) believe the egg contents to be virtually unchanged. The time which the egg spends in the isthmus is usually given as about an hour and a quarter (Romanoff, 1949; Sturkie, 1965) and the membrane is permeable to the soluble proteins of the albumen (Beadle, Conrad and Scott, 1938; McNally, 1934) - (see also Section 5.2), so the addition of a solution which may contain protein as well as salts is not impossible; it is usually considered however that most of such addition takes place in the shellgland, where the egg makes a longer sojourn. Sufficient analyses of eggs at the relevant stages were not obtained in this study for any conclusions to be drawn. No function has been ascribed to the tubular glands of the mid-isthmus, which contain granules, or those of the lower isthmus, which do not, but which present an appearance in electronmicrographs similar to that of known ion transport regions (G. M. Wyburn and H. S. Johnston,

personal communication). It is possible that secretions of this region could act on the egg in the shellgland, as there is no obstruction of the oviduct lumen between these regions.

Shellgland. When the egg enters the shellgland, the mammillary cores are complete and each is surrounded by a nucleus of calcium carbonate crystals, which are already taking the form observable in the completed shell (see Section 4.1). During the period which the egg spends in the shellgland, the egg is plumped, a process which involves the addition of a solution through the membrane. Analyses of eggs indicate that the main component added is water, about 15 g.; the other constituent of greatest importance is potassium. The process is discussed in more detail in Sections 4 and 5. It seems that the plumping mechanism may to a great extent depend on the osmotic force exerted by the outer layers of the thick white, and that the entry of water and salts from fluid present in the oviducal lumen continues until the calcification of the shell has formed a rigid layer which precludes further expansion of the contents.

Until the fusion of the crystalline masses surrounding the mammillary cores takes place, the egg fixed in the shell gland presents an appearance similar to that in the isthmus. (Figure17). The same peaking of the epithelium is observed, the same flattening of the ridges of the mucosa so that the egg is closely surrounded by the epithelium, and the same staining of contact cells by the von Kossa method. The peaking effect was described in the shell gland by Froböse (1928), who appears to have been the only worker to fix the egg in situ; apart from Richardson, who failed to confirm this observation, or, more probably, to recognise it as the same phenomenon which Richardson described as the mammillae being 'secreted from pits' in the epithelium. Richardson's photomicrographs, indeed, do not show this effect, which however in the present

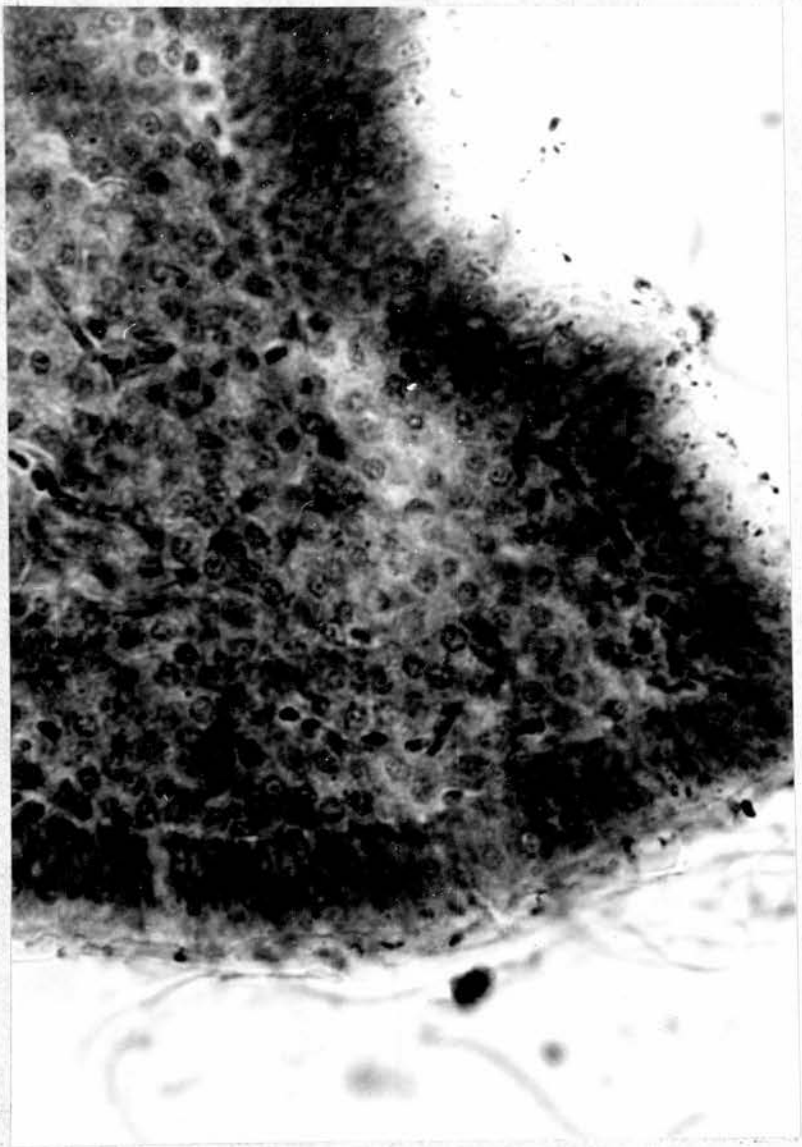


Fig.17 . Shellgland, near the junction with the lower isthmus, fixed in contact with a forming shell. Small particles stained by the von Kossa method may be seen among the cilia. Counter-stained with neutral red. x 640.

study was readily observed. This could possibly be explained by the fact that in a high intensity laying bird it is easier to catch the forming egg in the critical pre heavy calcification stage where 'peaking' is clearly observable.

When the mammillary crystals are almost fused, the von Kossa staining is very marked, and silver grains may be present in the mucosal layer for a depth of about 1 m.m. from the epithelium, scattered apparently at random. (Figure 17). At high power, grains are visible apparently arranged along the tips of the cilia from the grooves of the mucosa to the surfaces in contact with the shell. (Figure 17). Later stages of the process are more difficult to examine, as specimens containing hard-shelled eggs are almost impossible to section without prior decalcification. However, from the structure of the shell and a knowledge of rates of calcification, the probable course of events may be reconstructed. Calcification, and the simultaneous secretion of the organic matrix, which may act as chelating agent for the inorganic crystals continues at a steady rate, normally until the shell nears full thickness. The possible significance of the matrix is indicated by the observations of Simkiss (1961) who has demonstrated that metal ions can displace the cationic stain toluidine blue from the acid mucopolysaccharides of the matrix. (See also Simkiss and Tyler, 1958). The whole calcification process is usually estimated to take 16 - 18 hours in most birds, although some high-density light hybrids (Shavers) can lay fully calcified eggs 12 hours apart (P. E. Lake and A. B. Gilbert, personal communication), and so must be able to speed up the process. In the final two hours or so thereafter, the rate of calcification tails off (Burmester, Scott and Card, 1939; Bradfield, 1951). Possibly that of matrix secretion does not, as Simons and Wiertz, 1963, report a higher density of matrix fibrils in the outermost layer of shell. If the

shell is coloured, the pigment is usually only present in the superficial layer; fluorescent studies of the oviduct (Wells, personal communication) show that the porphyrins from which the pigments are derived are found in the region of the shellgland-vagina junction in the hen's oviduct. Porphyrin pigmentation is also present in the final layer of the egg, the cuticle. (Simons and Wiertz, 1966; Grini, 1939; Schoorl, 1965). This is a vesicular coating of organic material, generally described as being composed of mucin. It probably has a high proportion of mucopolysaccharides, but also contains proteins with many disulphide bonds and free sulphydryl groups (Simkiss, 1958; Simons and Wiertz, 1966) and keratin (Schmidt, 1961). It may be secreted by the transitional and vaginal regions. As the passage of the egg through the vagina is quite rapid, and accompanied by a bearing down reflex (Sykes, 1955) it has not proved possible to obtain a sample of this stage for histological examination.

3.6 CHEMICAL COMPOSITION OF OVIDUCT TISSUE

The composition of the tissue of the oviduct is not dissimilar to that of other soft tissues. Common (1938); (quoted by Romanoff and Romanoff, 1949) gives the general composition as:

Water 76%

Protein 18.6%

Fat 4.3%

Ash 1.1%

The results of the present study are comparable. In the same terms they are (approximately):

Water 80%

Fat 2%

Dry matter (protein).. 18%

Marked variation of the water, dry matter and fat content with functional state of the organ and between regions occurs. Variation in the fat values may reflect nothing more significant than the general state of the animal, as some hens kept in battery cages have large abdominal deposits of adipose tissue, or the inclusion in the sample of small quantities of fat attached to the organ. Although the dissection was performed carefully, it was not always possible to remove all such attachments. A store of fat as a metabolic reserve is not unlikely in an organ which has periods of intense synthetic and muscular activity, however; there is no pattern observable in the few results obtained here.

It may be seen from Table 10 that the water content of the regions becomes progressively greater in the isthmus and shellgland, with the exception of the inactive oviducts. The few oviducts which were analysed for chloride content, although the method was subject to considerable lack of reproducibility and the errors are rather large, could be interpreted as showing a parallel increase (Table 14). The

differences are not statistically significant; such a finding would if substantiated by further results imply that the increased water content is mainly extra-cellular, as the intra-cellular content of this ion is negligible. Assuming the extra-cellular fluid to be isotonic with blood plasma, the volume of such fluid must have increased, thus accounting for an increased water content of the tissue. This in turn could arise from increased blood flow, or increased permeability of capillaries such as may be produced by a rise in oestrogen level. An increase in extra-cellular fluid could be part of the mechanism whereby the watery solution which is ultimately added to the egg in the plumping process is mobilised.

The mineral composition of the tissue has been studied by Taylor and Hertelendy, (1961), and by Schraer and Schraer (1965), in relation to the reproductive cycle. The general pattern of their findings is similar to that of the present study, although an exact comparison is difficult, as the results of these authors are expressed in terms of the ashed weight of the samples, and the water contents are not given. Preliminary studies showed that good results could be obtained without ashing the samples, so this time-consuming step was omitted. Assuming a water content of 80%, the agreement is quite close.

The results of the mineral analyses of the three main regions of the oviduct are given in Table 10, together with the water contents. The overall values are within the ranges to be expected in other avian tissues (see Table 16). Analysis of variance shows highly significant differences in all minerals measured when the region ("Site") is considered, this being least marked for potassium, and quite significant (at the 5% level) difference with reproductive state ("Activity") for water content and sodium and potassium concentration. The slightly lower concentration of potassium in the shellgland region is perhaps not important; Schraer and Schraer (op. cit.) found this

ion to be constant throughout the length of the organ. The higher sodium in isthmus and shellgland is in agreement with their findings. It is interesting to note that in studies of developing muscles of chicks of various ages, a high sodium:potassium ratio was found to be a characteristic of tissue which probably had a high rate of protein synthesis; this is also found in the liver. (Table 16; Draper, 1968). Although sodium and potassium both rise significantly in all regions of the inactive oviduct save the shellgland, this may well be a reflection of a reduction of other tissue components.

It has recently been suggested that the electrolyte content of the pregnant mammalian uterus reflects the motility of the muscular layer (Hawkins & Nixon, 1958; Daniel, Hunt and Allan, 1960; Prasad, Gambhir and Sanyal, 1967). In the avian oviduct also there is a higher sodium:potassium ratio than in corresponding skeletal muscle. This suggests that changes in these minerals were due to the smooth muscle layers of the oviduct. These are so intimately connected to the other layers that separation is probably not feasible, and the possibility must remain in the realm of speculation. The effect in the human has been related to the action of ovarian hormones.

The calcium level in the avian oviduct tissues, in spite of the possibility of some degree of accumulation or of the presence of calcium-rich blood, is in all cases except the pre-secretory isthmus at or below the levels found in non-pregnant or inert mammalian tissues (see Tables 10 and 17). If the systems were comparable, such levels of calcium would, according to Prasad et al., be expected in circumstances of uterine malfunction only.

The calcium distribution is of more specific interest in relation to the process of shell formation. The level is highest in the isthmus, and the highest value is found in the oviduct taken

while an egg was in the magnum, that is when the isthmus would be expected to be preparing to receive it. This result is in agreement with the findings of Schraer and Schraer. Indeed these authors found an even greater increase at this time, to a level almost double that of the isthmus at other stages, and up to four times that of the magnum or shellgland. The accumulation of calcium by the isthmus and its discharge on the passage of the egg involves quantities which are not very great in absolute terms. 6g. of isthmus tissue (this was about the weight of the active isthmuses analysed), in which a drop of 3 m-moles/kg. of calcium is observed, represents only 18 micromoles of calcium. This could be enough to initiate the seeding of calcium crystals on the mammillae which was observed histologically. It is surprising that at no stage of the egg-laying cycle does the shellgland show even this small rise in calcium content, in spite of the comparatively huge quantities of calcium deposited on the egg while there.

The observation of an increased calcium concentration in the isthmus is not in agreement with the studies of Richardson (1935) and McCallion (1953), which indicated that if a concentration of calcium occurred anywhere, it was in the shellgland. In particular, the micro-incineration technique used by Richardson suggested a concentration in the surface epithelium. These results were not conclusive, and the quantities involved were small; in addition, the technique is not specific for calcium, but simply shows ash. The results of Schraer and Schraer indicate an increase in the ash content of the shellgland which is unconnected with the calcium level.

The fourth cation to be measured was magnesium. The concentrations of this ion are higher in the magnum than in isthmus or shellgland, and do not vary significantly with the functional state of

the organ, although there may be some indication of a fall in level when the oviduct is inactive. This result is also confirmed by Schraer and Schraer, who attribute it to the importance of the metal in protein synthesis; it is also possible that the ion is bound to or in some way associated with albumen, in which it is present at a concentration about ten times that of blood plasma while potassium and calcium are at about the same concentration in both. (Draper, 1967). Although the calcium:magnesium ratio in the initial seeding of the shell crystals is exceptionally low, less than 3 when the calcium deposited is less than 100 micromoles (see Section 4.7), no accumulation and discharge of magnesium is found in the isthmus to parallel that of calcium.

The composition of the three regions of the isthmus, upper, mid and lower, was further studied in a series of ten oviducts, all of which were in the active state. The results are shown in Table 15. These show that the increased water content of the whole isthmus is due almost entirely to the lower section. The dry weight of oviduct tissue is to a large extent due to protein; a higher water content therefore implies a lower percentage of protein in the tissue. On the basis of the functions ascribed to the various regions on mainly histological grounds (Section 3.5), this would be expected in the lower isthmus and shellgland. It is interesting that the sodium concentration does not vary significantly, between the sections, although the potassium variation is marked, being highest in the lower isthmus. This lack of significance in sodium levels may in part be due to the high variation of the individual results; for each section the coefficient of variation is more than twice that for potassium.

The high potassium in the lower region, in spite of the lack of overall significant variation, may possibly be related to the

transport of potassium by the lower reaches of the oviduct (Section 6.2). It is however to some extent in conflict with the high water content, which would suggest a high extra-cellular space, whereas the high potassium would, assuming the cellular composition to be approximately constant, imply a lower ratio of extra-cellular to intra-cellular space.

The lowered content of magnesium in the 'C' section (lower isthmus) may be related to the lowered protein content and the presumable comparative lack of protein synthesis in this region. The markedly higher calcium in the mid-isthmus, on the other hand, suggests the location of shell crystal seeding in this region. This is in agreement with the histological findings. (Section 3.5). The level of significance of this difference is fairly low (see Table 15), but the coefficient of variation within each group is higher than for any other component. The variation may be due to the difficulty in locating the upper-isthmus/mid-isthmus junction in particular, and the mid/lower junction to a lesser degree, under the conditions of dissection for analysis, where it is desirable to weigh the samples with a minimum lapse of time in which evaporation may occur. If the calcium-storage region is sharply defined, a slight error in the selection of the division line could give rise to a comparatively large departure from the true composition of the regions. On the other hand, the variability of the results may reflect the precise functional state of the organ (which was not recorded), or a functional overlap between the regions.

Other metals studied by Schraer and Schraer (1965), but not examined in the present work, were iron, copper, zinc and manganese. Iron was found in highest concentration in isthmus and shellgland tissue when the egg was in the isthmus, and was also high in immature

specimens, but did not fluctuate coincidentally with other ions. Schraer and Schraer considered it to be an indication of residual blood, and took the results to imply that contributions to measurements from this source were not important. Zinc content was not found to vary greatly, which is interesting as carbonic anhydrase is a zinc-containing enzyme; copper and manganese are present in greatest concentrations in isthmus and shellgland respectively, and decreases in these concentrations with the process of egg formation caused the authors to suggest that these metals are transferred to the egg in these regions.

In a later study, (1966), Hohman and Schraer attempted to localise intra-cellularly the calcium of the shellgland tissue. They found that Ca^{45} activity was greatest in the mitochondrial fraction, and, during the period of calcification, in the microsomal fraction of shellgland mucosal scrapings. However, no comment is made by the authors on the possibility of calcium exchange during the procedures of homogenisation and differential centrifugation.

TABLE 10

COMPOSITION OF OVIDUCT TISSUE:
WATER AND MINERAL CONTENT OF THE REGIONS

(Water as percentage of wet weight; minerals in m-moles/kg. wet weight. Means ± S.E.'s)

	Functional state	n	REGION		
			Magnum	Isthmus	Shellgland
WATER	Egg in magnum	1	77.00	78.15	-
	Egg in shellgland	11	73.77±0.84	78.60±0.55	81.71±0.35
	Active	1	72.99	78.41	81.75
	Inactive	3	80.06±0.36	80.20±0.31	81.07±0.59
SODIUM	Egg in magnum	1	64.6	67.1	-
	Egg in shellgland	11	53.4±2.4	71.1±2.2	73.4±2.4 (1)
	Active	1	58.1	66.4	81.9
	Inactive	3	75.7±2.0	92.4±6.6	77.8±0.2 (2)
POTASSIUM	Egg in magnum	1	72.6	71.5	-
	Egg in shellgland	11	67.0±1.8	69.1±1.9	64.1±2.0 (1)
	Active	1	68.0	69.3	56.3
	Inactive	3	71.4±2.2	80.4±1.2	61.1±0.6 (2)
MAGNESIUM	Egg in magnum	1	12.0	6.9	-
	Egg in shellgland	11	14.6±0.7	8.6±0.6 (3)	6.9±0.4 (1)
	Active	1	15.6	7.1	5.6
	Inactive	3	8.2±0.5	9.4±0.5	6.2±0.4 (2)
CALCIUM	Egg in magnum	1	3.7	12.0	-
	Egg in shellgland	11	2.8±0.2	8.9±0.8	4.6±0.3 (1)
	Active	1	2.4	9.1	3.8
	Inactive	3	5.6±1.6	9.0±2.6	6.4±1.8 (2)

(1) n = 9; (2) n = 2; (3) n = 10

TABLE 11

COMPOSITION OF OVIDUCT TISSUE:

SIGNIFICANCE OF DIFFERENCES IN WATER AND MINERAL CONTENTS OF THE REGIONS

(By analysis of variance)

	Water	Na	K	Mg	Ca
Activity	*	**	*	N.S.	N.S.
Site	***	***	*	***	***
Activity x Site	*	N.S.	N.S.	N.S.	N.S.

* Significant at 5% level

** Significant at 1% level

*** Significant at 0.1% level or better

N.S. Not significantly different

TABLE 12

OVIDUCT COMPOSITION: LIPID CONTENT
(g/100g. dry weight: Individual results)

Functional state	Region:		
	Magnum	Isthmus	Shellgland
Egg in shellgland	9.06	11.07	10.86
Active	6.16	8.02	10.92
Inactive	6.32	8.62	12.51

TABLE 13

OVIDUCT COMPOSITION: PHOSPHATE CONTENT
(m-moles/kg wet weight: Individual results)

Functional state	Region:		
	Magnum	Isthmus	Shellgland
Egg in magnum: above egg	0.12	-	-
Egg in magnum: below egg	0.14		
Egg in shellgland	0.17	0.14	0.08
Inactive	0.27	0.40	0.30
Inactive	0.40	0.63	0.34

TABLE 14

COMPOSITION OF THE OVIDUCT: CHLORIDE CONCENTRATIONS

(m-moles/kg wet weight. Individual results)

Functional state	Magnum	Isthmus	Shellgland
Egg in magnum	220.6	84.7	175.3
Egg in shellgland	129.6	251.6	148.5
Egg in shellgland	139.7	148.2	173.8
Egg in shellgland	132.7	119.4	166.5
Egg in shellgland	133.9	132.4	154.9
MEAN ± S.E.	151.3±17.4	147.3±56.2	163.8±10.5

(Means not significantly different)

TABLE 15

COMPOSITION OF OVIDUCT TISSUES

Water and mineral content of upper, mid and lower isthmus

(Water as percentage; minerals in m-moles/kg wet weight.
Means ± S.E.'s. n = 10 throughout)

Segment	Water	Na	K	Mg	Ca
Upper	77.40±2.11	64.0 ±5.3	64.7 ±1.7	8.1 ±1.3	8.7 ±1.2
Mid	76.89±0.60	53.9 ±3.2	64.7 ±1.9	7.4 ±0.3	12.1 ±1.3
Lower	81.37±0.31	67.7 ±2.9	70.0 ±1.4	6.6 ±0.2	7.9 ±0.7
Mean	77.98±0.57	61.32±3.06	65.36±1.61	7.39±0.24	9.57±0.87
Coefficient of variation					
Upper	2.88	26.14	8.36	17.24	44.75
Mid	2.46	18.87	9.50	12.56	33.93
Lower	1.21	13.35	6.27	7.18	27.82
All	2.29	16.57	8.16	11.49	30.02
Comparison of means for each segment ('Student's t-test)					
Upper & mid	N.S.	N.S.	N.S.	N.S.	*
Mid & lower	***	N.S.	*	*	**
Upper & lower	***	N.S.	*	**	N.S.

N.S. Not significantly different

Significantly different at:

10% * 5% * 2% **

1% ** 0.1% ***

TABLE 16

COMPOSITION OF OTHER TISSUES OF GALLUS DOMESTICUS

Water is given as a percentage, protein (= N x 6.25) is expressed as g/kg wet weight, fat as g/100g dry weight, minerals as m-moles/kg wet weight, and age of the chicken in days.

	AGE							
BREAST MUSCLE	0	9	16	24	37	59	70	450
Water	84.8	81.7	74.9	74.6	74.8	71.0	72.6	71.7
Protein	92	140	214	223	221	239	234	238
Fat	28.9	21.9	10.4	6.7	4.0	4.5	4.4	7.9
Na	81	104	49	34	32	28	36	30
K	36	73	101	112	109	119	115	98
Mg	4.6	9.6	13.5	13.4	12.4	13.5	13.3	-
Ca	-	10	9.3	5.7	5.4	10.0	-	-
THIGH MUSCLE								
Water	76.0	71.5	69.6	72.5	73.8	72.6	74.6	69.0
Protein	120	158	170	183	191	192	191	195
Fat	39.2	37.9	37.5	29.5	23.3	17.9	18.9	26.5
Na	66	80	65	45	45	36	45	46
K	47	71	84	100	96	106	103	85
Mg	7.1	9.7	10.5	10.9	10.3	9.7	10.6	-
Ca	-	8.4	7.2	12.0	5.3	6.0	-	-
LIVER								
Water	67.3	-	-	-	75.8	72.8	69.7	69.0
Protein	142	-	-	-	182	191	198	157
Fat	49.2	-	-	-	14.9	14.0	14.5	22.6
Na	73	-	-	-	71	60	67	62
K	67	-	-	-	67	75	89	72
Mg	-	-	-	-	8.0	8.9	9.6	-
Ca	-	-	-	-	6.2	10.2	-	-

TABLE 17

COMPOSITION OF SOME MAMMALIAN TISSUES

Water is expressed as g/100g., and minerals as m-moles/kg. wet weight

The figures for human uterus and skeletal muscle are recalculated from the data of Hawkins and Nixon (1958), and those for rat uterus from the data of Prasad, Gambhir and Sanyal (1967).

Tissue	Human uterus non-pregnant	Lower uterus at term	Lower uterus in inertia (Late 1st stage)	Lower uterus in premature labour	Skeletal muscle	Albino rat uterus
Water	79.5	82.8	84.1	82.8	72.5	-
Na	87.1	89.6	109.9	85.3	31.6	161.8
K	60.7	69.3	42.9	50.1	128.15	45.9
Ca	6.3	11.4	3.0	5.3	-	6.2
Mg	4.8	8.9	4.4	5.1	10.7	-
Cl	71.8	60.7	82.2	63.8	19.0	-

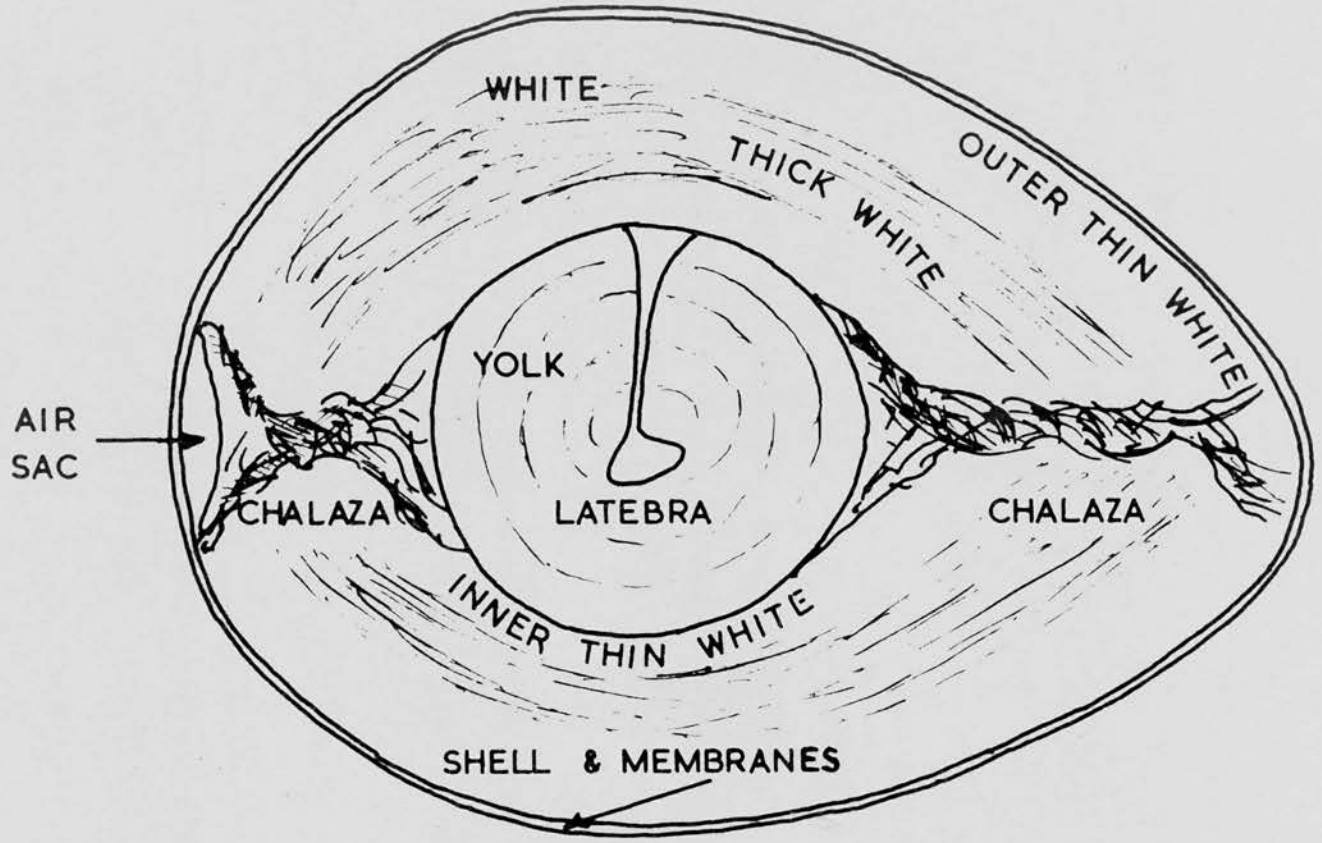


FIG 18: DIAGRAMMATIC REPRESENTATION OF THE STRUCTURE OF THE HEN'S EGG

SECTION 4

RESULTS: THE EGG

4.1 INTRODUCTION: STRUCTURE OF THE EGG

The experimental procedures used in this study were directed almost entirely towards the investigation of the mineral and water contents of the components of the egg. In order that the results obtained be seen in context, the structure of the egg is described.

Yolk. This is a slightly flattened sphere of diameter some 30 - 35 cm., and weight about 18 - 20 g. in an egg weighing 60 g. - about one third of the total, according to Romanoff and Romanoff (1949), 31.9%. It is built up by deposition of material from the centre outwards; if hens are fed at specified times rather than ad lib., a series of concentric spheres may be observed, deeply pigmented ones with large lipid globules representing the period after feeding, and narrow, pale ones of lower fat content representing the reduced deposition of the period when no food is available. The centre of the yolk is occupied by a particularly fluid layer, the latebra, from which a channel of the fluid material, the neck of the latebra, extends to the surface of the yolk, where it forms the nucleus of Pander, in which is situated the blastodisc of the egg. The material of the layers is a suspension of globules, of size from 0.025 to 0.150 mm. diameter; these in turn contain granules. The globules in the white yolk fractions are usually somewhat smaller, about 0.004 to 0.075 mm. diameter. About half of the mass of the yolk is water; the dry matter is composed mainly of fat and protein, according to Romanoff, about twice as much of the former as of the latter.

The whole highly structured mass is enclosed by the vitelline membrane. This is a thin, pliable, non-cellular envelope which arises during the last stage of follicular development. Wolken (1951)

was unable to determine its structure by electron microscopy; he assumed it to be composed of keratin, but the presence of mucin has also been suggested, and a variety of layered structures, dependent on technique of fixation. This probably also affects the thickness; Needham (1931) believed this to be about 24 microns in the natural state. In isolation, it is permeable to substances of low molecular weight, such as sugars, to water and ions, and to lipids, but not to proteins such as ovovitellin. Its permeability to water is influenced by the ions present and by the pH, and is lowest around the iso-electric point of ovalbumin (pH 4.6 - 5.0). It is not dissolved by organic solvents which do dissolve the yolk material (Osborne, 1931). In spite of its behaviour *in vitro*, it plays a part in inhibiting osmotic equilibration of the yolk and albumen.

More recently, Bellairs, Harkness and Harkness (1963) found a composition suggesting that the inner part of the vitelline membrane is of non-collagenous connective tissue protein, secreted in the ovary, and the outer (the chalaziferous layer) has a composition similar to egg-white proteins. In particular the analyses would fit a mixture of lysozyme and conalbumin. (Shenstone, 1968).

White. When the egg is laid, the albumen consists of three main layers: the inner thin white, the thick white, and the outer thin white. In addition to these there is the thin fibrous chalaziferous layer, and the chalazae themselves. It has been suggested (Section 3.5) that these are formed mechanically, as a result of the rotation of the egg in the oviduct, particularly in the shellgland, by the condensation of the insoluble ovomucin component of eggwhite. The albumen from which they are formed is probably initially similar to the thick white; the residue is the inner thin white. The outer thin white is probably formed during the plumping process, due to

the dilution of the outer layers of the thick white by the watery fluid passing through the membrane.

The thick white, and to a lesser degree the two thin white components, also display a structure of concentric layers, which are successively laid down during the passage of the egg through the magnum, and which owe their stability to the fibres of ovomucin, or possibly to the cross-linking of an ovomucin-lysozyme complex, (Brooks and Hale, 1959, 1961). Their formation is discussed in the previous section; they may be demonstrated histochemically (Cole, 1938).

Schaible, Moore and Davidson (1935) considered these fibres to be an artefact of the fixation procedure, but demonstrated the existence of layers, and possibly of membraneous sheets in the albumen by coagulation in distilled water. Forsythe and Bergquist (1951) explained the fibres as being portions of such a membraneous sheet, broken and curled up to give the appearance of a tubular-section fibre.

The chalaziferous layer, which surrounds the yolk, and may reinforce the osmotic resistance of the vitelline membrane, is continuous with the chalazae, the cloudy, twisted ropes of which spiral to the poles of the egg where the fibres disperse and become scarcely distinguishable from those in the body of the albumen. There is a tendency for the albumen around the poles to be thicker and this may be the diffuse termination of the chalazae. The chalaza at the narrow end of the egg is the larger; this end is caudal during egg formation. (Besch and Sluka, 1966).

Membranes. Two shell membranes are clearly distinguishable; various workers have described the existence of several layers in each. Most recently, Simons and Wiertz (1963) studied a section through all layers in one egg by electron microscopy. They confirmed

the description of the fibres from which the membranes are formed given by Masshoff and Stolpmann (1961). This is of an electron-dense core and a lighter mantle. The inner membrane complex was about 20 microns thick. Its constituent fibres were up to 1.5 microns in diameter and 23 microns long, and ran parallel to each other in three layers, each of which was parallel to the shell; the direction of the fibres in the middle layer was at right angles across that of the fibres in the other layers.

The fibres of this membrane intermeshed to some extent with those of the outer membrane. The fibres of the outer membrane complex were up to twice as thick as the inner membrane ones, but shorter, being around 15 microns long. All these fibre lengths apply to the core material: the mantle covering appears to be a continuous overlay. The outer membrane in the egg studied had six layers totalling some 50 microns in thickness, these again running at right angles to each other, alternately 'warp' and 'weft', approximately diagonal to the main axis of the egg. The total membrane thickness was 70 microns, and the mesh dimensions varied from about 1 micron in the most dense part of the inner membrane to about 10 microns in the coarser outer membrane. Wolken (1951), using a less critical technique, reported mesh holes of 1 - 2 microns.

The two membranes separate around the air cell, which is formed as a result of the contraction of the egg contents on cooling to the ambient temperature after oviposition. It usually occurs at the blunt end of the egg, where the porosity of the shell is greatest (Romanoff and Romanoff, 1949). The occasional formation of multiple air cells, the so-called "frogspawn egg", may be attributed to excessive porosity arising from faults in the shell or cuticle. (Hinton, 1968).

The fibres of the outermost layer of the membrane extend into the wax-like layer of the shell. According to Simons and Wierts

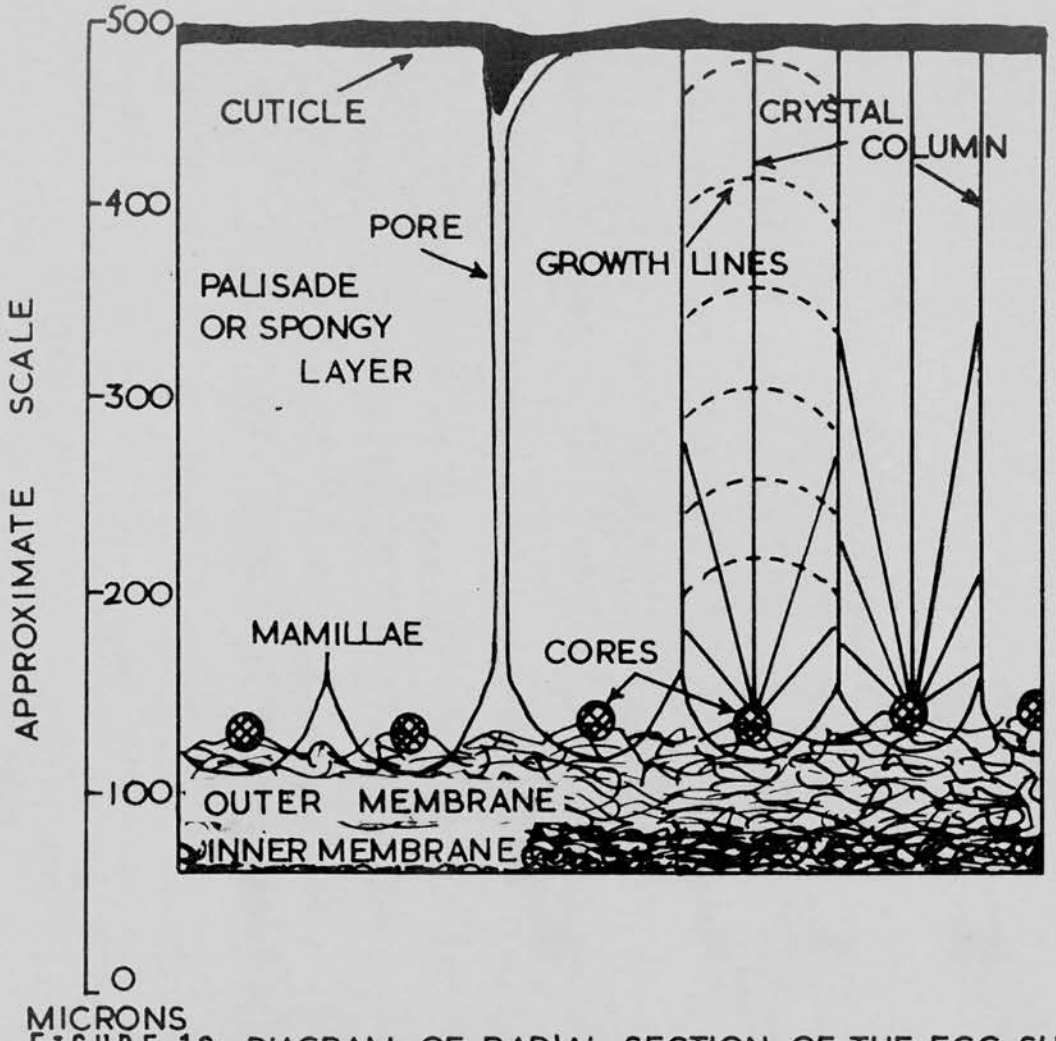


FIGURE 19: DIAGRAM OF RADIAL SECTION OF THE EGG SHELL

(1963), the mantle material of the membrane fibres is continous with that of the first protrusions of the organic material of the mammillae, and the core substance branches and fuses with the material of the mammillary matrix, forming a ring of loose meshwork in the lower part of the mammilla. Terepka (1963a) demonstrated just such a ring-like structure in fully decalcified shells.

Mammillae. The mammillary layer is usually considered to be the inner layer of the shell (see Figure 19), and here indeed calcification begins to occur. It is however more firmly attached to the membranes, and during incubation of the fertile egg, when, at about the sixteenth day, the membrane splits off from the shell, the mammillary tips and some of the calcium crystals attached to them remain with the membrane. (Terepka, 1963b). Structurally, the mammillae are composed of an organic core which is approximately spherical. A detailed description of the appearance of this in decalcified shells under electron microscopy is given by Simons and Wiertz (1963). A little higher than the level at which the membrane fibres enmesh with the mammillary core matrix, they described a region where the fibrils of this matrix take up the orientation which would be expected of the primary crystalline units of the shell in the model of Schmidt (1957). They also describe a layer of vesicular, irregular material at the upper limit of the mammillae, which they interpret as a weak zone at which the separation of the mammillary layer from the rest of the shell could be facilitated.

According to Terepka (1963a), the mineral mammillary core does not coincide exactly with the organic cores, but forms a ring round them. This they believe to be coincident with, or at least attached to, the ring of membrane fibre attachment, which they observed in decalcified preparations. From the evidence of Simons and Wiertz, it seems more likely that mineralisation occurs initially in the

region of matrix alignment slightly above this, but that crystal growth towards the centre of the egg continues until impeded by the membranes.

Shell. The calcified shell is usually divided into mammillary and spongy layers. Figure 19 is a diagramatic representation of the structure. The crystal planes imply growth radially from the mammillary cores until the crystalline masses from adjacent cores meet, and thereafter growth upwards in columns towards the surface of the egg. The orientation of crystalline elements as observed by X-rays and by polarised light is in agreement with this scheme (Terepka, 1963a) and growth lines, possibly due to gaseous inclusions, have been observed. (Schmidt, 1957, 1962). The evidence is reviewed by Simkiss, (1968). Simons and Wiertz (1963) describe an abrupt change in the appearance of the decalcified shell under electron microscopy. The irregular, vesicular matrix of the upper part of the mammilla gives way to a matrix of fibrils of up to 0.01 microns thick and 10 microns long, with attached vesicles about 0.4 microns wide. The fibrils anastomose, and this type of matrix continues until the outer zone of the shell is reached. In this region, the fibrils are thicker and more branched, and the zone culminates in a thin membraneous layer. This zone of denser matrix would seem to correspond to the translucent band found by Terepka (1963a).

The deposition of the crystalline and organic fractions of the shell takes place as has been stated, in the form of columns which meet and interlock with each other. In some cases, adjacent columns fail to meet, and the resulting channel remains as a pore. (See Figure 19). There are between 7,000 and 17,000 of these funnel-shaped openings in a single shell. (Stewart, 1935; Romanoff and Romanoff, 1949; Tyler, 1953), and each is from 15 - 65 microns wide at the mouth, tapering to 6 - 23 microns at the inner end.

(Tyler, 1956). It has been suggested that the pores remain open due to the passage of fluid through the shell during its formation (Tyler, 1956); Simkiss (1961) reports that sections of an egg taken two hours before it was due to be laid had pores filled with a sulphur-containing protein, which possibly represented albumen passing into the egg. In the incubating egg, they are responsible for gaseous exchange between the developing embryo and the environment.

According to various workers, the strength of the eggshell may be correlated with the quantity or density of organic matrix present. (Simons and Wiertz, 1963; Bronsch and Diamantstein, 1965; Mather, Epling and Thornton, 1962). None of these studies have been entirely satisfactory. Simons and Wiertz drew their conclusions from the study of only four shells; Bronsch and Diamantstein made no apparent allowance for the presence of membranes and cuticle and their contribution to the organic content. Mather, Epling and Thornton attributed different structures to the matrix in portions of decalcified shells, with no reference to the orientation of the sections; furthermore they estimated the quantity of matrix present by a thickness measurement in a section which had been decalcified, dried, and otherwise treated without regard for the effects of such treatment. Recent measurements suggest that the percentage of matrix present in the shell proper, that is in the spongy or palisade layer, is remarkably constant and bears no relationship to shell strength (T. C. Carter, personal communication).

Cuticle. The thickness of this final layer of the shell is usually stated to be about 10 microns (Romanoff and Romanoff, 1949; Simkiss, 1961, 1968). More recently, it has been recognised that this can vary considerably. Simons and Wiertz (1963) report variations from 1.7 to 12.8 μm in the cuticles of four eggs, with lesser variations between different areas of the same egg. Estimates of cuticle

thickness made during a study of the organic content of shells suggest that the cuticle may range from almost non-existent to some 100 microns thick (T. C. Carter, personal communication). Its structure is vesicular, with the smallest vesicles in the outer layer. Between the vesicles are air-spaces, which shrink as the egg ages. In some cases, there appear to be two layers, but this may be produced by changes initiated at the surface and progressing inwards. (Romanoff and Romanoff, 1949; Simons and Wiertz, 1963, 1966).

The cuticle is continuous over the openings of the pores, and may form a plug in the pore mouth (Figure 19). The number of pores which have this type of plug may determine the overall permeability of the shell; it is generally accepted that they play a part in protecting the egg against bacterial spoilage.

4.2 THE COMPOSITION OF THE EGG

The Yolk. In a 55 to 60 g. egg, the yolk may be expected to weigh about 18 gms., of which about half will be water. According to Romanoff and Romanoff (1949), the solids usually form rather the larger part; this is seen to be so in the Brown Leghorn eggs, see Table 21, but the Shaver eggs of Table 19 have a water content slightly over 50%. Almost all of the solids are organic, mainly proteins and lipids in a ratio of approximately 1:2, with about 2% carbohydrate and 2% inorganic constituents. The individual constituents are discussed by Parkinson (1966), and the composition is also reviewed by Shenstone (1968).

That some 31% of the lipids are phospho-lipids is reflected in the relatively high phosphorus content of the yolk. Typical yolk mineral concentrations are given in Table 18. Of the other minerals, calcium and sulphur may be to a large extent present in a bound form. It is apparent that sodium and potassium are present in approximately equal concentrations, the potassium being if anything slightly greater. In comparison with blood plasma, the sodium concentration is decreased by a factor of at least six, while that of potassium may be increased by the same factor. The considerable difference from blood, and also, as will be seen, from the concentrations found in egg albumen, imply that a barrier must exist to the free diffusion of ions, either due to the properties of the vitelline membrane, or to a considerable degree of organisation of the yolk materials, sufficient to effectively prevent the diffusion of ions, at least to a significant degree during passage of the egg through the oviduct. The available evidence has been reviewed by Shenstone (1968), who concludes that the structural properties of the yolk are of major importance in the control of diffusion, both of ions and of water. It is possible

TABLE 18

MINERAL CONCENTRATIONS IN THE YOLK

(Expressed as m-moles/kg. of water, means ± S.E.'s)

	SOURCE						Avian blood plasma
	(1)	(2)	I	II	III	V	
n	-	10	8	8	13	9	
Na	21	25±3.1	30±1.9	26±2.3	27±2.9	25±3.0	170
K	21	28±3.3	35±4.4	30±2.7	42±6.9	33±6.0	6
Ca	30	42±3.8	37±5.5	57±5.3	55±7.2	59±10	6.7 (1.2 Ca^{++})
Mg	47	5±0.6	6±0.7	6±0.4	7±0.8	6±1.0	1.2
Fe	1.27	-	-	-	-	-	-
S	43.3	-	-	-	-	-	-
Cl	32.5	-	-	-	-	155	-
P	148.0	-	-	-	-	-	-

Sources:

(1) Recalculated from the data of Shenstone (1968)

(2) Draper, (1966)

I - V Analyses of eggs from Bird 5284, P.R.C. Brown Leghorn J-line, from January 1964 to January 1965. (See Table 21)

Blood plasma. See Section 2.9

that an uptake of water from the white of the order of 1% does take place during the period between ovulation and oviposition, (Draper, 1966), and a greater uptake is known to occur during storage of the egg.

The White. In the completed egg, the albumen contains a high proportion of water. Romanoff and Romanoff (1949) give this as 88% in a 33 g. white, while Shenstone (1968) quotes 88.5%; the eggs shown in the accompanying tables have albumen water content of from 85% to 90%. The solids are almost entirely protein; according to Shenstone, 92%. There is also about 0.5% of free carbohydrate, more than 98% of which is glucose, and about the same quantity of inorganic constituents.

The composition and properties of the protein constituents have been recently reviewed by Parkinson (1966). More than half of contribution is due to ovalbumin, which was the first of the egg proteins to be isolated. This protein in the pure form contains all the essential amino acids, a single oligosaccharide group, and either one or two disulphide groups and four or five sulphydryl groups. The crystalline protein may be resolved electrophoretically into three components with differing phosphorus contents. The next most abundant protein is conalbumin, which forms 13% of the dry weight, followed closely by ovomucoid with 11%; 3.5% is lysozyme, and 1.5% the fibrous protein, ovomucin - a total of only 5% for those proteins believed responsible for the gel properties of the thick white. Of the minor proteins, some 8% are unidentified, but believed to be mainly globulins. Some of the components which make up less than 1% of the total have been identified through their biological properties; these are flavoprotein and apoprotein forms of riboflavin, in approximately equal amounts, ovoinhibitor, and avidin. (Parkinson, 1966).

. The membranes and shell. Approximately 95% of the shell is formed of inorganic crystalline material, and almost all of this is calcium carbonate as calcite. There are small quantities of magnesium and phosphate present together with a smaller residue consisting mainly of sodium, potassium and sulphur, which are present either as impurities in the crystal lattice, or in association with the organic matrix. The membranes are almost entirely organic. They contain about 20% water, while the shell usually contains only 1 - 2%. This is probably mostly in the pores and cuticle, and is to some extent dependent on the time and conditions of temperature and humidity under which the egg has been stored.

The evidence on the nature of the three organic components, membranes, shell matrix and cuticle, and on the ways in which the mammillary core differs from the shell matrix proper, is mainly histochemical and electron microscopical. There have been comparatively few chemical analytical studies carried out on the membranes and shell (vide Simkiss, 1968). Amino acid analyses have been reported by Baker and Balch (1962) and Frank, Burger and Swanson, (1965). The electron-dense core of the membrane fibres is believed to be a keratin-like protein and the less dense mantle material is believed to be a mucopolysaccharide. (Masshoff and Stolpmann, 1961; Simons and Wiertz, 1963). The cores of the outer fibres are embedded in the mammillae, and the mantle material is continuous with the lower part of the mammillary matrix, and may be formed from the same precursor (Robinson and King, 1968a), disulphide bonds being formed within the molecule in the case of the mammilla and between molecules in the case of the membrane fibre. This mammillary core is covered by a layer of soluble alcianophilic mucopolysaccharide, which merges into the matrix proper. This is homogenous throughout

the shell, and consists of acidic mucopolysaccharides, mainly chondroitin sulphates A and C. The matrix fibrils are more closely spaced in the outer layer of the shell, possibly corresponding to the slower rate of calcification in this stage of formation. There is a thin, irregular membrane probably of matrix material at the junction with the cuticle. (Simons and Wiertz, 1963). The cuticle is a mucin secretion containing proteins with many disulphide and sulphydryl groups (Simkiss, 1958), perhaps in the form of keratin (Schmidt, 1961), which would contribute to its stability and insolubility.

4.3 THE VARIABILITY OF THE COMPOSITION OF THE EGG

Table[19] shows the wet and dry weights and water content of the different components of a series of 20 consecutive eggs from a mature hen, a high-intensity light hybrid (Shaver). The relative constancy of the overall composition of the egg is obvious; quantitatively, this is examined in Table[20], which shows the coefficients of variation of all components for this bird and five others of the same strain and age. From this it is seen that, apart from the shell water content, which may be expected to vary most with time of collection, atmospheric conditions, and other external factors of which no account has been taken, the most variable part is the white dry weight. Thus in this complex system it is the most complicated process, synthesis of protein by the magnum, which is most variable. The overall egg weight, the weight of the shell, of the ovulated yolk, and perhaps most surprising of all, that of the water of the white, are comparatively constant in most of these birds - rather more so than would be expected for a biological system.

It is also apparent from the table that this statement is more true for some birds than for others, that is to say, that some birds seem capable of consistency in all stages of the production process, while others show considerable variability in all. This series of eggs was collected over a comparatively short period of time. The intention was to collect twenty eggs from each bird. In most cases, the birds were laying well, and the group was complete in less than a month. In the case of bird 682, collection was continued for two months, by which time only thirteen eggs had been laid. The largest variations in overall egg weight, yolk weights, and water content of the white were found within the eggs from this bird. The shell dry weight and the white dry weight were remarkably

TABLE 19

VARIABILITY OF THE COMPONENTS OF THE EGG

Weights (g) of the components of 20 consecutive eggs from Bird 560, (Shaver), September and October 1967

No.	Date	Egg wt.	SHELL			WHITE			YOLK		
			wet	dry	water	wet	dry	water	wet	dry	water
1	16. 9	55.1	6.9	5.5	1.4	30.9	4.2	26.7	16.9	8.1	8.8
2	17. 9	59.5	6.9	5.4	1.5	33.3	4.9	28.4	18.4	8.5	9.9
3	20. 9	56.2	6.6	5.1	1.5	31.0	3.8	27.2	18.5	8.4	10.1
4	21. 9	63.9	8.2	5.1	3.1	35.9	3.4	32.5	19.7	9.2	10.5
5	22. 9	56.9	6.5	5.0	1.5	31.1	3.3	27.8	18.6	9.3	9.3
6	23. 9	56.2	7.2	5.0	2.2	30.9	3.5	27.4	18.1	8.7	9.4
7	24. 9	57.7	6.8	5.2	1.6	32.7	3.5	29.2	18.2	9.1	9.1
8	25. 9	57.2	6.5	5.0	1.5	31.8	3.9	27.9	18.5	8.8	9.7
9	28. 9	56.9	6.7	5.1	1.6	31.7	2.6	29.1	17.8	7.6	10.2
10	29. 9	56.2	7.5	4.9	2.6	30.1	3.1	27.0	17.9	9.0	8.9
11	30. 9	54.1	6.5	4.6	1.9	29.9	3.1	26.8	17.2	8.4	8.8
12	1.10	52.4	6.1	4.7	1.4	28.5	2.3	26.2	17.4	7.1	10.3
13	2.10	58.0	6.6	5.1	1.5	31.6	3.9	27.7	19.2	8.2	11.0
14	3.10	55.6	6.1	4.9	1.2	31.5	4.0	27.5	18.0	7.9	10.1
15	4.10	56.6	6.4	4.9	1.5	31.1	4.0	27.1	18.1	8.7	9.4
16	5.10	55.7	6.3	5.0	1.3	31.7	3.8	27.9	17.5	7.7	9.8
17	7.10	54.1	6.2	4.8	1.4	30.6	3.7	26.9	17.3	7.6	9.7
18	8.10	53.3	6.5	4.7	1.8	28.1	3.0	25.1	17.3	8.1	9.2
19	9.10	54.1	6.1	4.7	1.4	29.9	2.8	27.1	17.5	8.3	9.2
20	10.10	53.3	6.0	4.8	1.2	29.6	2.8	26.8	17.0	8.0	9.0
MEAN:		56.15	6.63	4.98	1.66	31.10	3.48	27.62	17.96	8.34	9.62
±S.E.		±0.57	±0.12	±0.05	±0.11	±0.38	±0.14	±0.33	±0.16	±0.13	±0.14
S.D.		2.56	0.53	0.23	0.48	1.69	0.62	1.48	0.73	0.59	0.61

constant. It has been shown that the ovary and the oviduct, although both under the control of the sex hormones, are comparatively independent in function. For example, ligation or complete removal of the oviduct, or the congenital absence of part of the oviduct, does not affect ovulation. (Pearl and Curtis, 1914; Curtis, 1915). This suggests the possibility that the main source of malfunction in this bird was in the ovary. Both the deposition of yolk material, as evidenced by the variability of yolk size, and the control of ovulation, as evidenced by the low rate of lay are affected. The protein-secreting function of the oviduct seems to have been normal, as the white dry weight varies no more than in other birds. Here, however, the wide spacing of eggs could be a factor in that the resources of the magnum and other systems were not overworked. This could also apply to shell secretion. The variability of the white water may reflect a disturbance of timing during the plumping process, or an inadequacy of the outermost layer of the thick white: see Sections 5 and 7.

TABLE 20

COEFFICIENT OF VARIATION OF THE COMPONENTS OF THE EGG

Consecutive eggs from six birds, September to November 1967

Bird	507	563	682	560	617	557
No. of eggs	19	20	13	20	20	19
Egg weight	5.74	2.20	12.23	4.56	2.98	3.46
Shell weight	7.66	4.92	2.18	7.99	5.79	6.75
Shell dry	7.08	5.73	2.80	4.62	4.69	7.43
Water	17.42	19.84	26.19	29.00	15.74	28.18
White: wet	7.47	3.08	15.50	5.43	3.05	4.23
dry	16.67	20.57	17.05	17.82	18.74	13.53
water	7.69	3.94	16.50	5.36	3.73	4.88
Yolk: wet	5.59	3.49	10.51	4.06	5.60	4.77
dry	9.55	4.63	13.55	7.08	9.73	4.69
water	6.91	6.71	10.23	6.34	10.47	7.30

4.4 THE EFFECT OF THE AGE OF THE BIRD ON EGG COMPOSITION

Weight and water content of components. As well as the fluctuations over short periods in the different components of the egg, certain changes take place over long periods. To study these, eggs were collected from a group of Brown Leghorn pullets when they first came into lay, and at three-monthly intervals thereafter, the last group being collected when the birds had been laying for a year, and were about eighteen months old. The results are shown in Tables 21 and 22, for one of these birds. These are typical of the results obtained from animals which remained healthy.

From Table 21, it may be seen that the overall weight of the eggs laid increased steadily during the year, the mean weight having increased by over 25% in the final group, compared with the first eggs. Most of this increase took place in the second three-month period, there being comparatively little increase in the first three months of lay, and even less in the last six months of the year studied. The increase is not equally spread over the different parts of the egg, however. The dry weight of the white actually declines slightly, although this is probably not significant. A small initial decline in the water content of the shell also occurs; this too is unlikely to be significant, and indeed may merely reflect atmospheric conditions and the length of storage before analysis rather than a true variation. The greatest increase is in the yolk, which is almost half as heavy again after six months, and at the end of the year has increased by nearly 60%. Two-thirds of this increase is accounted for by the dry matter, in contrast to the white, in which the weight increase is entirely due to water, and indeed to the shell, where the contributions are in the opposite ratio.

TABLE 21

WEIGHTS OF THE COMPONENTS OF EGGS FROM BIRD 5284
From January 1964 to January 1965

(Weights in g, means ± S.E.'s; Increments as % of Series I)

Series	I	II	III	V*	Increments: I-II	I-III	I-V
Date	Jan. '64	Mar. '64	June '64	Dec. '64 & Jan. '65			
n	8	8	13	9			
Egg wt.	42.32 ±0.41	45.65 ±0.40	52.68 ±1.34	53.72 ±0.62	7.9	24.5	26.9
WHITE:							
Wet weight	23.69 ±0.33	25.38 ±0.32	27.86 ±1.01	27.51 ±0.45	7.1	17.6	17.1
Dry weight	3.28 ±0.08	3.18 ±0.02	3.23 ±0.18	3.18 ±0.15	-3.0	- 1.5	-4.0
Water	20.42 ±0.29	22.19 ±0.30	24.62 ±0.86	24.34 ±0.35	8.7	20.6	20.1
YOLK:							
Wet weight	11.51 ±0.24	13.68 ±0.20	16.97 ±0.28	18.37 ±0.25	18.9	47.4	59.5
Dry weight	5.87 ±0.15	7.21 ±0.14	9.38 ±0.31	10.79 ±0.39	22.8	59.8	89.6
Water	5.63 ±±0.28	6.46 ±0.11	7.59 ±0.27	7.59 ±0.46	14.7	34.8	29.3
SHELL:							
Wet weight	6.39 ±0.14	6.24 ±0.10	7.35 ±0.23	7.44 ±0.11	-2.3	15.0	17.7
Dry weight	4.57 ±0.05	4.64 ±0.08	4.93 ±0.12	4.99 ±0.07	1.5	7.9	11.8
Water	1.82 ±0.12	1.60 ±0.05	2.42 ±0.14	2.44 ±0.11	-12.1	33.0	32.4

(* Series IV, September 1964, was used for specific gravity measurements, and no weights are available)

From these results, some deductions may be made about the efficiency of the different mechanisms involved in egg formation. Firstly, it is clear that the ovary, initially capable of producing only small yolks, almost half of which are composed of water, improves steadily and dramatically throughout the year, producing yolks of greater size and with a higher proportion of solids in each series. The protein-secreting capacity of the magnum, on the other hand, is close to its maximum level immediately egg production begins, and indeed in some birds it may decrease slightly as the bird grows older. The small decreases found may not be significant because as mentioned previously the dry matter of the white has the greatest coefficient of variation. From Table 20, it can be seen that the coefficient of variation is several times greater in the twenty-egg groups than the decrease of the smaller groups of this experiment. The increase in addition of water to the white is significant; this could be partly accounted for by the increased surface area presented by the larger yolk surrounded by thick white. This will be discussed in relation to the phenomenon of plumping, see Section 5.4.

The increase in the shell dry weight may not necessarily be indicative of an increase in thickness. Using the formula:

$$\text{Surface Area} = 4.722 \log (W^{2/3}); \quad \text{(see Section 5.3)}$$

an increase in surface area between the first series of eggs, wet contents weight = 35.20 g. and series V, wet contents weight = 46.10 g., of $\frac{60.677 - 50.714}{50.714} = 19.6\%$ would be expected; the corresponding increase of shell wet weight is 17.7%, and of shell dry weight 11.8%, so that in fact the shell thickness would on this basis have decreased, although a greater amount of calcium was being transported and deposited on the shell. The actual decrease would

TABLE 22

Mineral content and concentrations in egg white from Bird 5284, from January 1964 to January 1965

(Minerals expressed as mg., micromoles, and m-moles/l. of water Means ± S.E.'s)

Element	SERIES			
	I	II	III	V
No. of samples	8	8	13	9
Na (mg)	18.01 ±0.26	23.61 ±2.44	29.24 ±1.94	38.29 ±1.72
K	14.46 ±1.93	20.60 ±2.63	25.09 ±2.19	29.93 ±1.33
Ca	0.54 ±0.20	3.12 ±1.05	7.28 ±1.26	3.80 ±0.69
Mg	1.42 ±0.15	1.85 ±0.14	1.99 ±0.13	2.20 ±0.07
Na (micromoles)	782.9 ±100.3	1026.7 ±105.9	1271.3 ±84.3	1664.6 ±74.6
K	369.9 ±50.5	526.7 ±67.3	641.6 ±56.1	765.3 ±34.1
Ca	13.4 ±5.0	77.7 ±26.3	181.7 ±31.5	94.8 ±17.2
Mg	58.4 ±6.2	76.0 ±5.6	81.8 ±5.3	90.5 ±2.7
Na (m-moles/litre)	38.4 ±5.1	46.3 ±4.9	52.6 ±4.1	68.7 ±3.8
K	18.2 ±2.5	23.8 ±3.1	26.5 ±2.6	31.5 ±1.4
Ca	0.66 ±0.25	3.5 ±1.2	7.6 ±1.4	3.9 ±0.7
Mg	2.87 ±0.32	3.4 ±0.3	3.3 ±0.2	3.7 ±0.1
Shell Ca (m-moles)	35.59 ±2.83	34.48 ±2.35	40.23 ±3.60	40.51 ±1.80

be of the order of $\frac{6.39}{50.714} - \frac{7.52}{60.677}$ g./sq. cm., that is from a density of deposition of 0.1260 g./sq.cm. to one of 0.1239 g./sq.cm. If the density of eggshell is taken to be 2.3, which would seem reasonable on the basis of the range of values quoted by Romanoff and Romanoff (1949) from the findings of several investigators, this corresponds to a decrease in thickness of approximately 9 microns, from 0.548 mm. to 0.539 mm. This decrease of about 2% is unlikely to make any practical difference, although the occurence of noticeably weak shells within the scatter of values to be expected would undoubtedly increase. The general tendency of the hen as it grows older to lay larger eggs with weaker shells, and to lay less of them, is of course well known; the fact that this increase is almost entirely due to an increase of yolk size, with a secondary and perhaps related effect of white water content, does not seem to have been commented upon previously.

Mineral content of White. The increased water content of the white is probably essential for the developing embryo; it is not improbable that an increase in white mineral content is equally necessary. The mineral content and concentrations of the same four series of eggs are shown in Table 22. The increases found in the quantities of sodium and potassium present are even greater than that of water. Both of these minerals double in the year. The sodium content increases by an approximately equal amount in each three months, while the greater part of the potassium content increase takes place in the first half of the year. Since the total volume of water in the albumen increases also, the behaviour of the concentration, expressed as millimoles per litre of water, is slightly different. However, a marked increase occurs in these also. Again, the sodium concentration continues to rise throughout the

TABLE 23

Mineral content of egg whites from Bird 5288 from January 1964 to June 1964*

(Weight in g. minerals in micromoles)

Series	Egg wt.	Shell dry wt.	White dry wt.	White water	Na	K	Ca	Mg
I	40.58	4.00	3.03	20.69	918.8	385.1	13.5	65.7
II	50.58	4.58	3.34	25.07	1189.3	548.9	114.7	75.5
III -1	64.21	5.90	5.78	30.94	246.5	283.5	492.0	58.0
2	43.44	3.81	2.24	19.74	1113.9	786.2	346.6	69.9
3	53.67	4.89	3.28	25.25	1176.1	661.6	368.5	81.8
4	45.28	4.20	2.30	20.24	164.3	184.0	343.3	38.2
5	48.64	4.25	2.50	22.00	282.6	454.3	246.8	88.4
6	45.08	3.67	2.41	20.41	1359.8	887.7	43.9	51.4
7	45.45	3.88	2.36	21.32	1116.1	639.4	78.1	92.1
8	44.50	4.08	2.32	20.44	783.9	466.6	113.5	55.9
9	44.59	3.06	2.34	20.81	1487.4	745.8	229.0	110.2
Mean (III)	48.32	4.19	2.84	20.81	859.0	567.7	251.3	71.8

* Died three days after ninth egg in series III

year; the potassium concentration, however, does not show any further increase after the first six months.

It has been shown by Draper (1966) that the newly hatched Brown Leghorn chick contains some 980 micromoles of potassium, and 1620 micromoles of sodium. On this basis, even the eggs of series V would be unlikely to be able to supply the minerals necessary for the development of the viable chick. Quantities in eggs from other breeds, and from breeding stock, are usually higher. The concentrations of the minerals found in the white by other workers are not however greatly dissimilar. Recalculation of the values quoted by Shenstone (1968) on the basis of 88.5% white water gives the sodium concentration as 56.6 m-moles/litre, and that of potassium as 31.7 m-moles/litre; these fall between the mean values of series III and series V for bird 5284.

The idea that the quantities of sodium and potassium which are desirable in the white are rather difficult for the hen, at least the P.R.C. Brown Leghorn, to produce, is borne out by the data of Table 23. This shows the weights and analyses for a bird of the same group as 5284, over the first three series. A few days after laying the ninth egg of the third series, the bird died. The reproductive tract was apparently normal. During the first two series, mean sodium and potassium levels were if anything slightly better than those of 5284. In the final series, wide oscillations occur in the values, particularly in the sodium content, and the overall mean of this drops below those of both the previous series and of 5284. Egg weight, shell dry weight and white protein also drop appreciably.

There is a comparatively high variation in the calcium content of the white, which may be a result of the contamination of some

samples with fragments of shell during the breaking out of the eggs. It may, on the other hand, be inherent; there is an appreciable quantity of calcium in the white of eggs taken from the magnum (see Section 4.5), where there is no such question of contamination. The increase in content and concentration of calcium is in any case significant, although at no time is there more than a fraction of the quantity present in the newly hatched chick. Most of the embryo's calcium requirements are supplied not by the white but by the yolk together with the mammillary layer of the shell, which is partially dissolved during incubation.

Even if all the calcium in the white were derived from chips of shell, there is still too little magnesium associated with this shell calcium to alter significantly the quantity of magnesium present. (The mean molecular ratio of calcium to magnesium in the shell is about 50:1, although variation occurs throughout the depth of the shell: see Section 4.7.) The magnesium content also increases as the bird develops, but not as markedly as the calcium. The magnesium present in the egg whites of series V, in combination with the 50 micromoles to be expected in the yolk, is only marginally adequate for embryonic development; this mineral too is available from the shell.

The concentration values for both divalent cations obtained in this study are comparable to those quoted by Shenstone (1968), which after recalculation are 3.6 (Mg) and 2.9 (Ca) m-moles/litre.

4.5 CHANGES IN THE MINERAL COMPOSITION OF THE EGG DURING FORMATION IN THE OVIDUCT

During the course of the present study, more than fifty eggs in different stages of formation have been obtained and analysed. The results of these analyses are shown in Table 24. These oviducal eggs are from several breeds and ages of birds, and for purposes of comparison, the composition of 26 oviposited eggs collected from a similarly mixed group of birds is included. Unfortunately, the equivalence of this group, which may be considered as a control, to all of the groups into which the oviducal eggs are divided, is not equally good, as variations between the groups are considerable. This is evidenced by the differences in mean yolk weight, and to some extent also the differences in white dry weight.

The division of the eggs into six groups was made on the basis of the presence or absence of a membrane and the quantity of calcium in the membrane. This gives a reasonable index of the stage of formation of the egg. While the quantity of calcium present will reflect the overall surface area of the individual egg in addition to the thickness of deposition, and while the rate of deposition follows a sigmoid curve, which can be considered to be essentially linear for most but not all of the time which the egg spends in the shellgland (Burmester, Scott and Card, 1939; Bradfield, 1951), it seemed to form a reasonable basis for making an initial classification. The groups chosen were as follows: first, those eggs which were found in the magnum region, and which had no membrane present (i.e. no membrane calcium): and groups (2) to (5), in which the ranges of membrane calcium were respectively: (2) 0 - 200 micromoles, (3) 200 - 2,500 micromoles, (4) 2,500 - 10,000 micromoles, and (5) 10,000 micromoles upwards, with oviposited eggs forming the

TABLE 24

COMPOSITION OF THE EGG AT DIFFERENT STAGES DURING FORMATION

(Weights in g, Membrane Ca in m-moles, white minerals in micromoles and m-moles/l. of water. Means ± S.E.'s)

	(1) (No membranes)	(2)	(3)	(4)	(5)	(6) (Ovi-posited eggs)
			(Membranes present)			
No. of samples	7	9	17	6	13	26
Egg weight	29.2 ±2.16 (n = 6)	32.73 ±1.83	39.46 ±1.50	58.26 ±2.42	54.01 ±1.37	57.53 ±1.58
Yolk weight	17.5 ±0.78	16.5 ±1.20	17.12 ±0.58	20.23 ±0.88	17.22 ±0.58	18.58 ±0.58
White dry wt.	2.06 ±0.22	2.68 ±0.27	3.21 ±0.16	4.34 ±0.14	3.37 ±0.14	3.45 ±0.16
White water	8.62 ±1.28	11.21 ±1.00	16.70 ±1.22	27.90 ±0.79	25.76 ±0.88	27.74 ±1.15
Membrane dry wt. (including calcification)	-	0.34 ±0.09	0.36 ±0.04	0.87 ±0.08	4.77 ±0.24	5.13 ±0.14
Membrane Ca	-	88. ±16	1230 ±133	5522 ±744	41613 ±2403	-
micromoles						
Na	1080 ±185	1398 ±136	2301 ±257	3778 ±384	2078 ±250	2599 ±120
K	85 ±15	153 ±28	377 ±82	561 ±150	1094 ±166	1415 ±50
Ca	71 ±19	133 ±18	140 ±12.5	168 ±8.9	142 ±15.1	95 ±13.8
Mg	107 ±13	143 ±11	157 ±16	198 ±23.7	129 ±13.9	202 ±9.3
m-moles/l	(n = 8)					
Na	127 ±14.5	127 ±10.0	134 ±8.5	134 ±11.8	78 ±8.0	93 ±1.5
K	10 ±1.2	14.8 ±4.0	20.5 ±3.6	20.0 ±5.7	40.7 ±5.2	52.2 ±1.8
Ca	8 ±3.3	12.0 ±1.6	8.6 ±0.72	6.0 ±0.3	5.5 ±0.6	3.4 ±0.4
Mg	13 ±1.2	13.2 ±0.9	9.5 ±0.9	7.1 ±0.7	4.9 ±0.4	7.5 ±0.3

final group (6). In terms of appearance, group (2) eggs are soft and unplumped, with little or no visual evidence of calcification, although microscopically crystals are visible around the mammillary cores. They are probably isthmal eggs rather than being found in the shellgland proper. Group (3) eggs have an average of 1.23mmoles of calcium on the membrane. This is approximately 50 mg., which would be visible as a light powdering of crystals over the whole surface of the membrane. In the eggs of group (4), the adjacent crystalline columns of the shell are either just fused or just about to fuse to form a rigid but very fragile shell; the egg is fully plumped. Group (5) includes all stages and thicknesses of hard shelled eggs, many of which would in fact be indistinguishable from oviposited eggs except perhaps by microscopical examination of the shell cross-section, and some of which were probably within an hour or less of being laid at the time of their removal from the oviduct.

In a genetically homogenous group of birds of the same age, the mean weight of the yolk would not be expected to vary greatly, as may be seen from Table 20. The variations in yolk weight between the groups of oviducal eggs may be taken as an indication of the overall variation which may be expected; that highly significant changes may nonetheless be demonstrated in the other components is indicative of the large scale of these changes, and of their independence of the effects of breed or age.

The major components of the white, that is the dry matter, mainly protein, and the water, are augmented between the first two stages, as would be expected as the yolk collects more albumen in its passage to the shellgland. There is a suggestion of a further upward trend in the dry weights during the period in the shellgland, which is more surprising. It may simply be due to

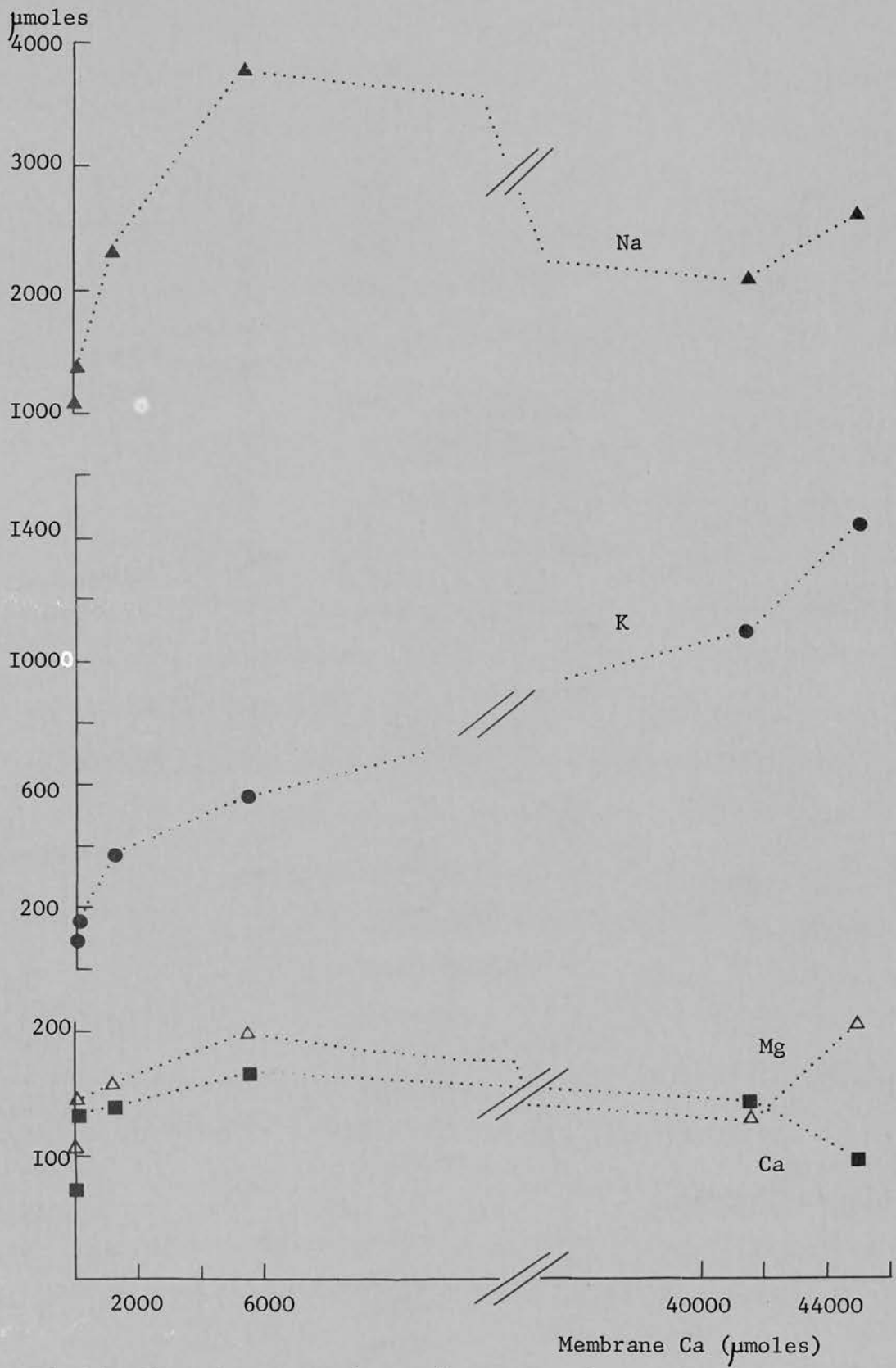


Fig.20. Mineral content (μmoles) of egg white during formation in the oviduct; the oviducal eggs are divided into 6 groups on the basis of quantity of calcium on the membrane, representing different stages in formation of the egg. (▲), Na; (●), K; (△), Mg; (■), Ca.

the variations in egg size, and indeed the increases seem to be related to increases in yolk weight; a small increase in albumen nitrogen in the shellgland has been suggested by some workers, (McNally, 1934; Hughes and Scott, 1936; Scott, Hughes and Warren, 1937; Beadle, Conrad and Scott, 1938). This has been denied by others (Conrad and Phillips, 1938). The results of McNally indicate a possible increase of 10% in the nitrogen content; this has been criticised on the grounds that such a difference could easily arise, and might in fact be expected, between the first egg of a clutch and the second, whether this was examined during formation or when complete. (Burmester, 1940), and Beadle, Conrad and Scott (1938) found a nitrogen concentration of only 0.06% in their analyses of shellgland secretion.

The white water content continues to increase rapidly as the egg plumps, but becomes effectively constant as soon as shell calcification has reached the stage at which the shell becomes rigid; until this point has been reached, calcification and the addition of water to the white proceed simultaneously. The membrane dry weight reflects the degree of calcification, and is included in order to express this in readily visualisable terms.

The mineral contents and concentrations at the different stages of Table 24 are displayed graphically in figures 20 and 21 respectively. The water content is included in Figure 21, as the inorganic ions are usually considered to be added to the white during plumping as an aqueous solution. (Hansen, 1933, Beadle, Conrad and Scott, 1938; Burmester, 1940; Hoover and Smith, 1958.)

In the plumping stage (up to and including group (4) of Table 24), it does indeed seem that all four of the ions determined are added to the white with the water, although the relationship is not linear, as may be seen from the concentrations graph. In parti-

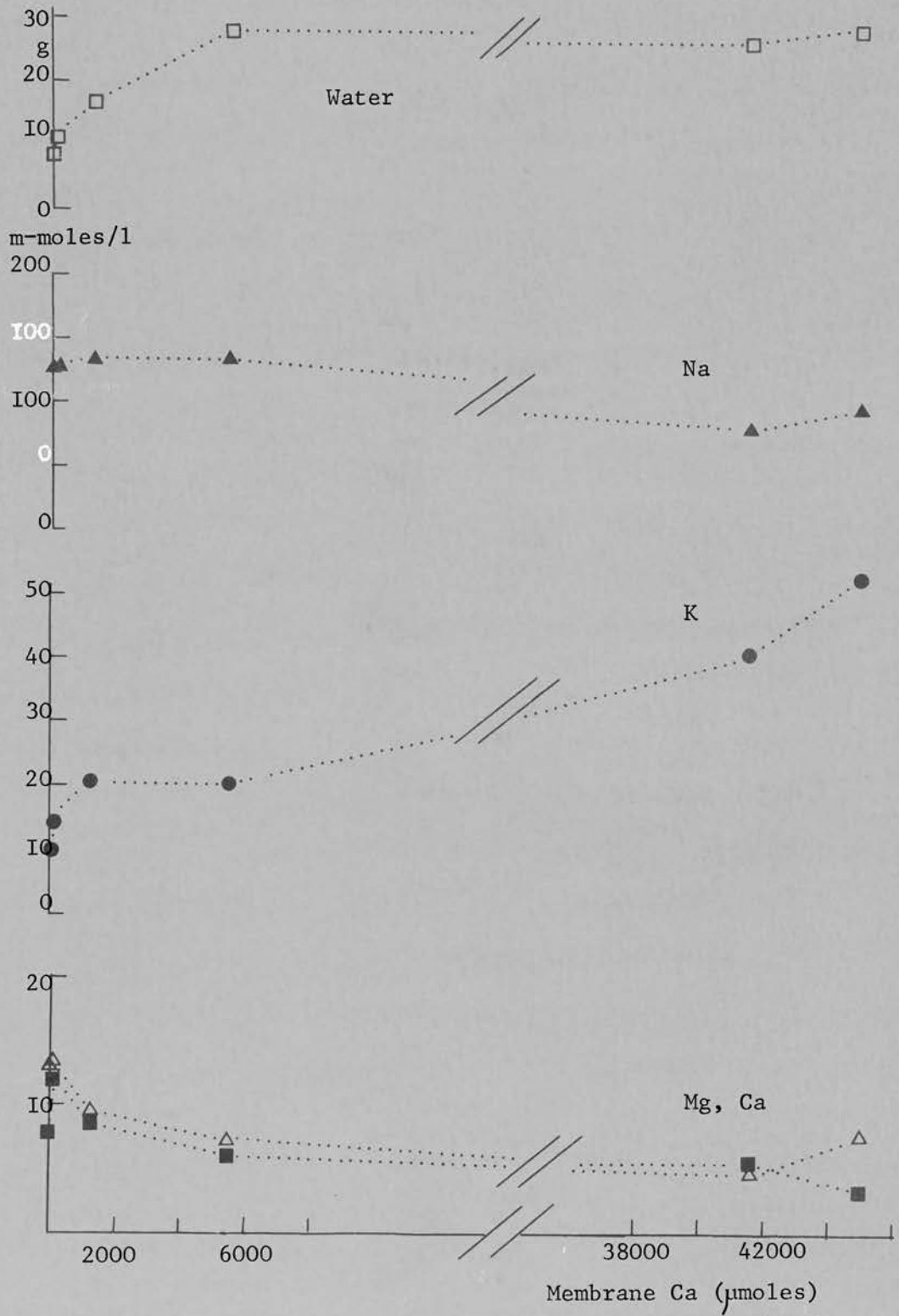


Fig.2I. Water content and mineral concentrations (m-moles/1) in egg white during formation. The eggs are again divided into 6 groups on the basis of the calcium on the membranes, the final group represent-ing oviposited eggs. (□),water; (▲), Na; (●), K; (△),Mg; (■),Ca.

cular, there is a noticeable influx of sodium and potassium, while the calcium and magnesium levels rise only slightly, so that their concentrations decrease. After the rigidity of the shell prevents the addition of more water, the concentrations of these two minerals remain fairly constant. The sodium concentration of the white, however, having reached a peak at the stage represented by group (4), decreases considerably during the rest of the period spent in the oviduct. The potassium content and concentration, on the other hand, continue to increase. Changes in the concentration of both ions taken together are comparatively small, and the plot of sodium plus potassium compared with that of the water content of the groups, (Figure 22), shows that the two curves are strikingly parallel. This suggests the possibility that an exchange mechanism may operate across the oviduct epithelium during this phase of egg formation. Regression of the mean values of (Na + K) for the six stages represented in the table gives a correlation of $r = 0.97$, which corresponds to a probability of $P < 0.001$, and a regression equation of:

$$y = -49.15 + 145.526\,x$$

where y is the sum of the quantities of sodium and potassium in micromoles, and x is the water of the white in grams.

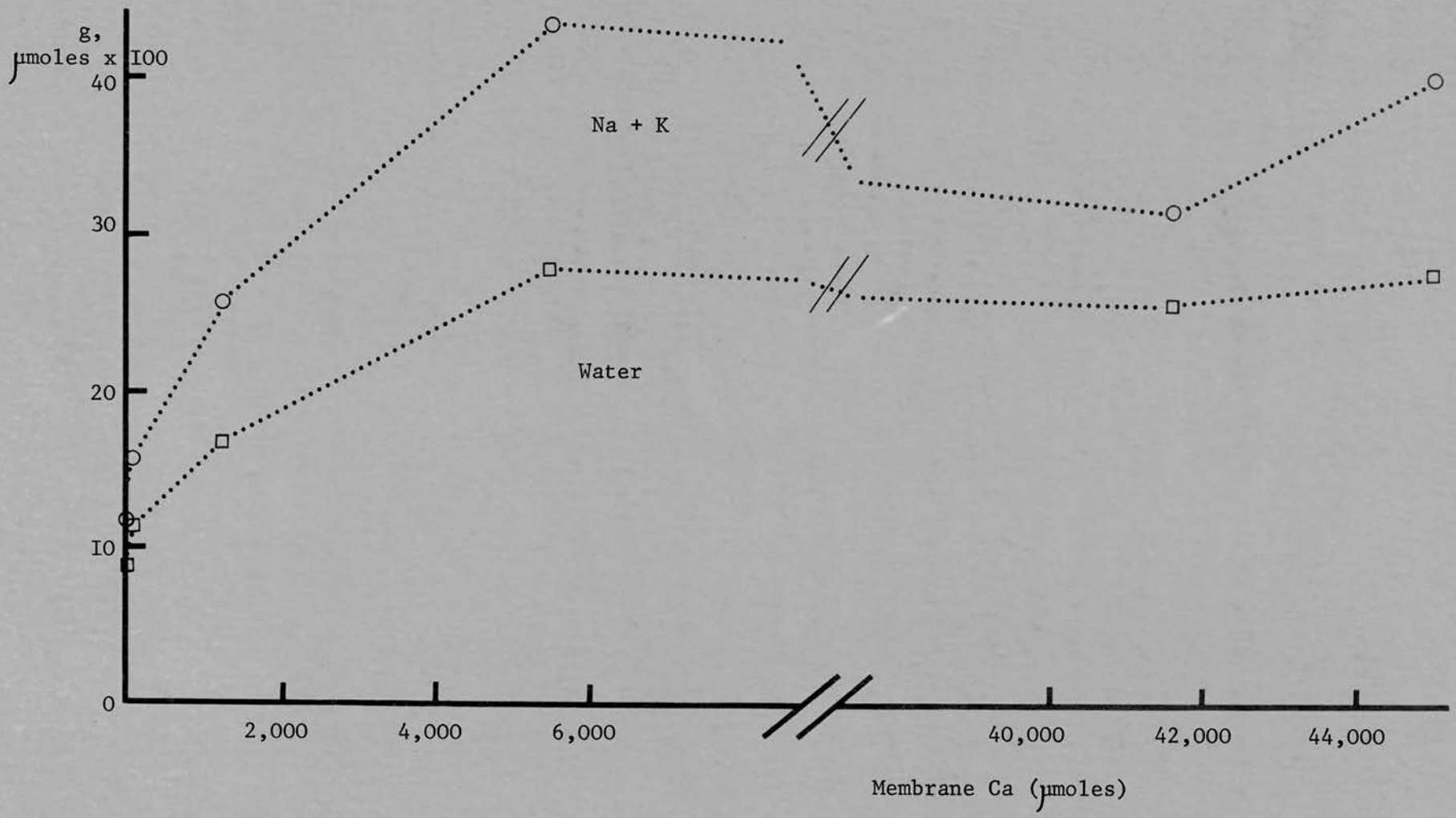


Fig.22. Water content (g) and sum of sodium and potassium contents (μmoles) of oviducal egg whites.

4.6 THE ENVIRONMENT OF THE FORMING EGG: COMPOSITION OF SHELLGLAND FLUID

The egg in the shellgland during formation is surrounded by a fluid secretion. It is from this fluid that the water, minerals, and protein (if any) must enter the egg. (Burmester, 1940). Attempts to determine the composition of this fluid produced rather varying results. (Beadle, Conrad and Scott, 1938; Hoover and Smith, 1958). In spite of the assertion of Hoover and Smith that the fluid is of the approximate composition of a plasma filtrate, their figures show a sodium to potassium ratio of about 1.6, whereas that of plasma is 27.3. Their statement that the fluid is not of the same composition as that of the addition to the forming white is supported by the finding that the minerals, and the water, are not added in a constant ratio. These contradictions are to some extent resolved by the finding of El Jack and Lake (1967), that the composition of the fluid varies greatly at different times of the egg-laying cycle; in the stage shortly after the egg has entered the shellgland, the sodium concentration was little lower than that of plasma, and the potassium about two-and-a-half times higher; within two hours of oviposition, the sodium had dropped and the potassium risen, together with the calcium and magnesium concentrations.

Thirteen of the oviducal eggs analysed were removed from the shellgland immediately before the fluid was collected by the method of El Jack and Lake. The analyses and osmotic pressures of these fluid samples, together with the corresponding oviducal eggs, are presented in Tables 25, and 26. Table 27 shows a corresponding analysis for an oviposited egg for each case. Each of these represents the mean of at least two eggs collected during the few weeks immediately preceding the operation, unusually small or large eggs and double-yolked eggs being excluded. Where analyses of

eggs from the individual bird were unavailable, those of eggs from a bird of the same breed and as nearly as possible the same age were substituted in order to maintain equivalence.

The results fall into two groups, approximating to the two stages of El Jack and Lake. The larger, comprising ten fluids and thirteen eggs, (it was not always possible to collect sufficient fluid for analyses), represent the early stages of calcification, with membrane weights of less than 1.2 g.; the smaller, of three fluids and corresponding eggs, represents the stage shortly before oviposition, with shell weights of greater than 4.4 g. Application of the Student's t-test shows the fluids from the two groups to be very significantly different in every respect measured save that of osmolarity. The most marked difference is the lowering of the sodium concentration from a mean of 138 mM. to a mean of 41 mM. with a corresponding rise in potassium level from 20 to 48 mM., in calcium from 3 to 20 mM., and in magnesium from 0.3 to 6.1 mM. The total cations have dropped from 161.4 to 115.3mM., but the osmolarity as measured by depression of the freezing point has not changed significantly, although most of the calcium in the late-period fluid is present in a suspended or precipitated form, which soon settles out unless the fluid is acidified with HCl. It thus seems likely that some of the cations are present in complex or undissolved form, and that the osmolarity is maintained at a value slightly below the mean value of the egg white.

Comparison of the concentrations of individual minerals in the egg-white and the corresponding shell-gland fluid shows that the sodium is higher in the fluid in a few of the early-stage cases, but very much lower in the three late-stage specimens; on average, the potassium is slightly lower in the fluid initially, and becomes slightly higher, although this stage of affairs is reversed in some

TABLE 25

IONIC CONCENTRATIONS AND OSMOTIC PRESSURE OF SHELL GLAND FLUID

(Age in months, membrane weight in g, minerals in m-moles/l, osmotic pressure in m-osmoles)

	Bird	Breed & age	Membrane dry wt.	Fluid: Na	K	Ca	Mg	Osmotic pressure
EARLY STAGE	1556	J 20	0.23	138	19	0.50	-	313.5
	912	Sh 20	-	-	-	-	-	-
	41	St 18	-	-	-	-	-	-
	P44	St 18	1.19	118	21	0.75	-	285
	P53	St 18	0.71	114	19	1.00		298
	P2	St 18	0.18	81	22	6.00	-	303.5
	1555	J 19	0.29	94	15	2.00	-	305
	P26	St 18	0.76	156	24	1.5	-	299
	7737	J 6	0.34	192	24	1.7	-	281.5
	P33	St 18	0.81	192	19	1.5	-	270
	1385	J 19	0.54	199	23	1.5	-	294
	B2269	J 20	1.02	97	17	13.0	0.3	275
	5352	J 13	0.33	-	-	-	-	-
	Mean			138	20	2.9	(0.3)	292.5
	±S.E.			±14	±1	±1.2		±4.5
LATE STAGE	7724	B 9	4.46	42	44	19.8	6.8	273
	1546	J 10	4.82	36	57	19.8	7.5	282
	621	Sh 16	5.43	45	42	21.2	3.9	298
	Mean			41	48	20.3	6.1	284
	±S.E.			±3	±5	±0.5	±1.1	±7.3
	Comparing early and late stages, P (t - test) less than			0.01	0.001	0.001	0.001	N.S.

Breeds: J, Brown Leghorn line J;
B, Brown Leghorn line B (see Blyth and Sang, 1960);
Sh, Shaver;
St, Sterling.

TABLE 26

COMPOSITION OF EGG WHITE OF OVIDUCAL EGGS COLLECTED WITH SHELL GLAND FLUID SAMPLES

(Age in months, weights in g, minerals as m-moles/l. of water and osmotic pressure as milliosmoles)

Bird	Breed & age	Membrane dry wt.	White: dry wt.	Water	Ionic concentrations				Osmotic pressure
					Na	K	Ca	Mg	
1556	J 20	0.23	3.23	14.97	155	26	15.03	17.57	-
912	Sh 20	-	3.26	10.68	191	44	8.38	25.00	-
41	St 18	0.50	2.53	7.17	176	45	20.50	18.41	-
P44	St 18	1.19	4.53	29.41	148	14	5.05	5.75	276
P53	St 18	0.71	3.88	25.63	152	47	6.91	6.91	268
P2	St 18	0.18	2.65	13.28	166	18	13.33	15.81	260
1555	J 19	0.29	3.06	15.60	161	28	12.24	13.46	264
P26	St 18	0.76	4.61	29.00	157	17	6.84	9.21	246
7737	J 6	0.34	4.05	20.45	147	17	7.92	12.66	250.5
P33	St 18	0.81	4.63	30.34	137	18	5.67	8.73	250.5
1385	J 19	0.54	3.90	23.16	160	15	9.50	11.36	286.5
B2269	J 20	1.02	3.92	26.32	134	18	6.53	7.22	-
5352	J 13	0.33	2.88	17.60	166	20	9.77	9.09	-
n		12	13	13	13	13	13	13	8
Mean		0.575	3.63	20.28	158	25	9.82	12.40	262.7
±S.E.		±0.094	±0.20	±2.12	±4.3	±3.4	±1.22	±1.56	±4.9
7724	B 9	4.46	3.49	26.00	97	36	6.00	6.31	-
1546	J 10	4.82	3.18	25.42	99	43	6.73	6.06	-
621	Sh 16	5.43	3.44	28.15	83	55	6.00	5.18	-
n = 3									
Mean		4.90	3.37	26.52	93	44	6.24	5.85	-
±S.E.		±0.28	±0.10	±0.83	±5	±5.4	±0.31	±0.43	

TABLE 27

COMPOSITION OF EGG WHITE OF OVIPOSITED EGGS, EQUIVALENT TO OVIDUCAL EGGS OF TABLE 26

(Age in months, weights in g, minerals as m-moles/l. of water. Numbers in brackets refer to the equivalent bird in Tables 25 and 26; where none is given the bird is the same.

Bird	Breed & Age	Shell dry.wt.	White dry wt.	Water	Ionic Concentrations			
					Na	K	Ca	Mg
2269	J 20	4.72	3.96	32.32	88	43	6.25	9.03
(1556)		4.81	3.05	24.52	96	67	8.91	9.71
992	Sh 19	5.94	4.20	28.99	96	54	8.67	9.57
(912)	(20)	5,90	3.95	28.57	93	51	1.92	9.20
P12	St 18	6.31	4.28	37.19	94	45	4.40	6.13
(41)		6.38	4.65	40.15	104	48	2.49	6.13
P12	St 18	6.31	4.28	37.19	94	45	4.40	6.13
(P44)		6.38	4.65	40.15	104	48	2.49	6.13
P12	St 18	6.31	4.28	37.19	94	45	4.40	6.13
(P53)		6.38	4.65	40.15	104	48	2.49	6.13
P12	St 18	6.31	4.28	37.19	94	45	4.40	6.13
(P2)		6.38	4.65	40.15	104	48	2.49	6.13
4425	J 19	5.19	3.53	30.15	91	52	1.06	6.90
(1555)		4.74	3.47	30.00	96	50	1.83	7.00
P12	St 18	6.31	4.28	37.19	94	45	4.40	6.13
(P26)		6.38	4.65	40.15	104	48	2.49	6.13
5352	J 13	4.67	3.40	23.61	97	50	1.74	7.83
(7737)	(6)	4.70	3.38	24.20	98	48	1.49	7.40
P12	St 18	6.31	4.28	37.19	94	45	4.40	6.13
(P33)		6.38	4.65	40.15	104	48	2.49	6.13
1385	J 19	5.19	3.53	30.15	91	52	1.06	6.90
		4.74	3.47	30.00	96	50	1.83	7.00
B2269	J 20	4.72	3.96	32.32	88	43	6.25	9.03
		4.81	3.05	24.52	96	67	5.91	9.71
5352	J 13	4.67	3.40	23.61	97	50	1.74	7.83
		4.70	3.38	24.20	98	48	1.49	7.40
7724	B 9	5.24	3.47	24.17	107	49	1.63	7.16
		5.20	3.61	26.43	105	44	1.49	7.08
5352	J 13	4.67	3.40	23.61	97	50	1.74	7.83
(1546)	(10)	4.70	3.38	24.20	98	48	1.49	7.40
621	Sh 16	5.29	3.88	32.00	80	52	6.37	5.34
		5.30	3.68	30.33	85	50	2.97	5.21
n = 32 Mean		5.50	3.90	31.62	96	49	3.26	7.13
±S.E.		±0.13	±0.09	±1.10	-+1.1	±1.0	±1.10	±0.23

of the individual samples. Those cases in which the fluid sodium is higher than or very close to that of the white (P26,7737, P33, 1385), all have a high white water, almost as high as that of the oviposited eggs, but a low membrane weight. They thus correspond approximately to Group (4) of Table 24, where the sodium concentration was maximal. Again, the values for albumen calcium are difficult to interpret as there is a possibility of contamination with shell fragments, or, in the case of the membraneous eggs, with loose crystals of calcite; it was not possible to analyse all fluid samples for magnesium, owing to the small volumes obtained. Those determinations which could be made suggest that the level in the fluid is never very great, but increases in the later stages to a concentration comparable to that of the white, possibly as a result of diffusion through the membrane.

The pattern of higher potassium concentration and lower sodium and magnesium concentrations, in the mature egg in comparison with the plumping stage egg, which was seen in the figures of Table 24, is confirmed in the individual birds of this group.

Three samples of shellgland fluid, collected without accompanying eggs and without regard to the stage of egg formation, were used for determination of nitrogen content by micro-Kjeldahl analysis. In all cases, the results obtained corresponded to a protein content of less than 1%. This confirms the findings of Beadle, Conrad and Scott (1938), of 0.06% nitrogen (0.375% protein).

TABLE 28

COMPARISON OF CONCENTRATIONS OF MINERALS IN SHELLGLAND FLUID AND IN EGG WHITES, BY APPLICATION OF STUDENT'S t-TEST

(P is the probability that the compared groups are essentially the same)

Comparing:	Mineral	t	Degrees of freedom	P
Early stage fluid with early stage eggwhite	Na	1.51	21	> 0.1
	K	1.18	21	> 0.1
	Ca	3.90	21	< 0.001
	Mg	5.49	2	$0.02 < P < 0.05$
	O.P.	4.48	16	< 0.001
Late stage fluid with late stage eggwhite	Na	9.40	4	< 0.001
	K	0.45	4	> 0.1
	Ca	26.65	4	< 0.001
	Mg	0.19	4	> 0.1
Early stage egg-white with eggwhite from oviposited eggs	Na	19.35	43	< 0.001
	K	9.29	43	< 0.001
	Ca	6.91	43	< 0.001
	Mg	5.07	43	< 0.001
Late stage egg-white with eggwhite from oviposited eggs	Na	0.81	33	> 0.1
	K	1.40	33	> 0.1
	Ca	2.59	33	$0.01 < P < 0.02$
	Mg	1.68	33	> 0.1
Early stage egg-white with late stage eggwhite	Na	6.93	14	< 0.001
	K	2.56	14	$0.02 < P < 0.05$
	Ca	1.37	14	> 0.1
	Mg	1.98	14	$0.05 < P < 0.1$

TABLE 29

Protein content of shell gland fluid

(Nitrogen expressed as micro g/ml. of fluid, protein (= N x 6.25) as mg/ml. and g/100 ml.)

Sample	Bird	Nitrogen	Protein (mg/ml)	Protein (g/100 ml.)
1	4217	350	2.19	0.22
2	4196	515	3.22	0.32
3	3824	226	1.41	0.14
Mean		364	2.27	0.23

4.7 THE RATIO OF CALCIUM TO MAGNESIUM DURING SHELL FORMATION

The relationship between magnesium content and shell strength was investigated by Brooks and Hale (1955), when they had found a significant difference in the quantities of this ion between groups of strong and weak shells. In the course of this work, they dissolved the shell in layers with a mixture of perchloric acid and alcohol, and reported a gradient of magnesium concentration, the level being highest in the outermost layer. During the present analyses of oviducal eggs, it appeared that the opposite was the case, as the very early membranes, containing little calcium, had a disproportionately high magnesium content. Measurements on a total of 45 partly-formed shells were obtained; again, total calcium was taken as the measure of maturity. The results are shown in Table 30, and displayed graphically in Figure 24. The results of Brooks and Hale, recalculated in terms of molar ratios, are also given. (Table 31). As they were quoted in terms of shell thickness, a direct comparison is not possible. An approximate comparison may be made by replacing the thickness fraction by a calcium fraction, assuming the calcium of a complete shell to be about 50 m-moles. The points obtained by this method are also shown on the graph.

The overall Ca/Mg ratio in m-moles for the shells of Brooks and Hale would be about 64; this is similar to the values found for nearly complete and complete shells in this study. Although there is a lack of measurements for the range from 10,000 to 40,000 micromoles of calcium, values for shells with more than 38,000 micromoles do show a tendency to decrease, supporting the proposal that magnesium is more abundant in the outermost layer. In the inner layer of the shell, however, there is a much greater concentration of magnesium than would be expected; according to Brooks and

TABLE 30

RATIO OF CALCIUM TO MAGNESIUM IN THE SHELL DURING FORMATION

(Calcium and magnesium expressed as m-moles)

Egg	Ca	Ca/Mg	Egg	Ca	Ca/Mg
39	0.012	1.46	P26	4.117	28
41	0.022	1.4	10	4.366	48.24
1	0.065	6.57	P33	5.364	36
3	0.082	7.96	2269	6.861	47.65
P2	0.087	4.2	P44	8.508	57
11	0.097	2.36	24	21.507	50.30
38	0.137	8.35	5	34.306	54.89
40	0.140	8.54	672	39.421	75.21
7	0.147	13.74	1546	40.544	48.67
9	0.362	22.07	541	41.167	49.75
4921	0.499	6.93	834	41.168	61.61
1556	0.524	20	7724	41.667	66.99
558	0.749	9.10	27	44.286	62.61
17	0.848	22.92	621	49.151	53.72
1555	0.923	25	745	50.150	53.61
8	0.998	22.08	769	52.146	54.85
25	1.015	9.49	26	52.395	55.89
18	1.330	64.56			
2	1.342	25.71	Laid eggs:		
7737	1.397	22	621	47.156	55.94
21	1.435	18.86	621	45.658	56.93
19	1.527	61.82	5288		
			Series I	28.69	73.75
22	1.647	19.06	Series II	38.192	78.63
832	2.046	30.63	Series III	39.92	63.47
5352	2.083	25.40	5049	46.16	51.23
1385	2.121	24			
4	3.044	48.70			
P53	3.917	40			

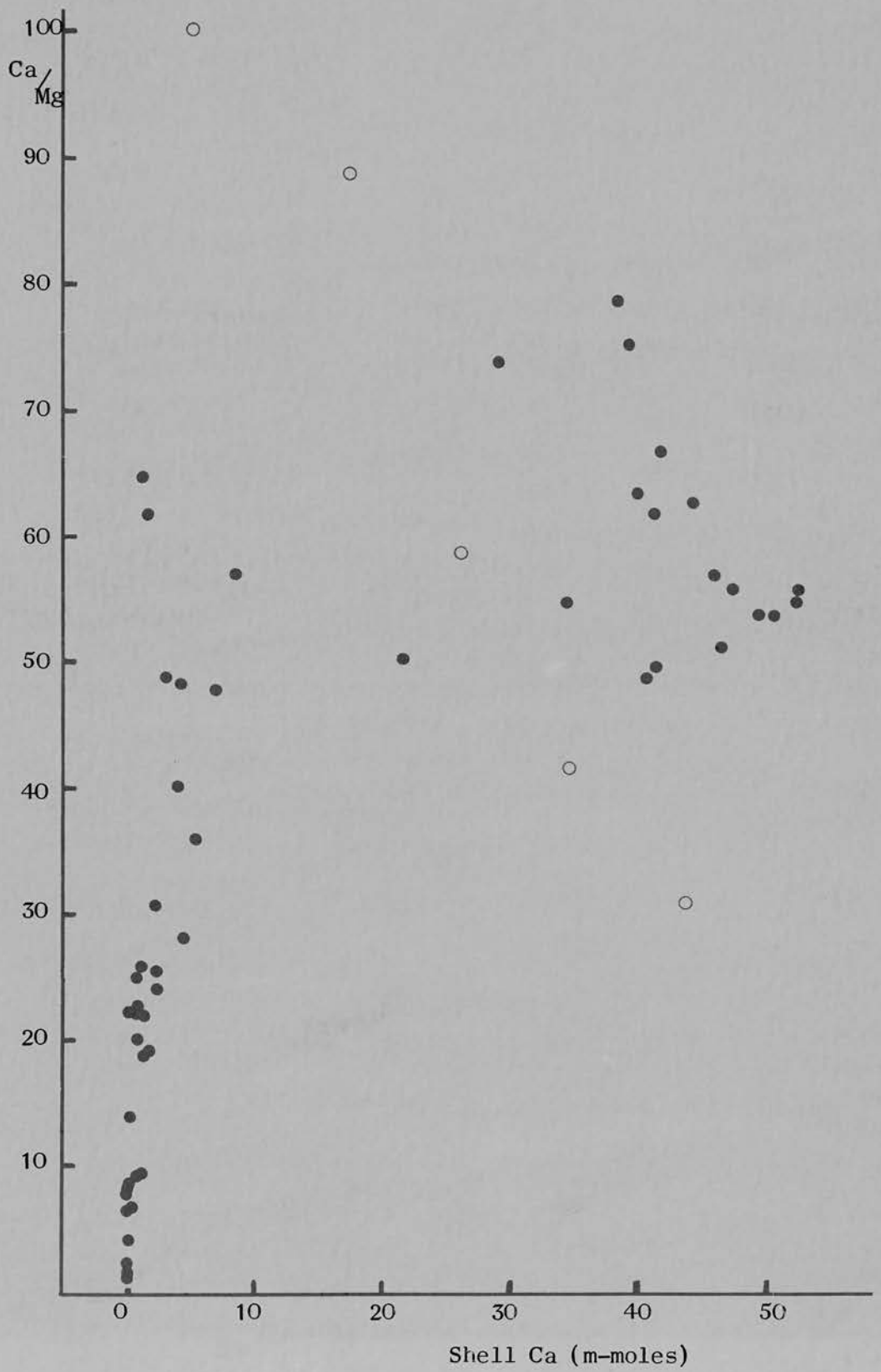


Fig.24. The molar ratio of Ca:Mg in the eggshell during calcification, plotted against total shell or membrane calcium in m-moles. The solid circles are values from eggs analysed for this study; the open circles represent values recalculated from the data of Brooks and Hale (1955).

Hale, this layer should have very little magnesium indeed. It seems that this magnesium may be in some way implicated in the seeding of crystallisation round the mammillae. The discrepancy in the findings could be explained if it is present in a form which is at least partly soluble. In this case, it could either be dispersed and lost before analysis of the complete shell is carried out, or it could be leached out through the pores of the shell, and would thus appear in the first dissolved fraction predominately, regardless of the location of this fraction.

The more gradual decrease in the calcium:magnesium ratio at the outer limit of the shell may reflect a reduced deposition of calcium without a corresponding fall in the magnesium; this would parallel the increased quantity of matrix in this part of the shell.

Smetsinger, Paul, and Mraz (1966) found an increase in magnesium concentration in the outer layers of the shell. They used the sequential dissolution method of Itoh and Hatano (1964), whose findings were similar, and in general not dependent on the direction in which the shell was dissolved; when the solvent was applied first to the inside of shells from which the membrane had been stripped, the highest concentrations of magnesium were still found in the outermost layer. Results of both directions of solutions showed also that the magnesium concentration was higher in the mammillary layer than in the intermediate layers; however, Itoh and Hatano did not consider this to be significant.

TABLE 31

RATIO OF CALCIUM TO MAGNESIUM IN THE SHELL: DATA OF BROOKS AND HALE (1955)

(Distance is expressed as % of shell thickness, calcium as m-moles; Mg/Ca ratio is by weight, Ca/Mg is molar ratio)

Distance from inside	Estimated corresponding Calcium	Mg/Ca	Ca/Mg
14	5.0	0.00605	100.3
38	17.0	0.00683	88.8
56	26.0	0.0103	58.9
73	34.5	0.0146	41.6
91	43.5	0.0196	31.0

SECTION 5

RESULTS: THE PLUMPING OF THE EGG

5.1 INTRODUCTION

There are four main steps in the formation of the tertiary membranes of the egg. The first of these is the secretion of the protein of the albumen; the second is the production of the fibrous shell membrane, also involving protein secretion. The fourth is the deposition of the calcified shell. During the initial stages of shell deposition, the third process, the phenomenon of 'plumping', occurs.

The unplumped egg is flaccid, and the membrane surrounds the egg contents loosely; by the time the membrane has acquired a coating of calcite thick enough to become rigid, the egg contents have filled out the membrane, or even slightly stretched it. The nature of the additions to the egg white may be deduced by reference to groups (2) and (4) of Table 24. The differences in mineral content between the various stages have been commented upon in Section 4.5. The most striking difference between these stages, representing the changes occurring in the course of plumping, is not the increase in sodium or potassium, proportionally large though these are, but the increase in water content. In this sample, it is 16.69g; this is perhaps exaggerated, as a result of inherent differences between the eggs of the two groups, but subtraction of the combined differences in the yolk weights and white dry weights from this value yields a residual 11.3 g., which may be considered a conservative estimate of the water gained. The time taken for this part of the process is probably about six hours (Warren and Scott, 1935; Burmester, Scott and Card, 1939;

Bradfield, 1951.) During this time, large quantities of water and ions must be transported, first into the lumen of the oviduct and then through the membrane, as it calcifies, into the egg white. In this section, some of the properties of the membrane and of the white which may be of relevance are examined.

5.2 PERMEABILITY OF THE EGG SHELL MEMBRANE

It has long been accepted that the eggshell membrane is permeable to water and ions (Burmester, 1940), and that unplumped eggs obtained from the oviduct could be 'plumped' by immersion in a saline solution (Burmester, Scott and Card, 1940). Opinion was however divided on the subject of the permeability of the membrane, at least in the process of egg formation, to substances of high molecular weight, especially the proteins of the albumen. McNally (1934) and Scott, Hughes and Warren (1937) held that a protein moiety is added to the white after the membrane is formed, and Beadle, Conrad and Scott (1938) believed the membrane to be protein-permeable, whereas Asmundson (1939) stated that all the protein had been added before the egg entered the isthmus. In order to test the permeability of the membrane, pieces of fresh membrane from soft-shelled or freshly decalcified eggs were interposed between samples of egg-white or solutions of albumen, and an aqueous, protein-free solution. When the system had been allowed to stand overnight, the aqueous solution was tested for the presence of protein. In all cases, regardless of the precise nature of the two components or the origin of the membrane, a positive result was obtained, showing the membrane to be permeable to the proteins of egg-white under these conditions.

The permeability of the membrane as demonstrated by the movement of protein across it overnight is shown in Tables 32, 33 and 34. The first of these, Table 32, gives the dried weight of TCA-precipitated protein found in the solution which was originally protein-free, for membranes from three sources and various combinations of solutions. The two right-hand columns, which should of course be zero, may be taken to indicate the size of the errors

TABLE 32

PERMEABILITY OF MEMBRANE, EXPERIMENT 1

Precipitate weights (g) of protein crossing membrane

Outer Solution	Membrane Source	Inner Solution: Egg white	Albumen Solution	D8 Solution	Distilled water
Distilled water	Natural soft-shelled egg	0.007 gms.	← Samples lost →		
	Decalcified egg	0.016	0.016	0.000	0.000
	Decalcified fertile egg	0.014	0.017	0.000	0.000
D8 solution	Natural soft-shelled egg	0.019	0.013	0.002	0.002
	Decalcified egg	0.019	0.015	0.002	0.002
	Decalcified fertile egg	0.026	0.023	0.002	0.002

which arise during weighing, or as a result of albumen adhering to the membrane. There is no obvious difference between the membranes from naturally occurring soft-shelled eggs, and those obtained by decalcification, either from fertile or infertile eggs. In all cases, a significant amount of protein is seen to have crossed the membrane.

The second experiment attempts to evaluate the extent of the protein transfer. Table 33 shows the results when the protein-containing phase, whether thick white (a), mixed white (b), or redissolved albumen solution (c), was in the small inner tube; for the results of Table 34, the outer tube contained the protein and the inner one distilled water (f) or saline solution, (d) or (e). Again in all cases protein crossed through the membrane. The dried weight of the precipitated protein which has moved across the membrane is in this table expressed as a percentage of the total dried weight of protein in the system.

If equilibrium were reached solely by the passage of protein, there being a volume of 1 ml. in the inner tube and 10 ml. in the outer, 90.9% of the protein for the results of Table 33 and 9.09% for those of Table 34 should have been transferred. In practice of course water and ions also cross the membrane, so that a lower result is expected. In fact, the results range from 20.6% to 84.6% (Table 33), and 1.23 to 2.72% (Table 34).

For the first seven results of Table 33, the volume in the small tube was not precisely 1 ml., as the substance in the tubes was thick white, which had been cut from the egg and weighed rather than pipetted as were the other samples. As a consequence, the total amount of protein present might be expected to differ more between individual tubes than in the other samples. This does not appear to be so, and should not in any case greatly affect the

TABLE 33

PERMEABILITY OF MEMBRANE, EXPERIMENT 2

Precipitate weights (g), protein moving outwards

Tube No.	Inner soln.	Outer soln.	Inside weight	Outside weight	% Change*	Total weight	Wt. thick white
1	a	d	+0.0751	+0.0195	20.6	0.0946	0.9716
1a			0.0299	0.0171	36.4	0.0470	0.8382
2			0.0873	0.0240	21.6	0.1113	1.4692
3	a	e	0.0439	0.0243	35.6	0.0682	1.1218
4			0.0351	0.0277	44.1	0.0628	1.0433
5	a	f	0.0375	0.0707	65.3	0.1082	1.0845
6			0.0381	0.0598	61.1	0.0979	1.1876
7	b	d	0.0087	0.0195	69.1	0.0282	
8			0.0357	0.0183	33.9	0.0540	
9	b	e	0.0165	0.0149	47.4	0.0314	
9a			0.0084	0.0213	71.7	0.0297	
10			0.0155	0.0095	38.0	0.0250	
11	b	f	0.0397	0.0148	27.2	0.0545	
12			0.0330	0.0191	36.7	0.0521	
13	c	d	0.0060	0.0330	84.6	0.0390	
13a			0.0175	0.0328	65.2	0.0503	
14			0.0163	0.0169	50.9	0.0332	
15	c	e	0.0149	0.0124	45.4	0.0273	
15a			0.0180	0.0187	50.9	0.0367	
16			0.0191	0.0208	52.1	0.0399	
17	c	f	0.0117	0.0448	79.3	0.0565	
18			0.0102	0.0526	83.8	0.0628	

* Theoretical change (see text) 90.9%

Solutions: a, thick white;
b, stirred mixed white
c, 100g/l albumen solution
d, saline solution, NaCl + KCl isotonic with oviducal egg white
e, saline solution, NaCl + KCl isotonic with oviposited egg white
f, distilled water

TABLE 34

PERMEABILITY OF THE MEMBRANE, EXPERIMENT 2

Precipitate weights (g), protein moving inwards

Tube No.	Inner soln.	Outer soln.	Inside weight	Outside weight	% Change*	Total weight
21	d	b	0.0089	0.4936	1.77	0.5025
22			0.0134	0.4795	2.72	0.4929
23	d	c	0.0043	0.2594	1.63	0.2637
24			0.0037	0.2959	1.23	0.2996
27	e	b	0.0088	0.4750	1.82	0.4838
28			0.0100	0.4180	2.34	0.4280
29	e	c	0.0075	0.2836	2.58	0.2911
30			0.0075	0.3447	2.13	0.3522
33	f	b	0.0092	0.4884	1.85	0.4976
34			0.0074	0.4284	1.70	0.4358
35	f	c	0.0058	0.2936	1.94	0.2994
36			0.0061	0.2759	2.16	0.2820

* Theoretical change (see text) 9.09%

Solutions: a, Thick white;

b, stirred mixed white;

c, 100g/l albumen solution;

d, saline solution, NaCl + KCl isotonic with oviducal eggwhite;

e, saline solution, NaCl + KCl isotonic with oviposited eggwhite;

f, distilled water.

percentage change. The weights of fresh thick white used are shown in the final column of the table.

The small number of experiments and the wide variation of the results do not permit the application of statistical procedures; however, certain differences seem to emerge. Protein seems to cross the membrane from the albumen solution most easily, but there is little difference between unstirred thick white and mixed white. This is surprising in view of the findings of section 5.4, and may be attributed to the fact that the thick white has been cut and inner layers exposed. More protein is transferred into the distilled water, which contains no osmotically active particles initially, than into either saline solution. There is little difference between the two concentrations of saline in the cases where the protein is in the smaller tube. Differences between results when the protein was in the larger tube were probably not significant.

In any case, the demonstration that the eggshell membrane is capable of permitting the passage of proteins of the type found in the egg white seems conclusive. Moreover, it does not matter in which direction the protein molecules cross the membrane. At first glance, it might seem that the differences between the results obtained when the protein was in the inner tube and when it was in the outer do suggest a directional difference in flow. This is almost certainly accounted for by the greater effect on the osmotic pressure of the movement of water when the protein is initially in the large tube. Whether any such movement of protein through the membrane takes place during egg formation is however a completely different question.

5.3 PLUMPING OF EGGS IN VITRO

The weight gained over a period of four hours by soft-shelled eggs immersed in distilled water or saline solutions is shown in Table 35. The behaviour of some typical individual eggs is shown in Figure 25. Generally, the egg is slightly shrunken as a result of evaporation at the beginning of the experiment. After an initial period of a few minutes in which the membrane takes up water and expands to allow free passage of the water to the white, the egg swells rapidly. When the water uptake is sufficient to fully extend the membrane (denoted by P, in Figure 25), the rate of this uptake is reduced. This is not unexpected, as work must now be done against the natural elasticity of the membrane to produce further swelling and weight gain. If the procedure is continued, the egg will continue to swell, increase in weight, and become more turgid, until eventually the internal pressure is so great that the membrane ruptures, and the contents flow out.

That this should happen when the egg is soaked in water is quite surprising, in view of the finding that the membrane is permeable not only to ions but also to the proteins of the white. Testing of the water or saline solution in which these eggs had been soaked using Biuret reagent produced negative results in every case, however. This indicates that there was less than 0.05g./100ml. of protein present, as in trials this concentration gave a clear positive reaction. This finding would suggest that the gel linkage of the white protein molecules is sufficiently strong to be undisturbed by the considerable osmotic forces which will act under these conditions. There is no significant difference between the swelling of eggs soaked in water and those immersed in saline solutions isotonic to early-stage shellgland fluid (a), early-stage egg-white (b), or late-stage egg-white (c). The

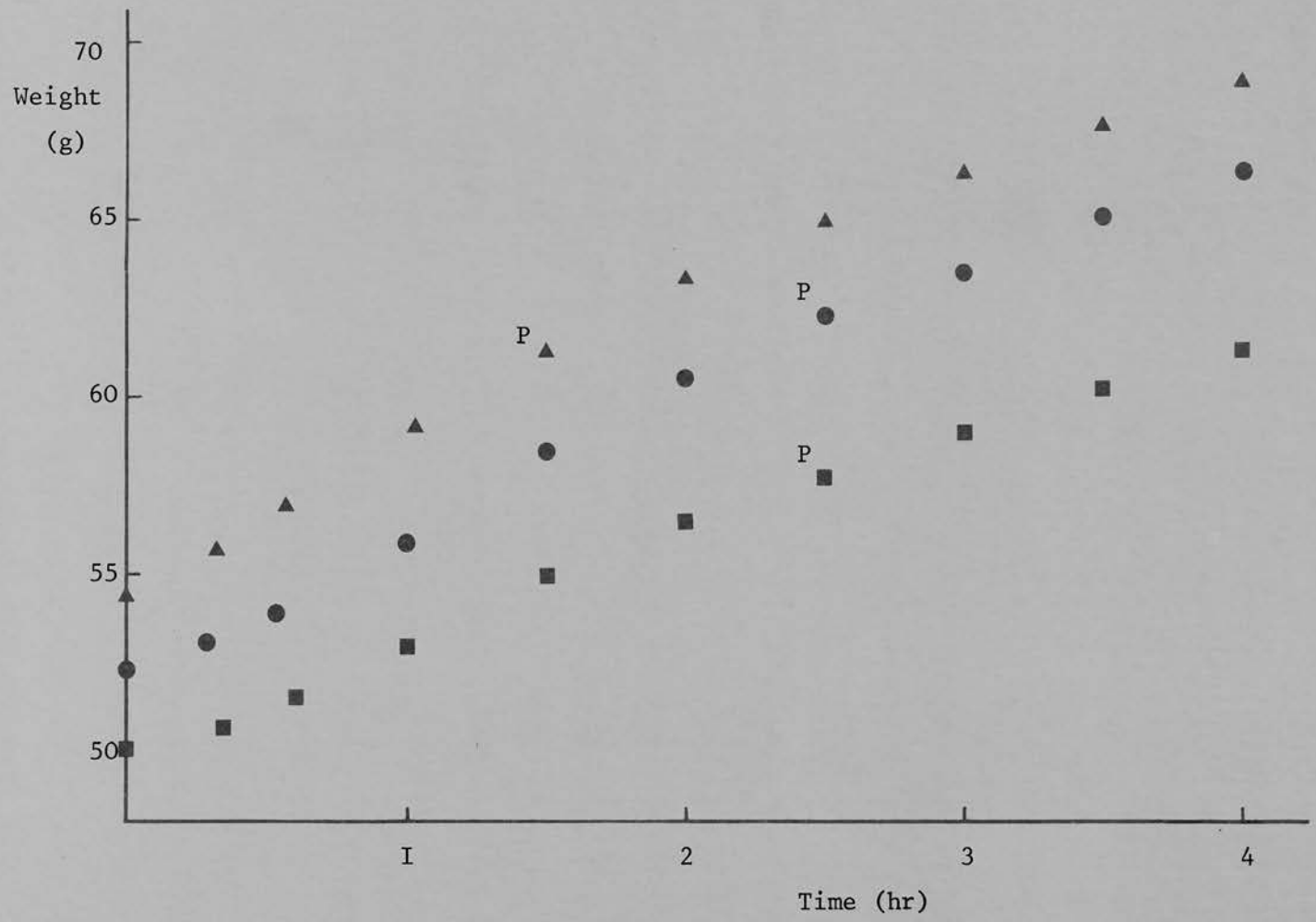


Fig.25. Weight gain (g) with time of soft-shelled eggs immersed in distilled water. P represents the point at which the egg was judged to be fully plumped without the membrane being stretched. The experiments are:

▲ 2/I; ● 3/9; ■ 3/I.

TABLE 35

PLUMPING OF EGGS IN VITRO

(Surface area calculated from S.A. = $4.722\ W^{\frac{2}{3}}$, where W = weight when just plumped)

Solution	Initial Egg wt.	White dry wt.	Surface Area	Membrane dry wt.	Membrane dry wt/	4-hr. wt. gain	Gain/ S.A.	Gain/g dry white
Distilled water	53.650	7.0897	72.996	1.0448	14.31	14.332	0.1963	2.0215
Water	64.271	4.8557	81.257	1.1564	14.23	13.776	0.1695	2.8371
Water	34.446	2.9001	52.803	0.4926	9.33	8.076	0.1529	2.7847
Water	55.406	3.3155	73.093	1.0284	14.07	13.745	0.1880	4.1457
Saline(a)	54.359	4.0680	71.63	0.6682	9.33	14.497	0.2024	3.5637
(a)	58.879	4.0367	79.32	1.0288	12.97	11.532	0.1454	2.8568
(a)	55.130	4.1762	75.69	0.7097	9.38	11.489	0.1518	2.7511
(b)	54.363	3.0607	74.46	1.1276	15.14	11.034	0.1482	3.6051
(b)	48.651	3.2427	69.04	1.1689	16.93	9.737	0.1410	3.0027
(b)	41.720	2.7707	65.44	1.0021	15.31	10.769	0.1646	3.8867
(c)	57.426 (D.Y.)	6.6840	80.17	1.5488	19.32	7.244	0.0904	1.0838
(c)	32.927	2.9515	58.79	0.8616	14.66	11.440	0.1946	3.8760
(c)	48.645	3.7659	73.99	0.9276	12.54	15.668	0.2118	4.1605
Water	50.053	2.809	69.46	0.839	12.08	11.319	0.1630	4.0295
Water	52.626	3.485	74.18	0.619	8.34	15.318	0.2065	4.3954
Water	31.152	0.802	53.31	C.570	10.69	(6.183) (2 hrs.)		
Water	49.258	3.012	71.54	1.196	16.72	17.968	0.2512	5.9655
Water	50.948	3.008	71.82	0.586	8.16	19.277	0.2684	6.4086
Water	46.036	3.439	70.74	1.284	18.15	11.887	0.1680	3.4565
Water	36.201	3.786	57.09	0.538	9.42	12.463	0.2183	3.2919
Water	49.315	1.425	64.55	1.250	19.36	(1.155) (3 hrs.)		
Water	52.387	3.5	72.86	1.046	14.36	14.012	0.1923	4.003
Water	53.716	3.082	77.77	0.624	8.02	22.993	0.2957	7.4604
Water	28.493	0.553	50.34	0.523	10.39	9.220	0.1832	16.6727
Water	36.094	2.037	57.33	0.541	9.44	10.762	0.1877	5.2833
Mean ± S.E.	48.103 ±1.98	3.406 ±0.25	69.165 ±1.82	0.8641 ±0.05	12.42 ±0.67	13.241 ±0.07	0.1909 ±0.0085	4.5664 ±0.64

*:- Estimate. White and yolk mixed
():- Membrane ruptured after indicated time
D.Y.:- Double yolked egg

experiments of Burmester, Scott and Card (1940), in which the solution used corresponded to the composition given by Beadle, Conrad and Scott (1938) for shellgland fluid, also showed swelling of the egg. It would be reasonable to suppose that the ionic concentrations of the solution and of the egg-white would reach equilibrium rapidly. This was demonstrated by immersing soft-shelled eggs in hypertonic saline solutions. Eggs were soaked for up to two days in $\frac{1}{2}$ M. solutions of a wide range of salts, including NaCl, KCl, NaBr, NaI, $NaNO_3$, $Na\ SO_4$, KBr, KI, KNO_3, $CaCl_2$, and choline chloride. In all cases the eggs swelled continuously; the same effect was observed if the solution used was of molar sucrose, after an initial period of shrinkage of about half an hour, and even a 15 or 20% solution of dried egg albumen caused an initial swelling, although this effect was later reversed (Figures 26, 27). The osmotic pressure of egg-white as measured by depression of the freezing point is only about 260 (m-osmoles), so the least concentrated of these solutions should have been almost double this.

The data of Burmester, Scott and Card showed that the rate at which the soft-shelled egg absorbed water from the immersing solution depended on the time which the egg had already spent in the shellgland - that is, on how much water it had already absorbed. Since no observations on this were possible in this study, the eggs being obtained when they happened to be laid rather than being removed from the shellgland of a bird which was under observation, this has been represented by the weight of the membrane per square cm. of surface area of the egg. The surface area was calculated by the formula quoted by Romanoff and Romanoff (1949), with the constant adjusted to compensate for the difference in specific gravity between a fully shelled egg, for which the formula was

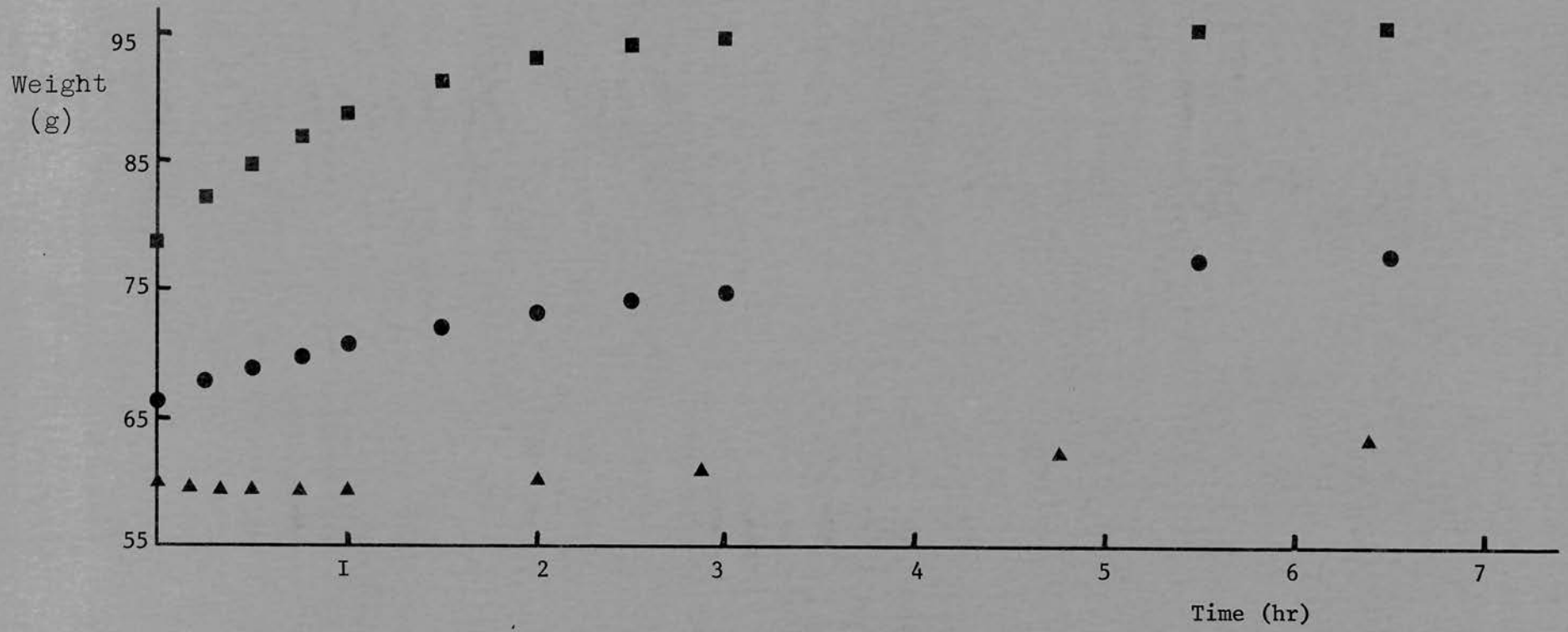


Fig.26. Changes in weight with time of soft-shelled eggs immersed in aqeous solutions.

■ $\frac{1}{2}$M KNO_3; ● $\frac{1}{2}$M $CaCl_2$; ▲ M sucrose.

intended, and the almost completely shell-less eggs of this experiment. No allowance can be made for differences in the membrane proper, but gross differences in the degree of calcification would be expected to be obvious.

Coefficients of correlation were calculated for four hour weight gain with: surface area of the egg at plumping, dry weight of the egg white, and stage of formation as represented by membrane dry weight/sq. cm. There is no correlation between weight gain and white dry weight or membrane dry weight/sq. cm., even at the 10% level of significance. The coefficients are 0.22 and -0.24 respectively, all results being considered regardless of the nature of the external solution. The coefficient of correlation for surface area, however, is 0.54, which is significant at the 1% level. Calculation of correlation for eggs soaked in distilled water only gave the same results, the coefficients being 0.22 for white dry weight, -0.11 for membrane dry weight/sq. cm., and 0.69 for surface area.

It is not entirely surprising that no correlation is found with membrane weight, as this is a most inaccurate measure of the stage of formation of the egg. It is at first sight more surprising that there is no apparent relationship of weight gain and the dry weight of the egg white; a simple osmotic pressure effect would be expected to depend on the quantity of osmotically active substance present. In this case, the osmotically active substance is egg white protein, which is closely equivalent to the dry matter. The implication is that it is only one part of the protein which is important; this may be a chemically distinct part, or it may be physically, in this case spatially, distinct. The

TABLE 36

HYDROSTATIC PRESSURES IN PLUMPED EGGS

Weights in g, pressure in cm. of water

Egg	Weight	Pressure	Method	Weight gained (after plumping)
2/1	69.865	10	T	10.758
2/4	65.4	1.5	M	2.816
2/6	52.835	0.2	M	1.239
2/8	44.712	0.1	M	0.815
2/11	65.887	0.1	M	0.494
2/9	68.780	> 3.0	M	6.761
A	78.305	9.2	T	9.189 *
B	56.193	34.6	T	9.839 *
C	69.276	11.4	T	19.005 *

M = readings taken by manometer

T = readings taken by pressure transducer

* Total weight gain.
Weight at plumping not noted.

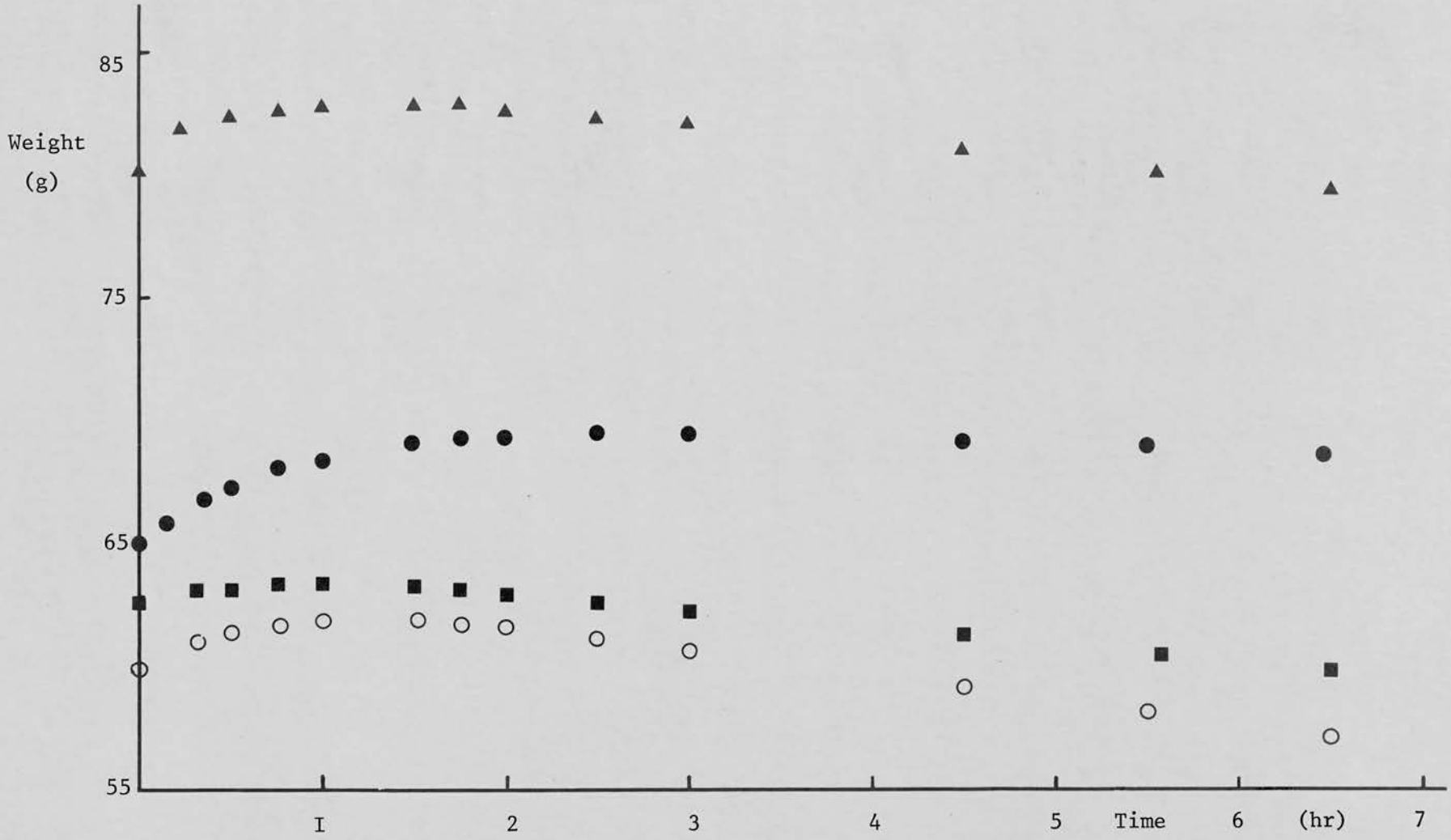


Fig.27. Changes in weight with time of soft-shelled eggs immersed in solutions of albumen.

▲ I0g/I00 ml; ● 5g/I00 ml; ■ I5g/I00 ml; ○ 20g/I00 ml.

existence of the surface area correlation suggests that the primary effect is of the latter type. The cross-linking of the layers of egg white (see section 4.1), which is, indeed, dependent on the chemical constituents, may be sufficiently strong to prevent all but the outermost layer of the thick white from being osmotically active; the area presented by this layer to the outside solution would then determine the 'pulling power' of the white. The lack of passage of protein from the white to the outside solution also suggests a considerable degree of binding.

The strength of the binding of the egg white proteins, and of the forces which are drawing water into the white, are indicated by the hydrostatic pressures which may be built up inside the egg, and which seem to be limited only by the mechanical strength of the membrane. The results of a few measurements of hydrostatic pressure which were made on swollen eggs are shown in Table 36. Most of the more turgid eggs burst before measurements could be obtained, and in others the contents were already leaking out by the time a reading of pressure was made. Three values of about 10 cm. water were obtained from eggs which did not feel exceptionally turgid, and the one egg with a pressure of 34.6 cm. water (Egg B, Table 36) seemed swollen, but not yet ready to burst. It is probable that higher pressures would be measured under suitable circumstances.

5.4 OSMOTIC BEHAVIOUR OF EGG WHITE: MISCELLANEOUS RESULTS

In order to examine the contribution of the membrane to the behaviour of the white during the process of plumping, it was first replaced by dialysis tubing, which was permeable to ions and small molecules, but not to the large protein molecules. The results obtained when samples of egg white enclosed by lengths of tubing were soaked in distilled water are shown in Table 37, and displayed graphically in Figures 28 and 29. In these experiments, since the tubing did not permit any great degree of expansion, the water taken up by the white was allowed to rise in an open-ended vertical tube. The uptake is thus opposed by gravity, and the rate of uptake decreases with time as a result. Nevertheless, a considerable column of water could be supported by the white; it is interesting that in the second experiment (Table 37), the greatest heights attained by the column of water exerted a pressure about the same as the highest hydrostatic pressure measured in a soft-shelled egg which had been soaked in water. The length of time involved was however much greater. The quantity of egg white protein present is only slightly less than that in an egg: indeed, the samples each represented almost the entire white of an egg.

This behaviour of a substance containing osmotically active molecules and enclosed by a semi-permeable membrane is not surprising. Much more interesting are the results shown in Table 38. These show the behaviour of soft-shelled eggs in which swelling was established, and then the membrane was punctured; and eggs which were removed from their membranes and transferred to a 'membrane' made of nylon stocking material. This has considerable extensibility, and the knitted mesh will permit the passage not only of small particles but of bulk substances.

TABLE 37

UPTAKE OF WATER BY EGG WHITE SAMPLES IN DIALYSIS TUBING

Weights are in g, time in hrs, heights in cm, and surface area in sq. cm.

1. Narrow tubing, 0.63 cm. diameter. Lengths: (1) 10.1 cm., (2) 11.2 cm., (3) 9.4 cm.

Time	Height of water column					
	Experiment 1		Experiment 2		Experiment 3	
	L	R 1	L	R	L	R
0	0	0	0	0	0	0
18	4.2	4.3	4.9	4.9	4.4	4.3
21.5	5.3	5.3	6.6	6.4	5.4	5.4
25.5	5.8	5.7	6.7	6.7	5.9	5.9
90	12.2	12.0	15.9	16.1	12.1	12.0
98	12.5	12.4	16.7	17.0	12.5	12.4
114	13.3	13.6	17.9	18.7	13.5	13.4

2. Wide tubing, diameter 2.5 cm.

Experiment	4	5	6	7	8	9
Approximate surface area	57	46	54	48	57	53
Wt. of white (wet)	32.31	25.60	30.54	26.90	32.32	29.65
Dry wt. of white	2.73	2.23	1.54	0.22	2.54	2.38
Time	Height of water column					
16	8.3	6.5	11.8	9.3	3.9	6.5
23½	13.2	7.6	17.0	13.9	9.1	7.5
40	19.3	13.7	24.5	21.5	15.3	11.0
48	21.8	15.4	26.4	23.6	17.0	12.7
64	27.4	19.8	29.2	28.3	21.1	17.0
72	29.1	21.1	31.3	30.0	22.5	18.7
88	33.4	24.1	34.4	34.2	24.8	21.6
96	35.1	24.7	34.8	34.3	25.6	22.7

TABLE 38

UPTAKE OF WATER BY PUNCTURED SOFT-SHELLED EGGS, AND EGG CONTENTS IN NYLON

Weights in g, time in hrs. minutes.
Eggs 2/10 and 2/11 punctured at 01.30 hours

Time	Experiment no.			
	2/10 (punctured)	2/11 (punctured)	2/12 (nylon)	2/13 (nylon)
00.00	41.401	49.794	-	-
00.15	43.442	51.901	-	-
00.30	-	-	44.120	37.955
00.32	44.775	53.275	-	-
00.45	-	-	46.934	38.022
01.00	46.581	55.128	49.284	37.933
01.30	48.308	56.833	51.041	37.394
02.00	49.844	58.502	51.520	35.826
02.30	49.505	60.201	49.752	36.152
03.00	49.427	61.910	48.195	35.635
03.30	50.102	63.338	46.878	35.968
04.00	49.364	64.419	46.209	34.718
04.30	50.062	65.393	-	-

One of the punctured eggs (2/10) had almost reached full 'plumping', at the time of puncture. That is to say, the membrane was completely extended, but not stretched. This egg gained a further 1 - 1½ g. of water, and then maintained a constant weight for the rest of the experiment. The small variations which did occur were probably the result of the manner of handling and drying the egg before weighing. The membrane of the other egg, (2/11), was still wrinkled at the time of puncture; it continued to gain weight at a rate comparable to that of eggs with intact membranes until the end of the experiment (Figure 30), in spite of several holes of about 0.5 mm. diameter in the membrane.

The eggs transferred to nylon also gained weight initially (Figure 31), although weighings were much more difficult and less accurate due to the difficulty of handling the 'egg'. At the end of the experiment, the egg white was partly coagulated, and the vitelline membrane had ruptured, releasing the yolk which was mixed with the white. It is possible that the breakdown was initiated when the sacs were removed from the water for weighing, when handling may have caused mechanical damage to the unsupported white gel. In later weighings, some of the albumen was lost in drying off excess water.

From these experiments the somewhat surprising conclusion seems to emerge that, although the membrane helps to preserve the integrity of the egg white as well as giving the egg its shape and being the foundation of the shell, it plays no primary part in the process of plumping.

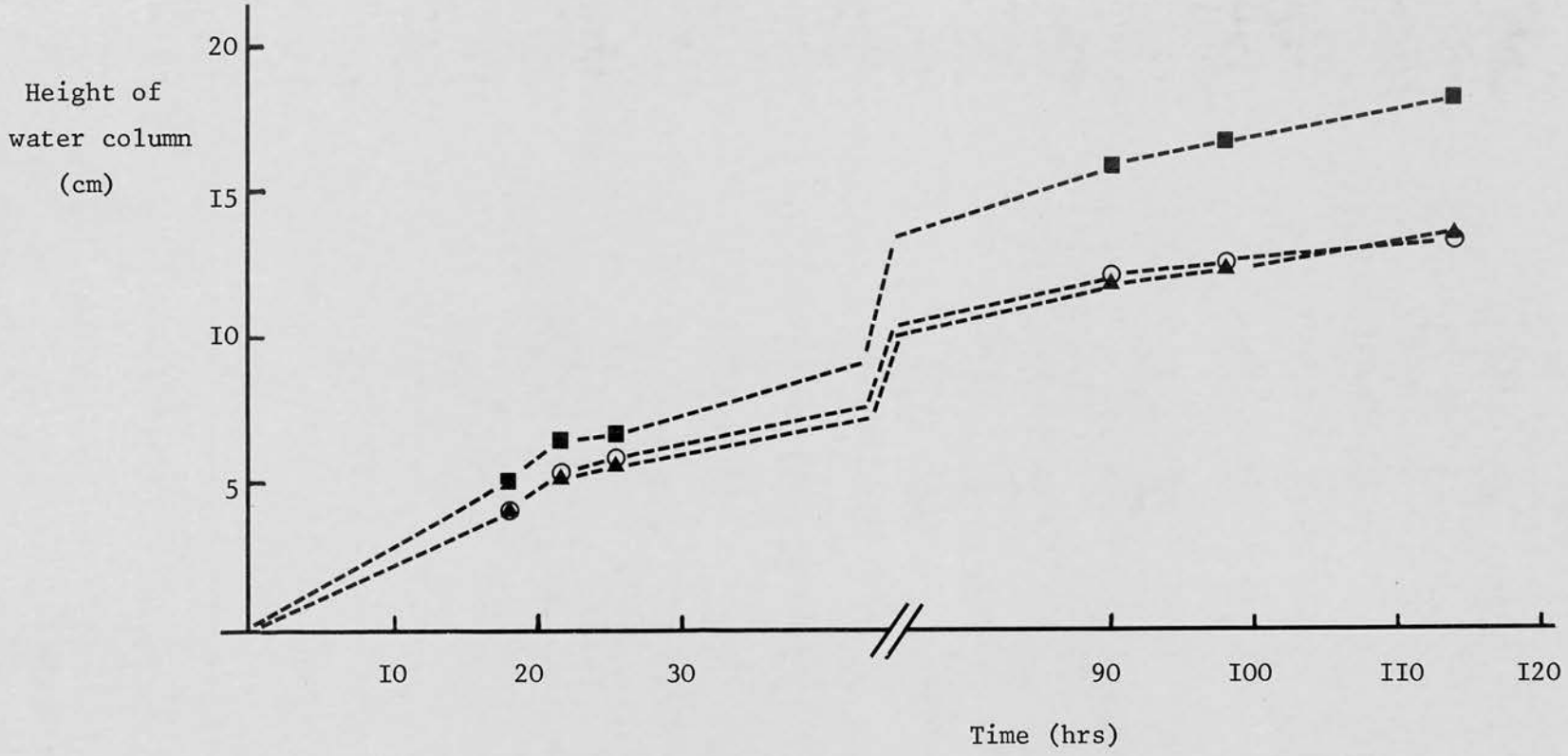


Fig.28. The uptake of water by egg white samples in narrow dialysis tubing.

○ Experiment I; ■ Experiment 2; ▲ Experiment 3.

SECTION 6

TRANSPORT OF MINERALS AND ELECTRICAL POTENTIALS ACROSS THE OVIDUCT WALL

6.1 INTRODUCTION

The results presented in the last two sections have centred on the egg, its composition and the changes which occur during formation, and the way in which it can take up water and aqueous solutions from its surroundings; section 3 described the appearance and defined the functions of the oviduct. But the greater part of the problem of egg formation lies in the apparatus which transports the constituents, and particularly the mineral constituents, to the forming egg. This apparatus must lie in the wall of the lower regions of the oviduct. In this section are described some investigations into the behaviour of these regions, both in isolation and during the process of egg formation in the bird.

6.2 THE TRANSPORT OF MINERALS ACROSS THE OVIDUCT WALL IN VITRO

When isolated sacs of oviduct, usually of the shellgland region, were set up as described in section 2.10 and maintained for an hour, no significant changes were found to occur in the concentrations of sodium, calcium, or magnesium in the surrounding avian equivalent solution, but a marked increase in the potassium concentration inside the sac occured in most cases. Table 39 shows the concentration of potassium by the isolated shellgland of the laying bird, expressed as the mean accumulation per minute over the hour of the experiment. The course of the concentration change with time for one such experiment is shown in Figure 32. The results of Table 39 were arrived at as follows: the regression equation, $[K^+] = a + bt$, for potassium concentration in mM., as measured by atomic absorption analysis, against time in minutes was calculated for each experiment. The coefficient b was then multiplied by the mean volume in litres of the internal solution to give the rate of accumulation which is shown in the table. For these experiments, the coefficient of correlation was in all cases significant at the 0.1% level.

Experiments using the isthmus region of the oviduct (and a smaller volume of internal solution) occasionally showed a slight increase in potassium concentration, but the correlation was extremely low, and the increase of doubtful significance. (Figure 33). Two experiments carried out using a solution with half the sodium and potassium concentrations of the D8 avian equivalent solution (described in sections 2.9) showed potassium accumulations of 1.30 and 1.75 micromoles/min., well within the spread of the results in experiments where D8 was used. When the internal solution only was replaced by a potassium-free solution (two experiments), the accumulation was in both cases 2.7 m-moles/min., also within the

TABLE 39

THE ACCUMULATION OF POTASSIUM BY THE ISOLATED SHELLGLAND

Mean values (micromoles/min) over 1 hour

Experiment No.	Physiological state of oviduct	Mean accumulation of K^+
11	Active, no egg	1.400
13	Active, no egg	1.085
17	Active, no egg	1.820
18	Active, no egg	2.450
Mean		1.689
21	Active, yolk just ovulated	1.575
10	Soft egg in shellgland	1.785
20	Hard thin-shelled egg in shellgland	3.185
12	Hard egg in shellgland	2.380
14	Hard egg in shellgland	1.330
Mean of all values:		1.890

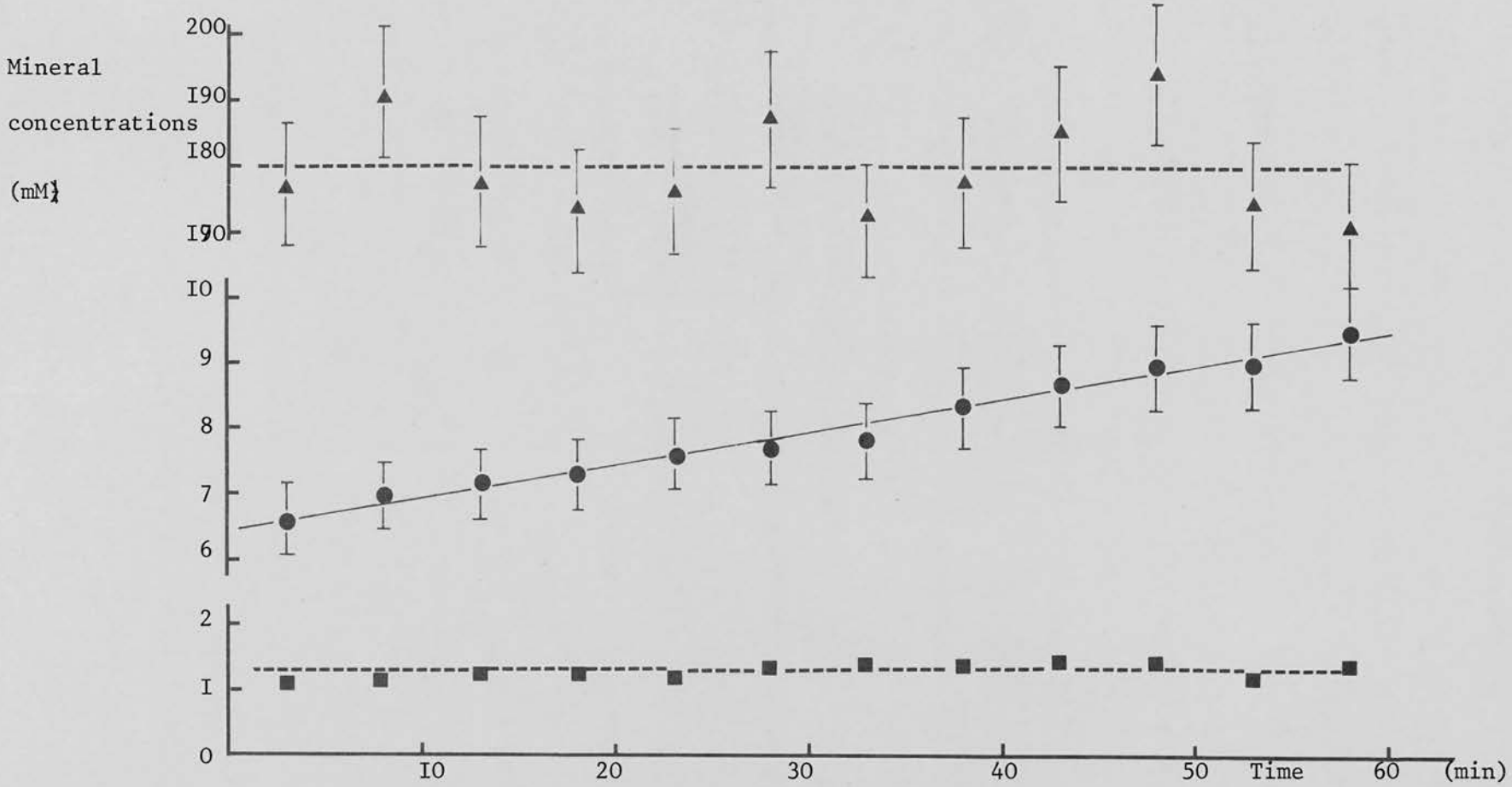


Fig.32. Changes in the concentrations of minerals in avian equivalent solution inside an isolated shellgland sac. The solid line represents the calculated regression equation for potassium (●), and the dotted lines the mean values for sodium (▲), and calcium (■) respectively. Bars represent mean standard deviations found in a separate experiment.

range of the results obtained when avian equivalent solution was on both sides of the tissue.

An accumulation rate of 1.89 micromoles /min. represents a total of 1,134 micromoles in a ten hour period, and 2,268 in twenty hours. From Table 24, the potassium content of the white of an egg at the stage of group (2), that is, with a membrane but very little calcification, is 153 micromoles; in late calcification (Group 5), the content is 1,094 micromoles, and in the oviposited eggs of group (6), 1,415 micromoles. The measured rate of accumulation of this ion is of the order needed to produce this. It is perhaps significant that the highest rate found (20, Table 39) was in a shellgland from which an egg in the stage of fastest calcification had been removed, as this could also be the time of greatest activity of mechanisms other than those of calcification.

In terms of the passage of potassium across the shell membrane, if an egg surface area of 70 sq. cm. is assumed, the flux is 450 picomoles/sq. cm./sec.

In the experimental arrangement used, the mean volume of the internal solution was 35 ml. Over 1 hour, the potassium concentration changed by approximately 50%. A change of the same absolute value in the sodium content of the internal solution would cause a concentration change of less than 2%. In a control experiment, in which eight samples were taken from a flask containing solution but no tissue, the coefficient of variation of the sodium determinations was 5.55%, and any movement of sodium which might take place in the same direction as the potassium transport would be undetectable by this method. A transfer of this order occuring in the opposite direction, into a volume of solution ten times as great, would have even less effect on the measured concentration.

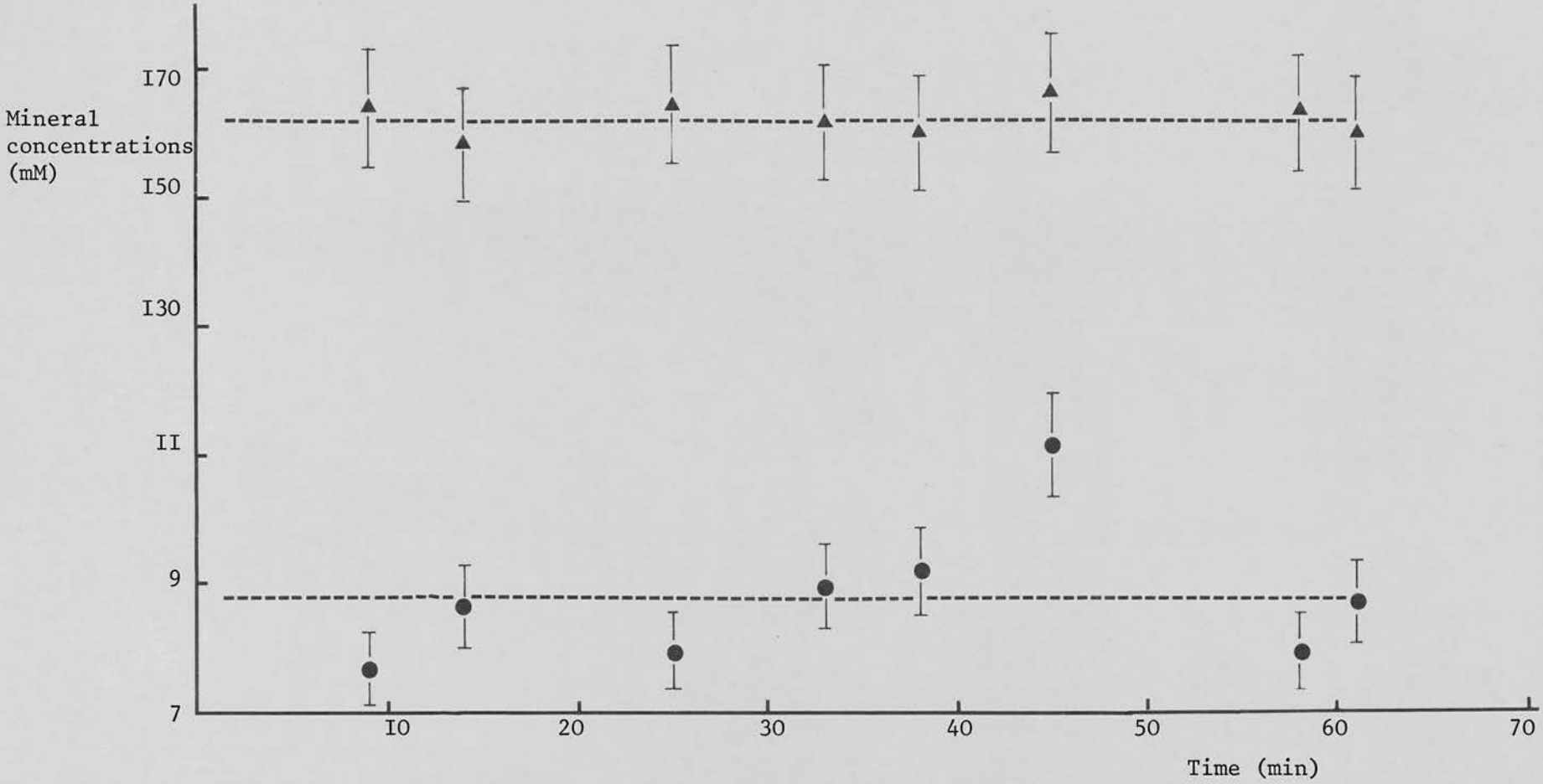


Fig.33. Concentrations of potassium (●) and sodium (▲) in avian equivalent solution inside an isolated isthmus sac over a period of time. In contrast to the shellgland of fig.32, the potassium concentration does not change significantly. Bars represent standard deviations, derived from a separate experiment, and dotted lines the mean concentrations.

The same argument does not apply to calcium, nor indeed to magnesium. It must be concluded that either any movement of these minerals which occurred under the conditions of this experiment was negligible, or that the solution was already saturated with them, and immediate precipitation occurred. No obvious signs of such precipitation were however seen.

TABLE 40

Potentials across the shellgland in vitro
(mV; mucosal side negative: time in min.)

Experiment no.	Functional State of Oviduct	Maximum potential	Time for potential to disappear	Energy source in bathing solution
3	Soft egg, plumping, in shell-gland	- 7.5	10	Glucose
5	Soft egg, plumping in shell-gland	- 2.3	32	none
7	Soft egg, plumping, in shell-gland	- 4.4	103	Glucose + ATP
8	Soft egg, plumping, in shell-gland	- 6.5	17	ATP
9	Soft egg, plumping, in shell-gland	-16.3	108	ATP (no glucose)
6	Thin-shelled egg in shellgland	- 6.9	83	Glucose + ATP
1	Hard-shelled egg in shellgland	-10.3	30	Glucose
2	Hard-shelled egg in shellgland	-13	45	Glucose
4	Hard-shelled egg in shellgland	-13.5	40	Glucose

6.3 POTENTIALS ACROSS THE OVIDUCT WALL IN VITRO

The massive amounts of calcium which are transferred from the blood, through the oviduct wall, to the forming shell, and also the smaller but not insignificant amounts of potassium which have been shown to be moved in the same direction, would both suggest the possible existence of a potential across the wall of the oviduct. The attempt to measure potentials at the same time as potassium concentrations was made and abandoned, and pieces of shellgland tissue were set up between two Perspex chambers, with avian equivalent solution D8 bathing both sides, as described in Section 2.11.

The initial potentials measured on pieces taken from shellglands which contained an egg at the time of death are shown in Table 40. Although the tissue was transferred as rapidly as possible from the animal to the apparatus, the solutions were thoroughly oxygenated, at a temperature similar to that of the bird, and contained 1 g./litre of glucose, the potential decreased rapidly to zero. In most cases, from a potential of perhaps 10 or 12 mV, zero was reached within 40 - 45 minutes. A typical graph of the behaviour of the potential with time is shown in Figure 34. In an attempt to correct this, it was decided to add a source of immediately available energy, in this case ATP, to give a final concentration of 10^{-7}g./l. The effect of this addition during the course of the experiment is shown in Figure 35. An immediate reversal of the potential fall occurred, but was short-lived, and in no case was the potential restored even to the starting level. The response to ATP, although partial, suggests that the rate of metabolism of the tissue is high, and that large supplies of energy are needed to support the potential. Addition of ATP later in the experiment had no effect, which suggests that an irreversible change had by

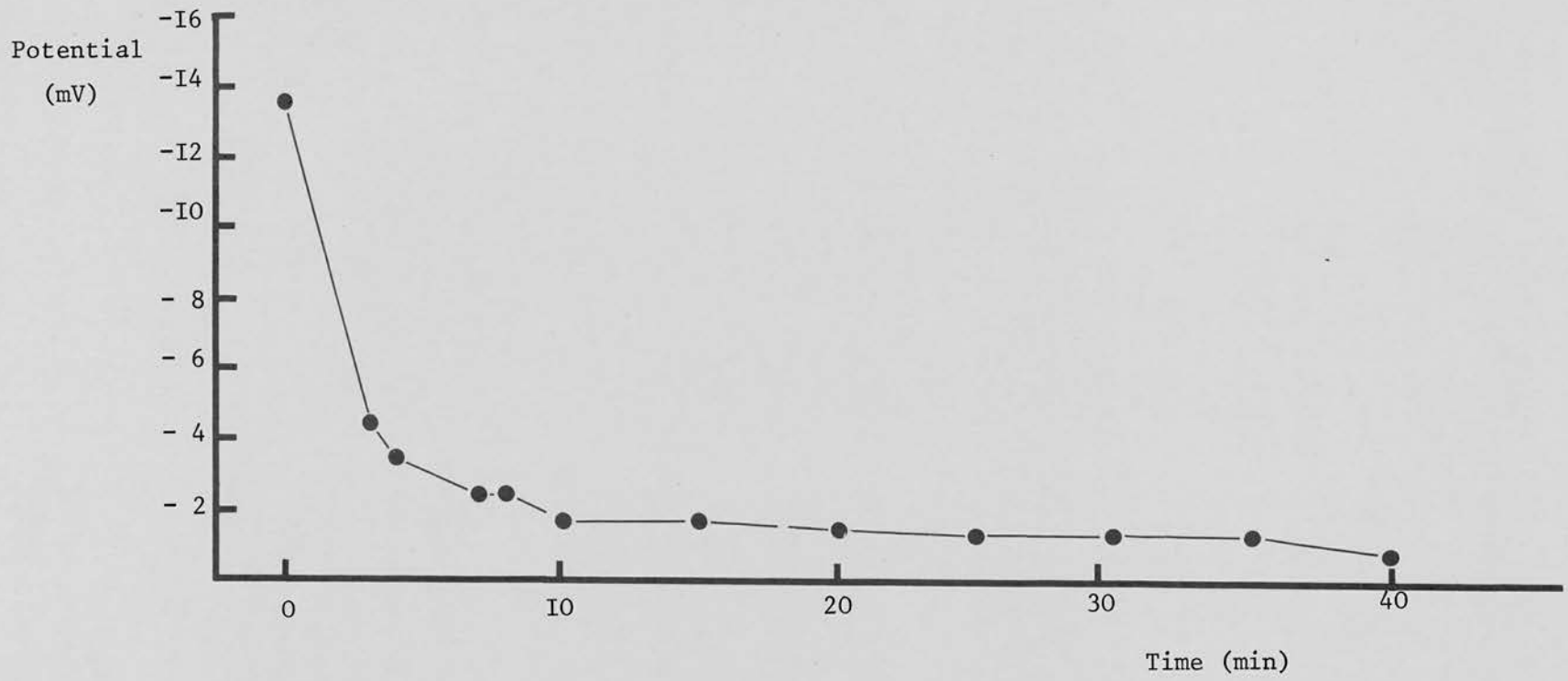


Fig.34. Potential across isolated shellgland tissue, mucosal side negative.

The rapid fall to insignificant levels is typical.

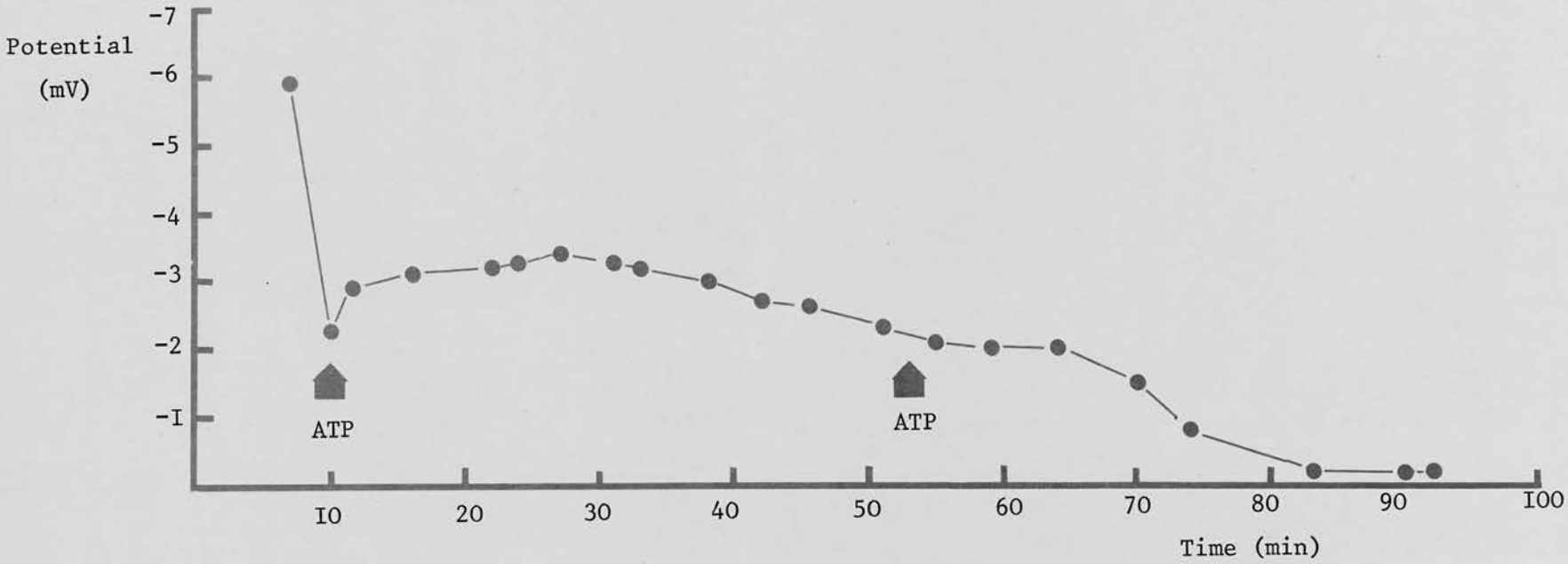


Fig.35. The effect of the addition of ATP to give a final concentration of $I0^{-7}$g/l to the incubation medium (avian equivalent solution) on the potential across the isolated shell gland tissue. On the first occasion, the fall in potential is temporarily reversed; on the second addition, the effect is less noticeable, and the potential eventually falls to zero.

this time (i.e. after about 25 minutes) taken place. It is thought that the effect may have been due to an inadequate supply of oxygen to the deeper tissue layers of this rather massive organ.

The highest potential recorded was -16.3 mV. (Experiment 9, Table 40). Although there may be a difference between different functional states, probably the source of most of the differences between potentials reflected the time taken to transfer the tissue to the apparatus. It was not possible to record this with any accuracy, as the time at which the bird 'died' from the point of view of oviduct function could not be determined.

The rate at which the potential is falling is initially high, and it is difficult to guess what the point of origin might be; probably, it was at least 20 mV., and perhaps as much as 50 or 60 mV., if extrapolation to the time of the fatal injection is made. The direction of the potential is the opposite to that which would be expected if active transport of either calcium or potassium, both of which form positive ions, were taking place into the lumen of the oviduct.

6.4 POTENTIAL MEASUREMENTS ACROSS THE OVIDUCT WALL IN VIVO

The measurement of potentials in the living bird proved to be difficult. It is not easy to be sure where precisely the electrode is positioned in the oviduct. The situation is further complicated by the fact that even after the electrode tip has been positioned, peristaltic movements of the oviduct and even the movements caused by the bird's respiration may shift it significantly. Both of these types of movement also caused a fluctuation, recognisable as it had the same periodicity, in the potential measurements obtained.

Because of these difficulties, the results set out in Table 41 are those where a consistent potential measurement was obtained for at least three successive determinations. It can be seen that the variations are considerable even when it is presumed that the measurements are made from the same place in an oviduct in the same functional state. Some general characteristics did however become apparent.

The potential of the magnum region was always small. In only one experiment, in which a forming egg was actually in the magnum at the time, a mean potential of 10 mV. was recorded; in all others, it was less than 5 mV. (lumen negative), with occasional swings positive. The one exceptionally high result may have been linked with the stronger than usual peristaltic contractions which were observed at the time.

In the mid isthmus region, the potential was slightly greater, usually fluctuating around -10 mV. The highest values were obtained from oviducts which contained a plumping egg. Potentials here reached 20 to 25 mV. at times.

Values for the area designated 'lower isthmus', and for the shellgland, are the most difficult to interpret. The position of the junction of mid and lower isthmus can only be guessed if the

TABLE 41

POTENTIALS ACROSS THE OVIDUCT WALL IN VIVO

in mV, lumen -ve. Means of 3 or more determinations

Experiment No.	Breed of bird	State of oviduct	Magnum	Isthmus (mid)	Lower Isthmus	Shell-gland	Vagina
7	Shaver	Egg in magnum				- 5.1	
15	Thornber 404	Egg in magnum	-10.25	-10.18	-10.98	- 2.14	
21	B.L.(J)	Egg in magnum	- 1.7	- 5.8	- 9.3	- 9.24	
25	Shaver	Egg in magnum	- 3.9	- 9.1	-15.76	- 9.2	
8	Shaver	Egg in isthmus			-16.5		
15	Thornber 404	Egg in isthmus	-10.25	-10.18	-10.98	- 2.14	
6	Shaver	Soft egg in s.g.			-43.6		
19	B.L.(J)	Soft egg in s.g.	- 3.9	-19.8	-11.1	14.0	
22	Shaver	Soft egg in s.g.	- 2.34	- 9.6	- 7.25	0.0	-20.19
9	Shaver	Egg just laid			-24.9		
10	Shaver	Egg just laid			-26.7		
14	Thornber 404	Egg just laid			-36.7		
16	Thornber 404	Egg just laid		- 7.2	-20.2	-20.75	
17	Thornber 404	Egg just laid	- 0.05	- 8.94	- 9.1	-22.1	
11	Shaver	Empty				-13.93	
12	Shaver	Empty				-16.9	
20	B.L.(J)	Empty	+ 0.1	- 3.6			
23	Shaver	Empty	- 0.51	- 5.2	- 8.51	-24.25	
28	B.L.(J)	Inactive (regressed)	- 5.5	- 3.76	- 0.05		

oviduct is to be left reasonably undisturbed in the body cavity; on the other hand, a bridge pushed far enough down the oviduct to be beyond doubt below this junction is in danger of entering the shellgland. Inspection of Table 41 shows that the results for the two regions taken together fall into three categories.

I. Those, mainly in the lower isthmus column, which are around 10 mV., similar to the potentials of the mid isthmus region.

II. Higher values, ranging from about 15 mV. to over 40 mV. Potentials of this kind often showed considerable and rapid variations, so that at times this group overlapped the 10 mV. group, while at others, peaks of 50 to 60 mV. appeared.

III. Very low values, found in the shellgland pouch only; these showed fluctuations around zero, although the mean was usually slightly negative.

The most remarkable examples of group II were found in those oviducts which contained an egg in the plumping stage, or from which an egg had just been expelled. In four of the five cases, this had happened when the bird was on the operating table, and may even have been hastened by the anaesthetic.

The interpretation of this grouping of results was indicated partly by observations of the behaviour of the potential with movement of the salt bridge, and of the position of the bridge, which could not in the circumstances be accurately determined. It seems that the following tentative scheme would explain the distribution of the results.

The potential of the upper and mid isthmus regions, around 10 mV., persists to and perhaps slightly overlaps the junction with the lower isthmus. In the latter region, but for a short distance only, there is an area of comparatively high potential, probably about 30 to 40 mV., or occasionally more. This is probably a

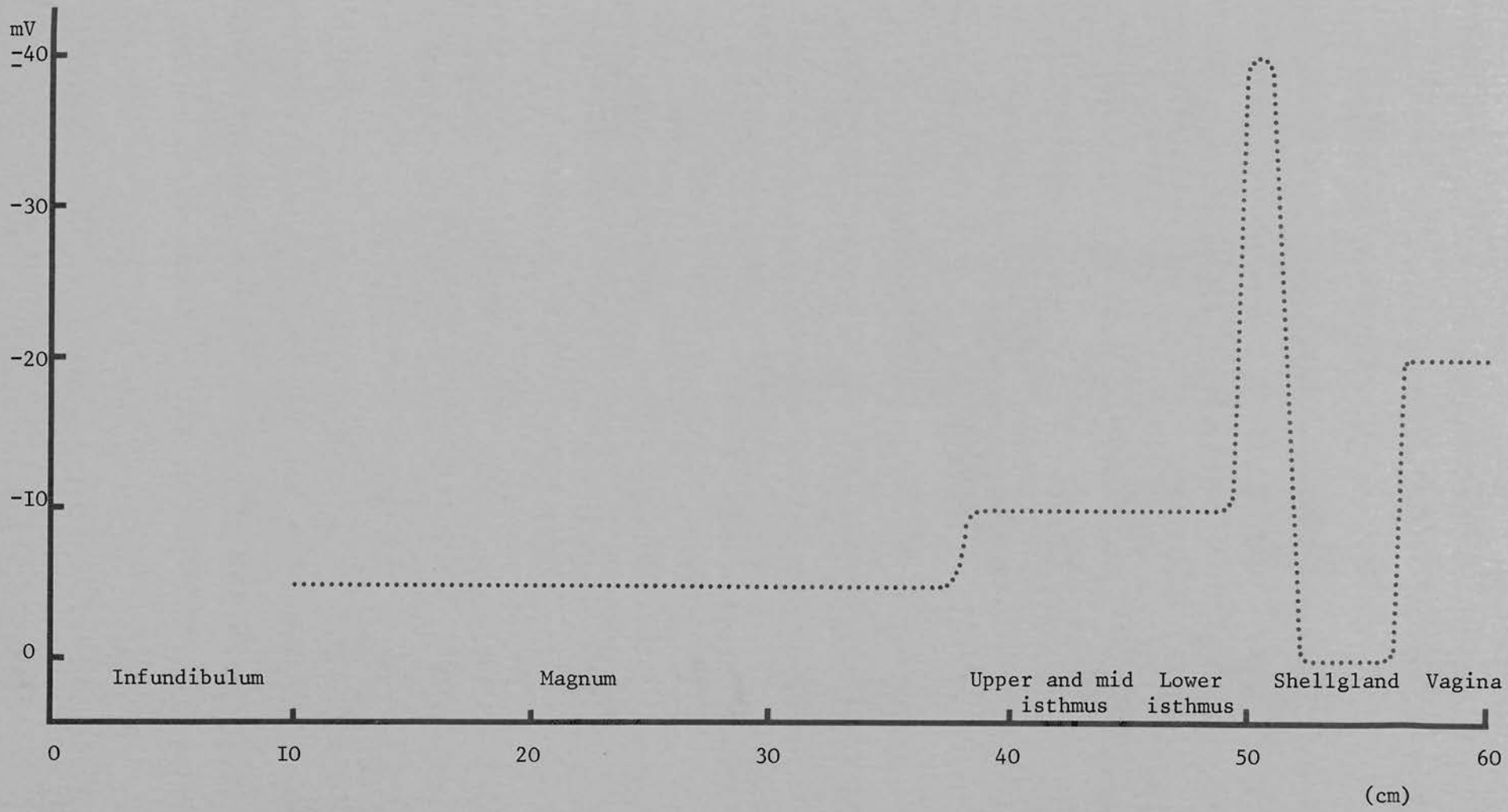


Fig.36. Suggested pattern of potentials in the active oviduct lumen. The highest potential is located in a narrow region near the lower isthmus - shell gland junction. The potential is always negative, except in the shellgland pouch, where it may be zero.

region of ion transport, and seems to correspond to the area in which electron microscopy shows cells of the type known to occur in such regions. (G. M. Wyburn and H. S. Johnston, personal communication). It may not extend for the whole length of the region which has been described on histological grounds as the lower isthmus. Frequently, these high potentials were observed when the bridge was estimated to be near the junction of the lower isthmus and the shellgland. A sharply delineated small area of high potential is compatible with the rapid and extreme fluctuations found in these readings, as comparatively little movement of the end of the measuring bridge would be needed to make a large change in the potential reading. This potential peak is highest at times when transport of minerals to the forming egg is thought to occur; at other times, it may be decreased. In some birds, it was not found at all.

When the bridge was carefully pushed into the pouch of the shellgland, well away from the isthmus junction, the potential found was usually of group III, and often was not significantly different from zero.

In the one experiment in which the vaginal potential was measured, a mean value of about -20 mV. (lumen negative) was obtained.

In the inactive oviduct (28, Table 41), the magnum potentials were of the same order as those in the active but empty oviducts, although slightly higher than those of the oviducts which contained an egg. No high-potential area was found in the lower isthmus; instead, the potential fluctuated around zero. No values were obtained for the shellgland.

The results of these experiments agree with the measurements in vitro to the extent that in both cases the mucosal side is

negative; however, the tissue portions used for the latter were taken from the pouch of the shellgland, where no significant potentials were found *in vivo*. The estimated initial potentials of these portions were comparable to those of either the mid or lower isthmus or the vaginal regions in the living bird. No explanation of this discrepancy is at present available.

SECTION 7

DISCUSSION

The results of the measurements and histological investigations on the oviduct, which are presented in Section 3, are in the main in agreement with the classical descriptions of this organ. The dimensions found correspond to the ranges reported previously, and the general characteristics of the regions are confirmed. The measurements on the regressed oviducts do draw attention to the striking rapidity of increase and decrease in length and mass of oviduct when the hen comes into or goes out of lay, even for a short period. But it appears from the measurements of surface area that this regression does not take place at a constant rate in all the regions. While the active oviduct had a greater surface area, corresponding to its greater secretory activity, than the inactive one, there was an even greater area in the magnum and isthmus of the bird which had recently stopped ovulating and was presumably pausing between clutches. A similar hypertrophy occurred in birds known to be internal layers, in which there had been ovulation but not oviposition, the yolk being reabsorbed from the body cavity rather than passing down the oviduct; and surprisingly in birds which had a thread in the shellgland and thus consistently produced soft-shelled eggs. This incomplete formation of the egg was first reported by Sykes (1953), and the subject has been reviewed by Lake and Gilbert (1964); the effect appears to be due to an increased motility of the oviduct which hastens the passage of the egg. This suggests that the hypertrophy, and thus the increased surface area, are due to the accumulation of secretions in these regions. This in turn supports the suggestion that the functions of the ovary and the

oviduct are partially independent, as ovulation continues apparently normally when the oviduct has been interefered with surgically, either by insertion of a thread or clip, or by partial or complete section. (Asmundson & Jervis, 1933; Asmundson and Burmester, 1938; Huston & Nalbandov, 1950; Juhn & Quigley, 1960).

The absolute values provided by this method of estimating the surface area are not very accurate. Since shrinkage may be supposed to affect different parts of the tissue comparably, and the ratio of two parts is used, errors from this source will not be great. But, as was shown, (see Section 2.4), there is a considerable variation between sections of the same tissue. In the example studied, the coefficient of variation was 18.3% for six sections, covering a region of the oviduct wall about a millimetre wide and a few centimetres long. The values of Table 9 for the surface area ratio strictly apply therefore only to the particular section measured, and the true surface area of the entire region may be larger or smaller. However as an indication of the order of magnitude of the area available, of the degree of change with functional state, of the difference between regions, and hence of the differences in glandular activity, the results are interesting.

In the case of the shellgland in particular, some calculations on the passage of calcium may be made. The completed egg shell contains some 2g., or 50 m-moles of calcium, almost entirely as the carbonate, and this is deposited over a period of 20 hours or less. (Romanoff & Romanoff, 1949; Warren & Scott, 1935; Bradfield, 1951). The anion may be accounted for by the carbon dioxide produced by the cells of the shellgland tissue (Hodges and Lorcher, 1967), and is in fact less than that which would be expected from a 10g. mass of shellgland (Draper, 1968). The calcium

is removed from the blood on its passage through the shellgland, as is shown by the drop in calcium levels in venous compared to arterial blood. (Winget, Smith and Hoover, 1958; Hunsaker & Sturkie, 1961.) The rate of this removal must be equivalent to that of shell deposition, since no storage of calcium can be demonstrated. This rate is 2.5 m-moles/hr., or 42 micromoles/min. For the bird in full lay, the surface area ratio R_a was approximately 40 (Table 9). If the serosal area is taken to be that of the egg, i.e. about 70 sq. cm., the corresponding mucosal surface has an area of approximately 2,800 sq. cm. This gives a value for the calcium transport of:

$$\frac{42 \times 10^6}{60 \times 2,800} = 250 \text{ p-moles/sq. cm./sec.}$$

with respect to mucosal surface area.

The effective serosal area may well be greater, as parts of the shellgland not in contact with the egg may still be active in the transport of calcium; if an area of 100 sq. cm. is taken, the figure becomes 175 p-moles/sq. cm./sec.; if the factor of 15 is used for R_a, (this is about the value found in sections of shellgland containing an egg), the rates are 670 p-moles/sq. cm./sec. for 70 sq. cm. of serosa, and 470 p-moles/sq. cm./sec. for 100 sq. cm. of serosa respectively. The rates per sq. cm. of serosa are of course greater by the factor R_a.

These calculations of fluxes are made on the basis of the surface area of the epithelium. It is probable that the tubular glands of the shellgland are active in the transport of calcium. The shellgland possesses a higher density of tubular glands than even the magnum, although it does not seem to secrete protein. It is difficult to think of any function other than calcium transport which this mass of mucosal tissue could be performing. As a result

of their immense complexity and close packing, estimates of the length and surface area provided by the tubular glands are difficult to make. Probably they increase the surface area by a factor of 100 at least.

Taking this into account, the flux of calcium is of the same order of magnitude as fluxes found in most biological systems. For example, those necessary to maintain the intracellular composition of muscle cells are about 4 - 10 p-moles/sq. cm./sec. There is one situation in which fluxes of 200 - 400 p-moles/sq. cm./sec. are found. This is the case of sodium and potassium in the nephron (Solomon, 1959, 1963). It would be surprising if the shellgland's movement of calcium were comparable to the kidney's rapid transport of these most important monovalent cations, and it seems much more likely that this movement takes place across the cells of the tubular glands. The epithelial cells may also be involved, or they may serve another function such as matrix secretion or potassium transport.

The greater part of the histological and histochemical study of the oviduct described in Sections 3.4 and 3.5 repeats and confirms the findings of other workers. It was felt that a comprehensive examination of the oviducts of modern light hybrid hens of the breeds to be used for other experiments was necessary, and the eversion and sectioning of the entire oviduct rather than the selection of small blocks of tissue from the different regions makes the study more complete than those already available. Partly as a result of this, the definition of the various regions and their junctions has been made with more confidence. In particular, it was possible to differentiate between three separate functional regions in the isthmus.

The results of the staining methods specific for mucopolysaccharides, although not new, are of interest in relation to the structure and composition of the organic components of the egg white. It is not always possible to distinguish precisely the different chemical classes of mucoid substances. However, the marked differences in the staining of the acidic and neutral mucopolysaccharides with Alcian blue make this distinction at least unmistakable. Neutral mucopolysaccharides are negative to this dye, as also to metachromatic dyes, mucicarmine, mucihaematein, and Hale's test, but will stain with the PAS procedure - as indeed will all mucopolysaccharides, with the exception of the acidic mucopolysaccharide, chondroitin sulphuric acid, where the reactive glycol groups may be substituted. Studies with ^{35}S autoradiographs suggest that the Alcian blue staining is in fact confined almost entirely to sulphated mucopolysaccharides (Casselman, 1959).

One may speculate that the extreme activity of the epithelial goblet cells in the lowest part of the magnum, the 'mucous-secreting region', bears a relation to the production of the outer layer of thick white. The stability of the thick white gel has been suggested to be a result of ovomucin-lysozyme cross-linking (Brooks and Hale, 1959, 1961), and ovomucin is a protein-sulphated mucopolysaccharide complex (Robinson & Monsey, 1966). In addition, the experiments of Asmundson and Jervis (1933) showed that resection of this part of the oviduct led to the production of small eggs, not by a significant reduction in the thick white, but by the accumulation of less outer thin white. The results of Section 5 suggest that this might be attributed to a change in the outermost layer of the thick white, reducing the forces moving the aqueous solution which forms the outer thin white, without causing an appreciable decrease in the absolute quantity of thick white.

Thus a decrease in plumping water could be the explanation of the production of smaller eggs.

Several attempts have been made in the past to locate calcium in the mucosa and epithelial layer of the isthmus and shellgland. Notable among these are the micro-incineration experiments of Richardson (1935), and the work of McCallion (1953), who used several techniques; that of von Kossa, the Cretin colour test, and tests with sulphuric and oxalic acid. This author claimed slight positive reactions in the shellgland with the Cretin test, but negative results with the method of von Kossa. In the present study, this method gave negative results for the everted oviduct, but when oviducts were fixed with a forming egg in the isthmus or shellgland region, a strong positive reaction was obtained around the mammillary cores and in those epithelial cells which were in contact with the membrane or shell. According to Casselman (1959), the results of the oxalate formation method are in any case liable to be misleading, and the microincineration method, although the only one which may demonstrate the presence of unreactive calcium, is non-specific and inconclusive. The von Kossa method has the merit of simplicity. The equations of the chemical reactions which occur are:

$$CaCO_3 + 2\,Ag^+ \rightarrow Ag\,CO_3 + Ca^{++}$$
$$Ag_2CO_3 + 2H^+ \rightarrow 2\,Ag + H_2O + CO_2$$

Unfortunately, as is clear from these equations, the positive reaction does not depend on the presence of calcium, but on that of the insoluble anion, carbonate, phosphate, or oxalate. In animal tissues, it is usually assumed that these are associated with calcium. Other sites which might be suggested to have an affinity for silver, for example acidic mucopolysaccharides, would be expected to give rise to background staining, which was not seen

except in cases where shell calcification was well advanced; in any case, von Kossa positive material and Alcian blue staining were not found in the same areas. The possibility that some other site is responsible for the staining, or that the reaction is caused by carbonate or phosphate which is not in association with calcium in the living tissue, should not however be overlooked. Pearse (1960) considers that none of the many substances which reduce silver nitrate to metallic silver is likely to cause confusion except perhaps uric acid or urates; he suggested removal of these by treatment with saturated aqueous lithium carbonate. When this was done, no difference in the distribution of staining was found. Carbonate and phosphate, and the enzymes carbonic anhydrase and alkaline phosphatase, are undoubtedly present in higher concentrations in the active shellgland than in other regions or at other times (Brown and Badman, 1961; Chakravorti and Sadhu, 1961; Misra and Kemeny, 1964a; Diamantstein and Schlüns, 1966). It is interesting that the histochemical method for the demonstration of alkaline phosphatase, that of Gomori, uses the von Kossa technique as the final step. This suggests the possibility that calcium dissolved in the fixative solution, or present in another form, could be converted to phosphate by alkaline phosphatase during the fixation. The fact that no staining appears except in cells in contact with the shell during the fixation procedure could be taken to suggest that some such process takes place; or alternatively, that in the absence of the shell, intracellular calcium is free to leave the epithelial cells. These problems may be resolved by the application of autoradiographic techniques; without further information they must remain a matter of conjecture.

The chemical analyses of the oviduct also confirm in general the findings of other workers. In the light of recent suggestions (Draper, 1968a) that cells actively engaged in the synthesis of protein tend to have a high sodium and a low potassium relative to other cells, the occurrence of almost equal quantities of these ions in the magnum and mid and upper isthmus regions in particular is interesting. In all regions, the sodium is noticeably higher than in mature skeletal muscle of either the bird or the mammal. The slightly increased concentration of calcium in the mid-isthmus region of the oviduct prior to shell crystal seeding is not accompanied by a similar accumulation of magnesium in the isthmus or of calcium in the shellgland. This suggests that these minerals, the magnesium in the seeding stage (where the Ca:Mg ratio is much lower than during the greater part of shell formation) and the calcium in later stages, must be removed from the blood supply at the time of their deposition in the shell. However, the conclusions which may be drawn from the consideration of gross composition of the multi-layered oviduct wall are necessarily limited, and must be confirmed by recourse to other techniques.

In contrast, the information provided by analysis of the egg, which is easily separable into its components, is more specific. In Sections 4.3 and 4.4 it was shown that while the most variable component of the egg over a short period was the dry matter of the white, over a period of a year or so greater changes took place in the weight of the yolk, particularly the dry matter, and in the water content and minerals of the white. It would seem that the protein-secreting apparatus of the magnum functions at full capacity from the earliest eggs laid, but that it is subject to fluctuations in output, possibly reflective of the interval between eggs during which albumen may accumulate, or alternatively of the difficulty and com-

plexity of the process itself. The ability of the bird to form a large yolk of high solid content, on the other hand, improves rapidly and steadily with the passage of time: in the example of Table 21, almost doubling in the year. The efficiency of shell formation, also, one may suppose, a process making considerable demands on the organism, changes little on either a short-term or a long-term basis. It could be suggested that the limitation here was not in the oviduct at all, but in the ability of the bird to absorb and mobilise the large quantities of calcium which are needed. The calcium metabolism of the bird is too large a subject to attempt to comment upon here; however, experiments performed in the P.R.C., as well as the large body of work on the subject (vide Simkiss, 1961, 1967), show that on reaching negative calcium balance, the normal bird will cease laying until positive balance is restored. This, and possibly the decrease in the proportion of calcium to magnesium and organic matrix in the outermost layers of the shell, may be taken to support the idea that shell formation is limited by the supply of calcium to the shellgland and not by its transport therein.

The increase in white water and minerals, like that of yolk size, is steady over the long term, whereas short term fluctuations are comparatively small. That part of the increase which takes place in accumulation of a passive nature, as the main part of the plumping would seem to be, may be attributed to an improvement in the quality of the outermost layer of thick white leading to an increase in magnitude of forces of an osmotic nature. Whether this improvement in quality represents an increase in concentration of ovomucin, if ovomucin is indeed the relevant substance, or in some other factor such as perhaps the degree of cross-linking, is a matter for conjecture. Another possible explanation of the

effect is that it is indirectly a result of the greater size of the yolk. This would cause the same quantity of thick white to present a larger surface to the surrounding fluid; if shell deposition is assumed to occur at a constant rate, this would, as well as increasing the quantity of osmotically active substance exposed, increase the time in which plumping may take place by delaying the formation of a rigid layer of shell.

If, as seems possible, the accumulation of minerals which takes place during the rest of the time which the egg spends in the shell-gland is partly or wholly a result of active processes, the increase in the quantities of all four ions studied, and in concentrations of sodium and potassium, probably implies an increase in the efficiency of the transport mechanism. Again, however, it could be a reflection of a longer stay in the shellgland. This seems less likely, as there is nothing to suggest that the overall duration of the process of shell formation increases with the age of the bird. This process takes up the greater part of the interval between one egg and the next, an interval which has been found to be remarkably constant for an individual bird.

At no stage in the formation of the egg is the mineral composition of the white the same as that of the hen's blood. The sodium concentration is always lower than the plasma level; in the oviducal egg analyses of Table 24, the highest concentrations, in groups (3) and (4), are 134 m-moles/l. of water, compared with 172 m-moles/litre of plasma water. On the other hand, even in the magnum eggs of group (1), the concentrations of the other three ions are much higher than those found in plasma. The quantities of calcium and magnesium do not seem to be increased after the formation of the membrane; as a result, the concentrations fall as water is added to the white during the plumping. It is also possible that

some of the calcium and magnesium from the white are involved in the seeding of shell crystals, and this would cause a further slight drop. The concentrations of these ions in the magnum white, then, are about ten times greater than the levels found in blood plasma. (Calcium taken as ionised calcium; the white concentration is only slightly higher than the total blood calcium.) Unless they are present in a bound form, calcium and magnesium must be moved into the white against a concentration gradient. This is also true of potassium, although the difference is not quite so marked for this ion, the concentration in the magnum being 10 m-moles/l. in comparison with a blood level of 6.3 m-moles/l. This concentration however increases steadily during plumping and shell formation.

The suggestion that accumulation of minerals in the later stages of egg formation at least may require active transport arises mainly from the analyses of oviducal eggs and of shellgland fluid presented in Table 24, Section 4.5, and Tables 25 - 28, Section 4.6. If the egg white only is considered, between the stage represented by group (3) of Table 24, at the end of plumping, and oviposition, the sodium content of the white falls while the potassium content rises, the sum being comparatively constant as is shown in Figure 22. As is to be expected from the extreme permeability of the shell membrane, the surrounding fluid reflects a similar change, but to a more marked degree. It may be supposed that it is the composition of the fluid which is actually being changed, and that the egg white then equilibrates with it. The effect may be explained as follows: The fluid in the lumen of the oviduct is probably initially a filtrate of blood. In the early stages of shell formation, its composition is fairly similar, although there is always a rather higher concentration of potassium. It is this fluid which enters

the egg during plumping. During the later stages, however, the sodium in the oviducal lumen is exchanged for potassium by a mechanism operating in the wall of the oviduct. From Figures 22 and 23, it would seem that the exchange could have a simple one-for-one ratio. As the potassium in the fluid increases and the sodium decreases, similar changes take place in the egg white to maintain equilibrium and remove the concentration gradients which are formed.

The occasional occurrence of eggs with very low sodium concentrations, 70 m-moles/l. or less, and shellgland fluid samples with even lower values, of 35 - 40 m-moles/l. in the later stages of egg formation, is in agreement with the suggestion of the outward transport of sodium from the oviducal lumen.

This is not the only possible explanation of the changes in white composition, and the small number and wide variation of the data leave scope for doubt as to its likelihood. The experiments on oviducal transport and potentials, which will be discussed later, seem to lend it some support.

Although a higher than average concentration of magnesium in the innermost part of the shell was indicated by the results of Itoh and Hatano (1964), it is rather surprising to find a calcium to magnesium ratio as low as 1.5 when there is about 20 micromoles of calcium. This is about the quantity of calcium that chemical analysis suggests is stored in the isthmus and transferred to the membrane to start calcification, but no corresponding storage of magnesium has been detected. According to Brooks and Hale (1955), the highest proportion of magnesium which can be accepted by the crystalline structure of calcite is 0.048, that is a Ca/Mg ratio of 20.83. The crystal structure of this part of the shell is not so ordered as is the spongy layer, and the individual crystal forms of $CaCO_3$ and $MgCO_3$ are not very dissimilar. It is even possible

that crystals of calcium magnesium carbonate, which occurs in nature as dolomite, may be formed.

The presence of magnesium in the mammillary layer of the shell, which is easily detached during incubation, may be of considerable value in meeting the requirements of the forming embryo for this mineral. There is no indication of the reason for the low Ca:Mg ratio, although it could be suggested that at this stage of egg formation calcium mobilisation processes were not functioning at maximum efficiency, and that similar quantities of the two minerals are available to a non-specific transport process. This too could explain the fall in the ratio towards the outer side of the shell, when reserves of rapidly-mobilisable calcium may be almost exhausted. The question invites further study, possibly again by labelling techniques.

The finding that the shell membrane is extremely permeable, and permits the passage of protein molecules as well as inorganic ions, is not surprising in view of its mesh structure (see Section 4.1). At first sight, however, this is difficult to reconcile with the artificial 'plumping' which has also been demonstrated. The experiments in which egg-white in a punctured membrane, or even in a loose-meshed nylon sac, was shown to take up water suggest the answer. The thick white of the egg has the nature of a gel. Many gels have the ability to absorb considerable quantities of water, swelling as they do so, before the cohesion between the molecules is broken down. Where the water is replaced by a highly dissociated solution with a pH well removed from the isoelectric point of the gel substance, this effect is enhanced by the interaction of the ions of the solution with the gel to produce ions which are bound in the gel structure. These ions cannot diffuse out of the gel, but the other ions of the system can move freely.

Thus a Donnan equilibrium is set up, and the resulting ionic distribution may cause an excess of osmotic pressure in the gel which will result in swelling. The theoretical treatment of such systems, first developed to explain certain processes used in the manufacture of leather by Procter, Loeb, and Wilson, is described by Bolam (1932). It is impossible to ascertain on the basis of the experiments of this study whether or not thick egg white is such a system. The pH of the oviduct, however, at around 7.5 (Ogasawara, Van Krey and Lorenz, 1964; Winget, Mepham & Averkin, 1965) is well removed from the isoelectric points of egg white proteins, most of which are around pH 4.5, with several below pH 4 (Parkinson, 1966). This makes it possible that such an enhancement does operate, rather than the repression of the swelling produced by neutral salts. In either case, the distribution of ions between the egg white gel and the surrounding liquid outer thin white and shellgland fluid will be affected. The repression can, in any case, not be very great, as it should otherwise be evident in the behaviour of the eggs soaked in $\frac{1}{2}$ M. salt solutions.

Although the plumping phenomenon can be accounted for in terms of the operation of physical forces, such as osmotic pressure or more likely gel hydration, in an entirely passive way, the accumulation of potassium by the shellgland which was demonstrated *in vitro* is less easily explained. This *in vitro* accumulation took place at a rate which was comparable to that required for the observed increase in the potassium content of the egg white during the stay of the egg in the shellgland. Under the conditions of the experiments, it was not possible to say whether the inward flux of potassium across the wall of the shellgland was accompanied by an outward flux of sodium, or indeed by the movement of any other ion present in either direction. In fact, there is no clear evidence that the

increase in potassium concentration measured is caused by a flux at all. It could be suggested that the potassium is provided, for example, by the rupture of the epithelial cells and the release of their contents into the oviducal fluid. It has been suggested that rupture of epithelial cells may take place during shell formation (Surface, 1912), and in fixed tissues they are certainly fragile. It seems unlikely that sufficient potassium could be provided by this method, as is shown by the following calculation.

If the surface area of the shellgland in contact with the egg is assumed to be approximately 70 sq. cm., and the average cell height is taken as 25 microns, the cellular volume is 175×10^{-3} ml. Suppose the intracellular concentration of potassium is 140 mM. This is around the concentration found in mammalian and amphibian muscle cells (128 - 152 m-moles/l. cell water) and in erythrocytes (112 m-moles/litre for the duck), although slightly lower than that in dog liver (161 mequiv/kg. cell water) and much lower than that of nerve axoplasm (320 - 380 mequiv/kg.) (Harris, 1960.) The amount of potassium in these epithelial cells will then be 24.5 micromoles. Now, the amount added to the egg is about 1500 micromoles over a period of approximately 20 hours, that is a rate of 75 micromoles/hr. This would mean that cells covering the entire egg surface would need to be ruptured every 24.5/75 hrs., i.e. every 19.6 minutes. If a surface area ratio of 40, the highest value found, is taken, the cells of the entire epithelium would have to be shed about every 13 hours, that is $1\frac{1}{2}$ times during the formation of the egg. Imondi and Bird (1966) give a period of about 48 hours for the turnover of intestinal epithelium in the chick, and rates in mammalian intestine are similar. It seems unlikely that turnover in the oviduct, if such a turnover occurs at all, would be much faster.

In contrast with the fluxes of calcium which were calculated to occur, the potassium flux of about 20,000 p-moles/sec., spread over a mucosal surface area of 100 sq. cm. (serosal area) x 40 (surface area ratio of active shellgland), giving a net flux of as little as 5 p-moles/sq. cm./sec., could quite reasonably be attributed to the cells of the surface epithelium of this part of the oviduct. These cells are probably involved in some energy consuming process, as electron microscopy shows them to contain numerous mitochondria. (G. M. Wyburn and H. S. Johnston, personal communication.)

Until recently, the idea of reproducing the function of the shellgland outside the living bird does not seem to have occurred to anyone; at least, no report of the attempt has been found. Since the present study was commenced, Ehrenspeck, Schraer and Schraer (1967) have published an account of a study using an experimental procedure rather similar to that described in Section 2.10 and employed to obtain the results presented in Section 6.2. The object of their experiments was to demonstrate the transport of calcium, and this they were able to do, using ^{45}Ca. The average net influx of calcium found for six shellglands which had contained an egg at the time of death was 0.03 micromole/sq. cm./hr., with respect to serosal surface area, or, in the units used in this study, about 8.33 p-mole/sq. cm./sec. This is much less than the 250 p-moles/sq. cm./sec. with respect to mucosal surface area with a ratio of $R_a = 40$, or about 10,000 p-mole/sq. cm./sec. with respect to serosal area, which is estimated to be necessary for shell formation, and would have caused a change in the calcium concentration of the solutions used in the experiments of this study which would be well within the experimental errors.

Ehrenspeck, Schraer and Schraer concluded that calcium movement in their experiments was dependent on oxidative phosphorylation and required the generation of phosphate-bond energy, and that it was predetermined by the physiological state of the shellgland. Similar conclusions are suggested by the behaviour of the potential across the oviducal wall, both *in vitro* and *in vivo*. The rapid disappearance of the *in vitro* potential suggests that this is produced by a mechanism even more sensitive to oxygen supply than the calcium transport of Ehrenspeck *et al*. This mechanism is clearly not that of potassium accumulation, as this continues at a steady rate for a much longer period. The response of the potential to the addition of ATP to the incubation medium, although partial, indicates that it, like the calcium transport, is dependent on phosphate-bond energy. Since measurements were attempted only on active shellgland tissue, it is not possible to say whether there is a dependence on functional state.

Such a dependence was however found in the *in vivo* measurements. Of particular interest among the results of these were the absence of potential difference in the pouch of the shellgland; this could of course only be effectively measured in the absence of an egg, but nonetheless suggests that calcium transport does not, if it occurs in this region as would be expected, involve such a potential difference; and the peak in potential found at about the bottom of the isthmus region. That this is an area where electron microscopy shows cells with the characteristics of an ion transport region might suggest that it is the area where potassium is moved across the oviduct wall, but again the continuation of potassium transport *in vitro* does not bear this out.

All these questions must await further investigation by more sensitive methods, or at the least many more measurements on more homogenous groups of birds, before definitive answers may be suggested. The complexity of the processes involved both in the addition of minerals to the white and in the formation of the shell appears to increase as they are studied. The work described here has set the stage for further investigation in depth. Clearly, the opportunities for further study of the avian oviduct are almost unlimited, and further work may identify biological bottlenecks of importance to the quality of both the white and the shell of the egg.

REFERENCES.

REFERENCES

Aitken,R.N.C. & Johnston,H.S. (1963). Observations on the fine structure of the infundibulum of the avian oviduct. J.Anat.97, 87 - 99.

Almquist,H.J. (1936). Formation of chalazae in the hen's egg. Poult.Sci. 15, 460 - 461.

Anfinson,C.B. & Steinberg,D. (1951). Studies on the biosynthesis of ovalbumin. J. biol. Chem. 189, 739 - 744.

Asmundson,V.S. (1931). The formation of the hen's egg. Scient. Agric. 11, 1 - 50.

Asmundson, V.S. (1939). Formation of the egg in the oviduct of birds: observations on turkeys. Proc.7th Wld's Poult.Congr.,96 - 99.

Asmundson, V.S. & Burmester, B.R. (1936). The secretory activity of the parts of the hen's oviduct. J. exp. Zool. 72, 225 - 245.

Asmundson, V.S. & Burmester, B.R. (1938). The effect of resecting a part of the uterus on the formation of the hen's egg. Poult. Sci. 17, 126 - 130.

Asmundson,V.S. & Jervis, J.G. (1933). The effect of resection of different parts of the oviduct on the formation of the hen's egg. J. exp. Zool. 65, 395 - 420.

Baker, J.R. (1966). Cytological Technique. 5th edition. London: Methuen & Co. Ltd.

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

Baker, R.C. & Stadelman, W.J. (1958). Chicken egg chalazae: strain and individual hen variations and their relation to internal quality. Poult. Sci. 37, 558 - 564.

Balch, D.A., & Tyler, C. (1964). Variation in some shell membrane characteristics over different parts of the same shell. Br. Poult. Sci. 5, 201 - 215.

Beadle,B.W., Conrad, R.M., & Scott, H.M. (1938). The composition of the uterine secretion of the domestic fowl. Poult. Sci. 17, 498 - 504.

Bellairs, R., Harkness, M., & Harkness, R.D. (1963). The vitelline membrane of the hen's egg: a chemical and electron microscopical study. J. Ultrastruct. Res. 8, 339 - 359.

Benesch, R., Barron, N.S., & Mawson, C.A. (1944). Carbonic anhydrase, sulphonamides, and shell formation in the domestic fowl. Nature,Lond., 153, 138 - 139.

Benzinger, T., & Krebs, H.A. (1933). Über die Harnsäuresynthese im Vogelorganismus. Klin. Wschr. 12, 1206 - 1208.

Besch, E.L., & Sluka, S.J. (1966). Blastoderm location in the avian egg. Poult. Sci. 45, 259 - 262.

Bligh, E.G., & Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37,911 - 917.

Blyth, J.S.S., & Sang, J.H. (1960). Survey of line crosses in a Brown Leghorn flock. 1. Egg production. Genet. Res. 1, 408 - 421.

Bolam, T.R. (1932). The Donnan Equilibria. London: Bell & Sons, Ltd.

Bradfield, J.R.G. (1951). Radiographic studies on the formation of the hen's egg shell. J. exp. Biol. 28, 125 - 140.

Bradley, O.C. (1928). Notes on the histology of the oviduct of the domestic hen. J. Anat. 62, 339 - 345.

Bradley, O.C., & Grahame, T. (1960). The structure of the Fowl. 4th ed. Edinburgh and London: Oliver and Boyd.

Brambell, F.W.R. (1925). The part played by the Golgi apparatus in secretion and its subsequent reformation in the cells of the oviducal glands of the fowl. Jl. R. Microsc. Soc. 45, 17 - 20.

Brandt, A. (1889). Anatomisches Allgemeines über die sog. Hahnenfedrig-keit undsoweiter bei Vögeln. Z. wiss. Zool., 48, 101 - 190.

Breen, P.C. (1966). The fine structure of the secretory glands of the uterus (shellgland) of the chicken. Anat. Rec. 154, 321.

Bronsch, K. & Diamantstein, T. (1965). Mucopolysaccharides as a localised factor of eggshell stability. Nature Lond. 207, 635 - 636.

Brooks, J. & Hale, H.P. (1955). Strength of the shell of the hen's egg. Nature, Lond. 175, 848 - 849.

Brooks, J. & Hale, H.P. (1959). The mechanical properties of the thick white of the hen's egg. Biochim. biophys. Acta 32, 237 - 250.

Brooks, J. & Hale, H.P. (1961). The mechanical properties of the thick white of the hen's egg. II. The relation between rigidity and composition. Biochim. biophys. Acta 46, 289 - 301.

Brown, W.O. & Badman, H.G. (1962). Respiration rate and alkaline phosphatase activity of the regions of the avian oviduct. Poult. Sci. 41, 654 - 657.

Brown, W.O. & Jackson, N. (1960). A study of the composition of the normal and hormone-stimulated oviduct of the common fowl, with special reference to its amino acid and nucleic acid content. Poult. Sci. 39, 602 - 611.

Bryant, R.L. (1944). A single comb White Leghorn pullet with two ovaries and oviducts. Poult. Sci. 23, 77 - 78.

Buckner, G.D.,Martin, J.H., & Hull, F.E. (1930). The distribution of blood calcium in the circulation of laying hens. Am. J. Physiol. 93, 86 - 89.

Buckner, G.D., Martin, J.H., & Peter, A.M. (1925). Chemical studies of the oviduct of the hen. Am. J. Physiol. 71, 349 - 354.

Burmester, B.R. (1940). A study of the physical and chemical changes of the egg during its passage through the isthmus and uterus of the hen's oviduct. J. exp. Zool. 84, 445 - 500.

Burmester, B.R. & Card, L.E. (1939). The effect of resecting the so-called 'chalaziferous region' of the hen's oviduct on the formation of subsequent eggs. Poult. Sci. 18, 138 - 145.

Burmester, B.R. & Card, L.E. (1941). Experiments on the physiology of egg white secretion. Poult. Sci. 20, 224 - 226.

Burmester, B.R., Scott, H.M. & Card, L.E. (1939). Rate of eggshell formation in the hen. Proc. 7th Wld's Poult. Congr., 99 - 101.

Burmester, B.R., Scott, H.M. & Card, L.E. (1940). The rate of plumping of uterine eggs immersed in an artificial uterine secretion. Poult. Sci. 19, 299 - 302.

Casselman, W.G.B. (1959). Histochemical Technique. London:Methuen & Co. Ltd. New York: John Wiley & Sons Inc.

Chakravorti, K.P. & Sadhu, D.P. (1961). Some aspects of the histochemical and histological studies on the oviduct of the hen. Proc. zool. Soc. (Calcutta) 14, 27 - 32.

Chappelier, A. (1913). Persistance et developpement des organes genitaux droits chez les femelles adults des oiseaux. Bull. Scient. Fr. Belg. 47, 361 - 372.

Cole, R.K. (1938). Histology of the oviduct of the fowl in relation to variations in the condition of the firm egg albumen. Anat. Rec. 71, 349 - 361.

Common, R.H. (1933). Observations on the mineral metabolism of pullets, I. J. Agric. Sci. 23, 555 - 569.

Common, R.H. (1936a). Observations on the mineral metabolism of pullets, II. J. Agric. Sci. 26, 85 - 100.

Common, R.H. (1936b). Serum phosphatase in the domestic fowl. J. Agric. Sci. 26, 492 - 508.

Common, R.H. (1938). Observations on the mineral metabolism of pullets, III. J. Agric. Sci. 28, 347 - 366.

Common, R.H. (1941a). Observations on the mineral metabolism of pullets, V. Acid-base equilibria and reproductive activity. J. Agric. Sci. 31, 281 - 294.

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

Common, R.H. (1943). Observations on the mineral metabolism of pullets, VII. The calcium requirement of the laying bird. J. Agric. Sci. 33, 213 - 220.

Conrad, R.M. & Phillips, R.E. (1938). The formation of the chalazae and inner thin white. Poult. Sci. 17, 143 - 146.

Conrad, R.M. & Scott, H.M. (1942). The accumulation of protein in the oviduct of the fowl. Poult. Sci. 21, 81 - 85.

Coste, M. (1847). Histoire Générale et particulière du développement des corps organisees. Tome 1. Paris.

Cotlove, E., Trantham, H.V. & Bowman, R.L. (1958). An instrument and method for automatic rapid accurate and sensitive titrations of chloride in biological samples. J. Lab. clin. Med. 51, 461 - 468.

Curtis, M.R. (1910). The ligaments of the oviduct of the domestic fowl. Ann. Rept. Maine Agr. Expt. Stat., Bull. 176, 1 - 20.

Curtis, M.R. (1914). A biometrical study of egg production in the domestic fowl. IV. Factors influencing the size, shape, and physical constitution of eggs. Arch. Entw.-mech. Org. 39, 217 - 327.

Curtis, M.R. (1915). Studies on the physiology of reproduction of the domestic fowl. XII., On an abnormality of the oviduct and its effect upon reproduction. Biol. Bull. 28, 154 - 163.

Cushny, A.R. (1902). On the glands of the oviduct in the fowl. Am. J. Physiol. 6, xviii - xix.

Daniel, E.E., Hunt, J. & Allan, D. (1960). Electrolytes in human myometrium. Am. J. Obstet. Gynec. 79, 419 - 427.

Danielli, J.F. (1953), Cytochemistry - A critical approach. New York: John Wiley & Sons, Inc. London: Chapman & Hall, Ltd.

David, D.J. (1960). The application of atomic absorption to chemical analysis: a review. The Analyst, 85, 779 - 791.

Diamantstein, T. & Schlüns, J. (1966). Behaviour of carbonic anhydrase in the uterus of hens during moult. Nature,Lond. 209, 304.

Dittmer, D.S. (ed.) (1961). Blood and other body fluids. Biological Handbooks: Federation of American Societies for Experimental Biology, Washington.

Draper, M.H. (1966). The accumulation of water and electrolytes in the egg of the hen. pp. 63 - 74 in Physiology of the Domestic Fowl. B.E.M.B. Symposium I. Edinburgh & London: Oliver & Boyd.

Draper, M.H. (1967). The transport of minerals to the white of the hen's egg. XIIIth Wld's Poult. Congr. Proc., 333 - 336.

Draper, M.H. (1968a). Changes in mineral and protein content of skeletal muscles from growing chickens. J. Physiol. 196, 85 - 86P.

Draper, M.H. (1968b). Discussion on Part 1 of Egg Quality. B.E.M.B. Symposium IV. Edinburgh & London: Oliver & Boyd.

Ehrenspeck, G., Schraer, H. & Schraer, R. (1967). Some metabolic aspects of calcium movement across the isolated avian shellgland. Proc. Soc. Exp. Biol. Med. 126, 392 - 395.

El Jack, M.H. & Lake, P.E. (1967). The content of the principal inorganic ions and carbon dioxide in uterine fluids of the domestic hen. J. Reprod. Fert. 13, 127 - 132.

Finlay, G.F. (1925). Studies on sex differentiations in fowls. Brit. J. Exp. Biol. 2, 439 - 468.

Fisher, R.B. & Parsons, D.S. (1950). The gradient of mucosal surface area in the small intestine of the rat. J. Anat. 84, 272 - 282.

Forsythe, R.H. & Bergquist, D.H. (1951). The effect of physical treatment on some properties of egg white. Poult. Sci. 30, 302 - 311.

Foster, M. & Balfour, F.M. (1874). Elements of Embryology. London: Macmillan & Co.

Frank, F.R., Burger, R.E. & Swanson,M.H. (1965). The relationships among shell membrane, selected chemical properties, and resistance to shell failure of Gallus Domesticus eggs. Poult. Sci. 44, 63 - 69.

Frank, F.R. & Vohra, P. (1964). Synthesis of egg shell matrix in the hen uterus. Poult. Sci. 43, 1319.

Frobose, H. (1928). Die mikroskopische Anatomie des Legedarmes und Bemerkungen über die Bildung der Kalkschale beim Huhn. Z. Mikosk-anat. Forsch. 14, 447 - 482.

Fujii, S. (1963). Histological and histochemical studies on the oviduct of the domestic fowl with special reference to the region of uterovaginal juncture. Archvm. histol. jap. 23, 447 - 459.

Fuwa, K. & Vallee, B.L. (1963). The physical basis of analytical atomic absorption spectrometry: the pertinence of the Beer-Lambert Law. Analyt. Chem. 36, 942 - 946.

Giersberg, H. (1922). Untersuchungen über Physiologie und Histologie des Eileiters der Reptilien und Vögel, nebst einem Beitrag zur Fasergenese. Z. wiss. Zool. 120, 1 - 97.

Gilbert, A.B., Lake, P.E. & Wood-Gush, D.G.M. (1966). Aspects of the physiology of the transport of the ovum through the oviduct of the domestic hen. pp. 11 - 13 in Physiology of the Domestic Fowl. B.E.M.B. Symposium I. Edinburgh & London: Oliver & Boyd.

Godfrey, G.F. (1948). Some histophysiological aspects of the uterus in the domestic fowl. Poult. Sci. 27, 664 - 665.

Greenwood, A.W. (1935). Perforation of the oviduct in the domestic fowl. Trans. Dynamics of Development 10, 81 - 92.

Greenwood, A.W. & Blyth, J.S.S. (1938). Experimental modification of the accessory sexual apparatus in the hen. Q. Jl. exp. Physiol. 28, 61 - 69.

Grini, O. (1939). Luminescence test for eggs. Proc. 7th Wlds. Poult. Congr. & Exposn, Cleveland. 496 - 498.

Gutowska, M.S. & Mitchell, C.A. (1945). Carbonic anhydrase in the calcification of the egg shell. Poult. Sci. 24, 159 - 167.

Gutowska, M.S., Parkhurst, R.T., Parrot, E.M. & Verburg, R.M. (1943). Alkaline phosphatase and egg formation. Poult. Sci. 22, 195 - 204.

Hansen, C.H. (1933). On the formation of the hen's egg, with especial reference to the white. Proc. 5th Wld's Poult. Congr.,2, 587 - 593.

Harris, E.J. (1960). Transport and accumulation in biological systems. 2nd ed. London: Butterworths.

Hawk, P.B., Oser, B.L. & Summerson, W.H. (1947). Practical Physiological Chemistry. London: Churchill.

Hawkins, D.F. & Nixon, W.C.W. (1958). The electrolyte composition of the human uterus in normal pregnancy and labour and in prolonged labour. J. Obst. Gynaec. Br. Commonw. 65, 895 - 910.

Heller, V.G., Paul, H. & Thompson, R.B. (1934). Changes in the blood calcium and phosphorus partition during the life cycle of the chicken. J. Biol. Chem. 106, 357 - 364.

Hertelendy, F. & Taylor, T.G. (1961). Changes in blood calcium associated with eggshell calcification in the domestic fowl. I. Changes in total calcium. Poult. Sci. 40, 108 - 114.

Higginson, T.W. (1863). Out-door Papers. Boston: Ticknor & Fields. Cited by Romanoff & Romanoff, 1949.

Hinton, H.R. (1968). Storage of eggs. pp. 251 - 261 in Egg Quality. B.E.M.B. Symposium IV. Edinburgh & London: Oliver & Boyd.

Hodges, R.D. (1966). The functional anatomy of the avian shellgland. pp. 191 - 198 in Physiology of the domestic fowl. B.E.M.B. Symposium I. Edinburgh & London: Oliver & Boyd.

Hodges, R.D. & Lörcher, K. (1967). Possible sources of the carbonate fraction of egg shell calcium carbonate. Nature, Lond. 216, 609 - 610.

Hohman, W. & Schraer, H. (1966). The intracellular distribution of calcium in the mucosa of the avian shell gland. J. Cell. Biol. 30, 317 - 331.

Hoover, G.N. & Smith, A.H. (1958). Secretion of fluid by the shellgland of the laying hen. Poult. Sci. 37, 467 - 471.

Hoover, G.N., Smith, A.H. & Abbott, U.K. (1962). Some mechanical properties of the avian shell gland. Poult, Sci. 41, 1137 - 1145.

Hughes, J.S. & Scott, H.M. (1936). The change in concentration of ovoglobulin in egg white during egg formation. Poult. Sci. 15, 349 - 351.

Hughes, J.S., Titus, R.W., & Smits, B.L. (1927). The increase in the calcium of hen's blood accompanying egg production. Science, 65, 264.

Hunsaker, W.G. & Sturkie, P.D. (1961). Removal of calcium from the uterine blood during shell formation in the chicken. Poult. Sci. 40, 1348 - 1352.

Hurwitz, S. (1964). Calcium metabolism of pullets at the onset of egg production, as influenced by dietary Ca level. Poult. Sci. 43, 1462 - 1472.

Huston, T.M. & Nalbandov, A.V. (1950). The effect of injury of the oviduct on the ovary. Poult. Sci. 29, 764.

Imondi, A.R. & Bird, F.H. (1966). The turnover of intestinal epithelium in the chick. Poult. Sci. 45, 142 - 147.

Itoh, H. & Hatano, T. (1964). Variation of Magnesium and Phosphorus deposition rate during egg shell formation. Poult. Sci. 43, 77 - 80.

Johnston, H.S. (1962). Nuclear inclusions in the epithelium of the hen's oviduct. Z.Zellforsch. mikrosk. Anat. 57, 385 - 389.

Johnston, H.S., Aitken, R.N.C. & Wyburn, G.M. (1963). The fine structure of the uterus of the domestic fowl. J. Anat. 97, 333 - 344.

Juhn, Mary, & Quigley, G.D. (1960). Molt in the hen with the oviduct transected as a pullet. Anat. Rec. 137, 368.

Kar, A.B. (1947). Studies on the ligaments of the oviduct in the domestic fowl. Anat. Rec. 97, 175 - 195.

Kyes, P. & Potter, T.S. (1934). Physiological marrow ossification in female pigeons. Anat. Rec. 60, 377 - 379.

Lake, P.E. (1968). The maintenance of spermatazoa in the oviduct of the domestic fowl. in Reproduction in the Female Mammal.Ed. G.E. Lamming & E.C. Amoroso. Proc.13th Easter Sch.agric. Sci. Univ. Nott., 1966. London: Butterworths.

Lake, P.E. & Gilbert, A.B. (1962). Factors affecting uterine motility in the domestic hen. J. Reprod. Fert. 4, 211 - 212.

Lake,P.E. & Gilbert, A.B. (1964). The effect on egg production of a foreign object in the lower oviduct regions of the domestic hen. Res. Vet. Sci. 5, 39 - 45.

Landois, H. (1865). Z. wiss. Zool. 15, 1 - 31. (1878). Zool. Gart. 19, 17 - 24. (1892). Jber. westf. ProvVer. Wiss. Kunst. 20, 34 - 35. Cited by Romanoff & Romanoff, 1949.

Lewis, P.R. (1962). Histochemistry in biology. in Viewpoints in Biology, 1, 50 - 89. London: Butterworths.

Ljungkvist,H.I. (1967). Light and electron microscopical study of the effect of oestrogen on the chicken oviduct. Acta Endocr. Copenh. 56, 391 - 402.

Masshoff,W. & Stolpmann, H.J. (1961). Licht- und Elektronmikroskopische Untersuchungen an der Kalkschale des Hühnereies. Z. Zellforsch. mikrosk. Anat. 55, 818 - 832.

Mather, F.B., Epling, G.P. & Thornton, P.A. (1962). The microscopic structure of the egg shell matrix as influenced by shell thickness and environmental temperature. Poult. Sci. 41, 963 - 970.

Mazel,P. & Holland, W.C. (1958). Acetylcholine and electrolyte metabolism in the various chambers of the frog and turtle heart. Circulation Res. 6, 684 - 688.

Mehring, A.L. & Titus, H.W. (1964a) The effects of low levels of calcium in the diet of young laying chickens. Poult. Sci. 43, 1405 - 1414.

Mehring, A.L. & Titus, H.W. (1964b). Levels of calcium and phosphorus in the diet of young growing chickens (0 - 10 weeks). Poult.Sci. 43, 1474 - 1484.

Meldrum, N.U. & Roughton, F.J.W. (1933). Carbonic anhydrase. Its preparation and properties. J. Physiol. 80, 113 - 142.

Misra, M.S. & Kemeny, A. (1964a). Studies on the oviduct and serum of fowls. I. Oxygen uptake, alkaline phosphatase activity, concentration of phosphorus, calcium and magnesium in adult Hungarian Yellow hens. Acta vet. Hung. 14, 389 - 397.

Misra, M.S. & Kemeny, A. (1964b). Studies on the oviduct and serum of fowls. II. Oxygen uptake by the regions of the oviduct and effect of calcium ion on it, the concentration of inorganic phosphorus, calcium and magnesium and the level of alkaline phosphatase activity in serum of Hungarian Yellow pullets. Acta vet. Hung. 14, 443 - 454.

Moran,T. & Hale, H.P. (1936). Physics of the hen's egg. J. exp. Biol. 13, 35 - 40.

Morgan, C.L. & Mitchell, J.H. (1938). The calcium and phosphorus balance of laying hens. Poult. Sci. 17, 99 - 104.

Morgan, V.E. & Chichester, D.F. (1935). Properties of the blood of the domestic fowl. J. biol. Chem. 110, 285 - 298.

Mueller, W.J. (1962). Carbonic anhydrase, diuretics, and egg shell formation. Poult. Sci. 41, 1792 - 1796.

Mueller, W.J. & Schraer,H. (1960). The role of the diffusible and non-diffusible calcium in egg shell formation. Poult. Sci. 40, 1435.

McCallion, D.J. (1953). A cytological and cytochemical study of the shellgland of the domestic hen. Can. J. Zool. 31, 577 - 588.

McNally, E. (1933). Relative amount of mucin in thick and in thin egg white. Proc. Soc. exp. Biol. Med. 30, 1254 - 1255.

McNally, E.H. (1934). Passage of ovoglobulins through the shell membrane. Proc. Soc. exp. Biol. Med. 31, 946 - 947.

Nathusius, W. von (1867). Arch.mikroskop.Anat.Entwmech. 46, 654 - 692. (1868). Z.wiss. Zool. 18, 225 - 270. (1869). Z. wiss. Zool. 19, 322 - 348. (1871a). J.Orn. Lpz. 19, 241 - 260. (1871b). Z. wiss. Zool. 21, 330 - 355. (1874). J. Orn. Lpz. 22, 1 - 26. (1878). Z. wiss. Zool. 30, (supp) 69 - 77. (1879), J. Orn. Lpz. 27, 225 - - 261. (1882a). J. Orn. Lpz. 30, 255 - 314. (1882b) J. Orn. Lpz. 30, 129 - 161. (1885). J. Orn. Lpz. 33, 165 - 178. (1893). Z. wiss. Zool. 55, 576 - 584. (1894). Zool. Anz. 17, 440 - 445, 449 - 452. (1895). Arch. mikroskop. Anat. EntwMech. 45, 654 - 692. Cited by Romanoff & Romanoff, (1949).

Needham, J. (1931). The relations between yolk and white in the hen's egg. V. The osmotic properties of the isolated vitelline membrane. J. exp. Biol. 8, 330 - 344.

Ogasawara, F.X., Van Krey, H.P. & Lorenz, F.W. (1964). Hydrogen ion concentration of the oviduct of the laying domestic fowl. Poult. Sci. 43, 3 - 6.

Olsen, M.W. & Byerly, T.C. (1936). Multiple turning and orienting eggs during incubation as they affect hatchability. Poult. Sci. 15, 88 - 95.

Osborne, W.A. (1931). A note on the permeability of the egg shell. Aust. J. exp. Biol. med. Sci. 8, 239 - 240.

Owen, R. (1866). On the anatomy of vertebrates. vol.2,Birds and mammals. London: Longmans, Green and Co. Cited by Romanoff and Romanoff, 1949.

Parkinson, T.L. (1966). The chemical composition of eggs. J. Sci. Fd Agric. 17, 101 - 111.

Pearl, R. & Surface, F.M. (1909). The nature of the stimulus which causes a shell to be formed on the bird's egg. Science, N.Y., 29, 428 - 429

Pearl, R. & Curtis, M.R. (1912). Studies on the physiology of the domestic fowl. V. Data regarding the physiology of the oviduct. J. exp. Zool. 12, 100 - 132.

Pearl, R. & Curtis, M.R. (1914). Studies on the physiology of reproduction in the domestic fowl. VIII. On some physiological effects of the ligation, section, or removal of the oviduct. J. exp. Zool. 17, 395 - 424.

Pearse, A.G.E. (1960). Histochemistry, theoretical and applied. 2nd ed. London: J.& A. Churchill,Ltd.

Peters, J.A. & Smith, L.M. (1964). The composition of the liver lipids of sheep and the effect of early Sporidesmin poisoning. Biochem. J. 92, 379 - 385.

Prasad, S., Gambhir, S.S. & Sanyal, A.K. (1967). Electrolytes and uterine motility: an experimental study on albino rats. Indian J. med. Res. 55, 459 - 462.

Quinn, J.P., Burrows, W.H. & McNally, E.H. (1939). A Rhode Island Red pullet with two oviducts. Poult. Sci. 18, 381 - 384.

Richardson, K.C. (1935). The secretory phenomena in the oviduct of the fowl, including the process of shell formation examined by the microincineration technique. Phil. Trans. R. Soc. B, 225, 149 - 195.

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

Robinson, D.S. & King, N.R. (1968). Mucopolysaccharides of an avian egg shell membrane. Jl. R. Microsc. Soc. 88, 13 - 22.

Robinson,D.S., King, N.R., & Bowen, D.J. (1968). The occurrence of neutral and acidic mucins in the reproductive tract of the laying hen. Histochemie 13, 97 - 104.

Robinson, D.S. & Monsey, J.B. (1966). The composition of ovomucin. Biochem. J. 100, 61 - 62P.

Romanoff, A.L. & Romanoff, A.J. (1949). The Avian Egg. New York: Wiley; London: Chapman & Hall.

Sacchi, M. (1887). Contribuzione all'istologia dell'Ovidotto dei Sauropsidi. Atti Soc. ital. Sci. nat. (Milano) 30, 273 - 308.

Schaible, P.J., Moore, J.M. & Davidson, J.A. (1935). A note on the structure of egg white. U.S. Egg Poult. Mag. 41,(12), 38 - 39.

Scheib-Pfleger, D. (1955). Contribution biochemique à l'étude des processus de différentiation des canaux de Müller chez l'embryon de poulet. Bull. Biol. Fr.Belg. 89, 403 - 490.

Schmidt, W.J. (1957). Über den Aufbau der Schale des Vogeleies nebst Bemerkungen über kalkige Eischalen anderer Tiere. Ber. oberhess. Ges. Nat.-u. Heilk.,(N.F.), 28, 82 - 108.

Schmidt, W.J. (1961). Über das Shleimhäutchen der Vogeleischale, insbesondere bei Phoenicopterus, Aptenodytes, und Spheniscus. J. Orn. Lpz. 102, 456 - 457.

Schmidt, W.J. (1962). Liegt der Eischalenkalk der Vögel als submikro -skopische Kristallite vor? Z. Zellforsch. Mikrosk. Anat. 68, 194 - 204.

Schoorl, P. (1965). Red fluorescence of eggs in U.V. light after washing. Congr. Internat. Inst. Refrigeration, Karlsruhe. Cited by Simons & Wiertz, 1966.

Schraer, R. & Schraer, H. (1965). Changes in metal distribution of the avian oviduct during the ovulation cycle. Proc. Soc. exp. Biol. Med. 119, 937 - 942.

Scott, H.M. & Huang, W.L. (1941). Histological observations on the formation of chalazae in the hen's egg. Poult. Sci. 20, 402 - 405.

Scott, H.M., Hughes, J.S. & Warren, D.C. (1937). Augmentation of nitrogen to the egg white after the formation of the shell membranes in the fowl. Poult. Sci. 16, 53 - 61.

Scott, H.M., Jungherr, E. & Matterson, L.D. (1944). The effect of feeding sulfanilamide to the laying fowl. Poult, Sci. 23, 446 - 453.

Sharma, G.P. (1950). Some observations on the formation of the hen's egg with special reference to the chalaziferous layer and the chalazae. Res. Bull. E. Panjab Univ.,Zool., 7, 83 - 89. Cited in Biol. Abstr. 26: 2: 3854.

Shenstone, F.S. (1968). The gross composition, chemistry, and physico-chemical basis of organisation of the yolk and white. pp. 26 - 58 in Egg Quality. B.E.M.B. Symposium IV. Edinburgh & London: Oliver & Boyd.

Simkiss, K. (1958). The structure of the eggshell with particular reference to the hen. Ph.D. thesis, Reading University.

Simkiss, K. (1961). Calcium metabolism and avian reproduction. Biol. Rev. 36, 321 - 367.

Simkiss, K. (1967). Calcium metabolism in the laying bird. Chapter 12 of Calcium in reproductive physiology. London: Chapman & Hall, New York: Reinhold.

Simkiss, K. (1968). The structure and formation of the shell and shell membranes. pp. 3 - 25 in Egg Quality. B.E.M.B. Symposium IV. London & Edinburgh: Oliver & Boyd.

Simkiss, K. & Tyler, C. (1958). Reactions between eggshell matrix and metallic cations. Q. Jl. microsc. Sci. 99, 5 - 13.

Simons, P.C.M. & Wiertz, G. (1963). Notes on the structure of membranes and shell in the hen's egg. An electron microscopical study. Z. Zellforsch. mikrosk. Anat. 59, 555 - 567.

Simons, P.C.M. & Wiertz, G. (1966). The ultra-structure of the surface of the hen's egg in relation to egg-cleaning. Poult. Sci. 45, 1153 - 1162.

Snetsinger, D.C., Paul, H.S. & Mraz, F.R. (1966). Variations in location and quantity of selected mineral elements in egg shells. Poult. Sci. 45, 1125.

Solomon, A.K. (1959). Ion and water transport in single proximal tubules of the necturus kidney. In The method of isotopic tracers applied to the study of active ion transport. Proc. 1er Colloque de biologie de Saclay, July 1968. London, New York: Pergamon.

Solomon, A.K. (1963). Single proximal tubules of Necturus kidney. VII. Ion fluxes across individual faces of cell. Am. J. Physiol. 204, 381 - 386.

Spector, W.S. (ed.) (1956). Handbook of Biological Data. Philadelphia & London: W.B. Saunders Co.

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

Sturkie, P.D. (1965). Avian Physiology. 2nd ed. New York: Cornell University Press.

Surface, F.M. (1912). The histology of the oviduct of the domestic fowl. Maine Agr. Expt. Sta. Bull. 206, 395 - 430.

Sutton, J.B. (1885). Diseases of the reproductive organs in frogs, birds, and mammals. J. Anat. Physiol. Lond. 19, 121 - 149.

Sykes, A.H. (1953). Premature oviposition in the hen. Nature, Lond. 172, 1098.

Sykes, A.H. (1955). Further observations on reflex bearing-down in the fowl. J. Physiol. Lond. 128, 249 - 257.

Sykes, A.H. (1967). The effect of a uterine irritant on the composition of the egg in the fowl. J. Endocr. 37, 103 - 104.

Tarchanoff, J.R. (1884). Über die Verschiedenheiten des Eiereiweisses die befiedert geborenen (Nestflüchter) und nackt geborenen (Nesthocker) Vögeln und über die Verhältnisse zwischen dem Dotter und dem Eierweiss. Pflüger's Arch. f. Physiol. 33, 303 - 378.

Taylor, T.G. (1966). The endocrine control of calcium metabolism in the fowl. pp. 199 - 202 in Physiology of the domestic fowl. B.E.M.B. Symposium I. Edinburgh & London: Oliver & Boyd.

Taylor, T.G. & Hertelendy, F. (1961). Changes in blood calcium associated with egg shell calcification in the domestic hen. II. Diffusible calcium. Poult. Sci. 40, 115 - 123.

Tegetmeier, W.B. (1867). The Poultry Book. London: Routledge. Cited by Romanoff & Romanoff, 1949.

Terepka, A.R. (1963a). Structure and calcification in avian egg shell. Expl. Cell Res. 30, 171 - 182.

Terepka, A.R. (1963b). Organic-inorganic interrelationships in avian egg shell. Expl. Cell Res. 30, 183 - 192.

Tyler, C. (1940). Studies of calcium andphosphorus metabolism in relation to the chemical structure of bone. I, experiments with laying birds.

Tyler, C. (1946). Studies in the absorption and excretion of certain minerals by poultry. J. Agric. Sci. 36, 263 - 274.

Tyler, C. (1953). Studies on egg shells. II. Marking and counting pores. J. Sci. Fd Agric. 4, 266 - 272.

Tyler, C. (1956). Studies on eggshells. VII. Some aspects of structure as shown by plastic models. J. Sci. Fd Agric. 7, 483 - 493.

Tyler, C. & Simkiss, K. (1959). Studies on eggshells. XII. Some changes in the shell during incubation. J. Sci. Fd Agric. 10, 611 - 615.

Ussing, H.H. & Zerahn, K. (1951). Active transport of Na as the source of electric current in the short-circuited isolated frogskin. Acta physiol. scand. 23, 110 - 127.

Walsh, A. (1953). Australian Patent Specification 163,586 (23,041/53).

Walsh, A. (1955). The application of atomic absorption spectra to chemical analysis. Spectrochim. Acta 7, 108 - 117.

Warren, D.C. & Scott, H.M. (1935a). The time factor in egg formation. Poult. Sci. 14, 195 - 207.

Warren, D.C. & Scott, H.M. (1935b). Physiological factors influencing the rate of egg formation in the domestic hen. J. Agric. Res. 51, 565 - 572.

Warren, R. (1939). Serosal and mucosal dimensions at different levels of the dog's small intestine. Anat. Rec. 75, 427 - 437.

Wentworth, B.C. (1960). Fistulation of the hen's oviduct. Poult. Sci. 39, 782 - 784.

Wilcox, F.H. (1966). Effect on performance of selection for high level of alkaline phosphatase in serum. Poult. Sci. 45, 776 - 784.

Wilcox, F.H. & Cloud, W.S. (1965). Alkaline phosphatase in the reproductive system of the hen. J. Reprod. Fert. 10, 321 - 328.

Williamson, J.H. (1965). Cystic remnants of the right Müllerian duct and egg production in two strains of white leghorns. Poult. Sci. 44, 321 - 324.

Winget, C.M., Mepham, C.A. & Averkin, E.G. (1965). Variations in intrauterine pH within a circadian rythm (Gallus Domesticus). Am. J. Physiol. 208, 1031 - 1035.

Winget, C.M., Smith, A.H. & Hoover, G.N. (1958). Arterio-venous differences in plasma calcium concentrations in the shellgland of the laying hen during shell formation. Poult. Sci. 37, 1325 - 1328.

Witschi, E. Sex and secondary sexual characters. (1961). Chapter XVII of Biology and comparative physiology of birds. ed. A.J. Marshall. New York & London: Academic Press.

Wolken, J.J. (1951). Structure of hen's egg membranes. Anat.Rec. 111, 79 - 84.

Worden, A.N. (1964). p. 546 in A New Dictionary of Birds. ed. A, Landsborough Thomson. London: Nelson.

Yarrell, W. (1827). On the change of plumage of some hen pheasants. Phil. Trans. R. Soc. Lond. 1, 268 - 275.

Zarrow, M.X., Greenman, D.L. & Peters, L.E. (1960). Inhibition of the Bursa of Fabricius and the stilboestrol-stimulated oviduct of the domestic chick. Poult. Sci. 40, 87 - 93.