PHYSIOLOGICAL STUDIES OF FOWL SPERMATOZOA AND THEIR ENVIRONMENT IN THE OVIDUCT

A Thesis submitted for the degree of Doctor of Philosophy of the University of Edinburgh

by

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November 1965

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CONTENTS.

INTRODUCTION

Fowl (Gallus domesticus) spermatozoa have proved difficult to maintain *in vitro* for more than a few hours and yet can remain viable in the oviduct for one or two weeks. These results contrast greatly with those obtained with cattle, where spermatozoa can be maintained *in vitro* for a few years and yet their life in the oviduct extends for only a few hours. It would be an advantage to future studies on the metabolism of fowl spermatozoa, or to investigations of fertility problems in the fowl, to be able to maintain the spermatozoa *in vitro* for prolonged periods. Knowledge of the type of environment within the oviduct, where spermatozoa reside, would be an advantage in this work. The period of survival of fowl spermatozoa in the oviduct is often shortened leading to poor fertility. This could be due to adverse conditions appearing in the *milieu* of the residence sites within the oviduct from which spermatozoa travel periodically to the infundibulum to fertilize eggs. However, nothing is known about the composition of normal secretions in those parts of the oviduct and thus the basic physical and chemical conditions for their survival here are unknown.

Little information is available on the physiological changes which occur in spermatozoa associated with their

rapid loss of activity *in vitro*, or on the basic chemical and physical conditions of the environment in the male reproductive tract which favours the survival of spermatozoa.

Electrolytes, especially some inorganic ions, play an important role in metabolic processes concerned with the proper functioning of somatic or germ cells. They also play a general role in preserving the physical integrity of cells and tissues. The state of cell membranes can often be judged by the intracellular content of certain inorganic ions. The balance of inorganic ions in biological fluids is constantly maintained for the contained cells to survive satisfactorily. Nothing is known about the inorganic ion content of fowl spermatozoa or how far the spermatozoon resembles a somatic cell in this respect. Much information is available on the composition and metabolism of somatic cells but how much of it can be applied to a solution of the problems of maintaining spermatozoan cells *in vitro* must remain uncertain until we have more knowledge of the chemistry of spermatozoa.

The main object of this work has been to study the intracellular content of the principal inorganic ions of fowl spermatozoa and to examine the findings in relation to the composition of extracellular fluid in the male tract and a selected part of the oviduct in the vicinity of which

spermatozoa are sustained for long periods. Progress in techniques of artificial insemination and the development of long-term storage of fowl semen partly depends upon gaining a thorough knowledge of the normal chemical and physical structure of the spermatozoon in relation to natural physiological environments in which it is known to be maintained in a viable stage, e.g. in the vas deferens and oviduct.

To make a direct comparison with a somatic cell it was decided to include a study of the avian red blood cell from a group of males used for semen studies.

REVIEW OF LITERATURE

REVIEW OF THE LITERATURE

A. Storage of Fowl Spermatozoa *in vitro*

It has already been mentioned that the majority of mammalian spermatozoa, in comparison with those of the domestic fowl or turkey have a short functional life within the oviduct. However, the situation is quite reversed when the problem of storing spermatozoa *in vitro* is considered. Bull spermatozoa can be stored for several years *in vitro* without losing their fertilizing ability. The subject of artificial insemination in cattle has been reviewed by Melrose (1962) and it is realised how the ability to store bull spermatozoa *in vitro* relatively easily has revolutionized breeding in Russia, the U.K., the U.S.A. and Europe. Paradoxically, the prolonged storage of the semen of other domestic species of animals has not proved successful e.g. sheep, horses and pigs.

Bull semen can be stored for about one week at 5°C in several different diluents, e.g. egg yolk-citrate, egg yolk-phosphate and boiled milk (Melrose, 1962), and it can be diluted about 100-fold without any apparent detrimental effect on the survival of the spermatozoa. Bull semen has to be stored at sub-zero temperatures (-196°C) to preserve the spermatozoa for several months or years and this

technique has proved useful for sending semen around the world from proven sires in Britain and America.

Attempts to store fowl spermatozoa *in vitro* for more than 24 hrs have not yet met with very great success and the problems have been reviewed by Lorenz (1959, 1964) and Lake (1962a). However, the task of preserving fowl spermatozoa *in vitro* demands a higher degree of success than for bull spermatozoa because after insemination the spermatozoa must be capable of surviving in the oviduct for several days to fertilize a succession of eggs laid by the hen; in cattle only one ovum, or occasionally two, require to be fertilized within a few hours.

(1) Dilution of semen and immediate insemination.

The dilution of fowl and turkey semen for dissemination among several females within a short time, e.g. 15 mins to one hour, is feasible. Several physiological saline solutions and other fluid media suffice for this purpose. Details of dilution rates, holding time of neat semen, temperatures of holding diluted semen and insemination dosages using a variety of diluents have been reviewed by Lake (1962a).

The degree to which semen is diluted would appear to be a most important factor in the success achieved with

fowl semen diluted for immediate insemination. Weakley and Shaffner (1952) used fowl seminal plasma as a diluent and varied dilution ratios from three- to eightyfold. Fertility was recorded for two weeks following a single insemination (0.1 ml) and semen diluted up to tenfold gave fertility that compared favourably with that from fresh, undiluted semen. Greater dilutions resulted in a pronounced drop in fertilizing capacity of spermatozoa which was inversely proportional to the dilution rate. The concentration of spermatozoa in fowl semen (3.5 - 6.0 x 10^6 per cmm) is greater than that of the bull (about one million per cmm) and one might have assumed that fowl semen could be diluted to a larger extent. However, this is not the case, presumably because insufficient spermatozoa would be placed in the hen to maintain fertility for a week or more. Rowell and Cooper (1957, 1960) used glycine in distilled water and seminal plasma as diluents for fowl semen and varied dilution ratios from 1:1 to 1:3.38. Fertility decreased linearly as the dilution rate increased and 1:1 dilution resulted in fertility which did not differ from that obtained with fresh, undiluted semen. It was pointed out that the principal cause of the decline in fertility was the decreasing number of spermatozoa inseminated. Munro (1938c) earlier reported that fertility in the hen was influenced by the number of spermatozoa inseminated and was

particularly poor when less than 10^8 were introduced into the hen. Dilution had a particularly adverse effect on semen obtained from cocks of poor fertilizing capacity presumably because of the low, initial numbers of spermatozoa in their semen.

Wales and White (1961) reported that the motility of fowl spermatozoa was depressed at relatively high dilutions but that the use of diluents of high tonicity helped to alleviate the detrimental effect. Kan (1962) diluted fowl semen in the ratios 1:3, 1:15 and 1:31 with Tyrode solution at room temperature and found that there was a general decline in fertility with increasing dilution. Dilution ratios between 1:3 and 1:15 resulted in fertility comparable to that of the undiluted semen.

(2) Long-term Storage of Semen

Dilution of fowl semen for long-term storage of spermatozoa is still an unsolved problem. It has been difficult, or even impossible, to keep undiluted cock semen *in vitro* for more than a few hours without a drastic loss of fertilizing ability. Schindler, Weinstein and Moses (1955) found that diluted or undiluted fowl semen retained its full fertilizing capacity for 4 hrs at 10°C. Only a small number of fertile eggs could be obtained with undiluted semen after 24 hours storage at 10°C, or with semen diluted

with whole milk after 24 hours storage at 4°C.

Cock seminal plasma is inadequately buffered against acid production by spermatozoan metabolism during storage *in vitro*. Wilcox and Shaffner (1958) found that with phosphate buffers (pH ranging from 6.5 to 7.8) it was possible to produce reasonable fertility after storing fowl semen for 24 hours at 10°C. Antibiotics were included in the diluent to reduce bacterial contamination and a combination of 90 µg oxytetracycline and 90 µg dihydrostreptomycin per ml proved to be most satisfactory in helping to preserve the fertilizing ability. It was later found that oxytetracycline was beneficial not because of its antibacterial action but because of its chelating properties (Wilcox, 1959a). It was suggested that heavy metal ions were being prevented from exerting a deleterious action on spermatozoan metabolism. Since storage of semen results in a progressive decrease in viable spermatozoa Wilcox and Shaffner (1958) and Wilcox (1959b, c; 1960) adopted a procedure by which spermatozoa, after being stored, were reconcentrated by centrifugation. Seminal plasma and diluent was removed and the spermatozoa resuspended in phosphate buffer to the original semen volume before dilution. The addition of fructose to the resuspension fluid gave improved fertility results although it was not considered necessary in the actual medium used during the storage period. Motility, as well as fertilizing

ability, of spermatozoa was also improved by adding a variety of reducing sugars to the resuspension fluid. Such reconstituted semen, after 48 hours storage at 10°C, was inseminated at a dose of 0.1 ml per hen and produced 60-70% fertility during the first week following artificial insemination. No fertility was obtained if semen was diluted tenfold and the storage period was extended to 3 days. However, moderate fertility was obtained if the semen was originally stored at a dilution of 1:2 or 1:4 which indicated that above fourfold dilution of fowl semen increased damage is sustained by the spermatozoa.

Wilcox and Clark (1962) used a sodium phosphate-buffered diluent containing oxytetracycline and/or 2,5 dimethylbenzimidazole and dihydrostreptomycin for storing fowl semen *in vitro*. All diluted semen samples were centrifuged and resuspended to the original volume of the ejaculate before insemination. Greater dilution ratios than 1:5 during storage resulted in no subsequent fertility.

Lake (1960) adopted another approach to the problem of diluting fowl semen for a prolonged period of storage *in vitro*. He formulated a diluent containing the proportions of inorganic ions and free glutamic acid that are found in the type of seminal plasma collected without contamination from 'transparent' fluid i.e. semen ejaculated from the vas deferens (Lake, 1957a). It was found essential to use semem

uncontaminated with 'transparent' fluid for the best preservation of viability of spermatozoa. Such semen was diluted threefold with a glutamate-containing saline solution with added fructose. Fertility was 64% and 47% during one week post-insemination following the insemination of 0.1 ml diluted semen stored at 2°C for 24 and 48 hrs respectively. The diluent did not include calcium, and chloride concentration was low compared with that found in undiluted seminal plasma. Fertility was significantly lower in pullets inseminated with semen stored in a fructose-free diluent.

Yamane, Tsukunaga and Takahashi (1962) suggested that hypertonic solutions were more beneficial as storage media for fowl spermatozoa. These authors experimented with citrate-glucose solutions with a freezing point depression (Δ) of -1.03°C. Freshly-diluted semen, gave an average fertility of 72.2% during a period of two weeks after a single insemination into each of 12 hens. Diluted semen, after 4 days storage at 2.5°C, gave an average fertility of 35.5% for two weeks after a single insemination into each hen. However, the results reported were from a few hens selected from their experimental animals.

Blackwood and Harris (1960) explored a very promising approach to the storage of semen by using a metabolic

inhibitor hoping to reduce the metabolic rate and thus prolong the life span of the spermatozoa. One group of metabolic inhibitors, 2,5-alkylbenzimidazoles, were found to prolong the life-span of fowl spermatozoa significantly (Harris, Wilcox and Shaffner, 1961). Under exceptional conditions, 75% fertility was obtained after fowl semen had been stored for 5 days at 2°C. However, more extensive tests gave average fertility results of 57%, 46% and 2% for semen diluted two-, four- and eightfold during storage respectively. Turkey semen could only be stored for 6 hours in which case there was no difference between two- and fourfold dilution; an average fertility of 50% for one and 56% for another group of turkey hens was obtained during the first four weeks after insemination. With both fowl and turkey semen it was found necessary to wash out the inhibitor after storage before insemination and the washed spermatozoa were resuspended in a fructose-containing diluent to the original volume of semen. Fertilizing ability was prolonged only by the addition of amounts of inhibitor which did not inhibit fructolysis.

The fowl and turkey possess a cloaca, which is a common opening for the rectum, ureters and vas deferens, and there is a great risk of the semen containing varying amounts of faeces and urine when it is collected by massage.

Thus bacterial contamination of semen often presents a problem. Smith (1949) found a great increase in the bacterial population, particularly of coliform bacilli, in diluted or undiluted semen stored for 24 hours at room temperature or 48 hours at 2°C. Streptomycin in concentrations of 50, 100 and 200 µg/ml effectively checked bacterial growth but was ineffective in improving motility or fertilizing power of stored diluted semen. Sulphathiazole in amounts capable of checking bacterial growth was toxic to fowl spermatozoa. Wilcox and Shorb (1958) investigated the effect of different levels of seven antibiotics on bacterial number, motility of spermatozoa and fertility of fowl semen stored at 10°C. A combination of dihydrostreptomycin with either penicillin or oxytetracycline was the most effective in reducing bacterial number. There was a remarkable improvement in fertility of semen diluted with a buffer containing 90 µg/ml oxytetracycline and 90 µg/ml dihydrostreptomycin when compared with buffer without antibiotics or buffer with penicillin and dihydrostreptomycin separately. It was found that the beneficial effect of oxytetracycline and dihydrostreptomycin was far more superior than was accounted for by their anti-bacterial properties alone and Wilcox (1959a) suggested that the beneficial effect of adding oxytetracycline to the diluent

was due to its property of chelating heavy metals. Wales and White (1960) investigated the toxicity of some antibiotics to fowl spermatozoa and found that penicillin and sulphanilamide at the level of 1 mg/ml were relatively toxic at a 1 in 20 dilution in a medium having a similar tonicity to the fowl seminal plasma. Gale and Brown (1961) identified the bacteria contaminating ejaculated turkey semen and staphylococcal and coliform organisms were the most abundant. Various antibiotics were tried and a combination of dihydrostreptomycin and penicillin or dihydrostreptomycin and oxytetracycline offered the best control for the bacteria present in turkey semen. However, it was not known whether these antibiotics improved survival of spermatozoa *in vitro* and subsequent fertility.

It is evident that many problems remain to be answered with regard to prolonging the storage time of fowl and turkey spermatozoa *in vitro*.

(3) Influence of ions, various chemical compounds, osmotic pressure, temperature and light on semen *in vitro*.

The effect of several physical and chemical conditions of particular *in vitro* environments on the activity of spermatozoa have been studied with the object of discovering the reasons for the difficulty of maintaining the

spermatozoa *in vitro*. Dilution of semen, which is essential for storage, quite possibly results in a leakage of essential substances from the spermatozoa which becomes evident after a long period *in vitro* by a marked reduction in their viability.

The suitability of experimental diluents or *in vitro* conditions of storage of fowl semen has often been judged by the degree of motility of spermatozoa maintained after varying periods of time. It should perhaps be more widely recognised that if spermatozoa display great motility *in vitro* it does not necessarily imply that they would be capable of surviving in the oviduct for the requisite period of time after storage. Studies on the storage of fowl semen *in vitro* should include an examination of the period of survival of spermatozoa in the oviduct.

Wales and White (1958b) investigated the action of various inorganic ions on the motility of fowl spermatozoa and comparisons were made with various mammalian spermatozoa. Potassium, magnesium and calcium each improved the motility of fowl spermatozoa *in vitro* when added to a phosphate buffer. Additive effects were given by potassium in combination with magnesium or calcium. However, calcium ions in concentration greater than 0.3 mM caused agglutination of the spermatozoa. Washing more than once was harmful to fowl spermatozoa and the effect

of potassium was more beneficial on washed than unwashed cells. The importance of potassium and magnesium for the maintenance of full motility of fowl spermatozoa was demonstrated; calcium in limited amounts proved beneficial in contrast to its action on ram spermatozoa. The beneficial action of potassium was independent of the presence of magnesium or calcium and it was suggested that the divalent ions probably acted at a different site from potassium in the metabolism of spermatozoa and that magnesium and calcium had a similar role since they were interchangeable in their action. No fertility tests were made by Wales and White (1958b) in their experiments.

Wilcox (1959b) and Wilcox and Wilson (1961) reported that the addition of small amounts of magnesium, potassium and sodium to a phosphate diluent resulted in no changes or small reductions of fertility, while higher concentrations resulted in a pronounced reduction in fertility. Large amounts of sodium chloride had no deleterious effect on fertility provided insemination was carried out immediately. Wales and White (1961) added potassium and magnesium, either alone or in combination, to a diluent composed of $Na_2HPO_4.12H_2O$, $NaH_2PO_4.2H_2O$, NaCl and variable amounts of glucose to make low, medium and high tonicity fluids. Motility at low spermatozoan concentration was

maintained best in diluents of high tonicity containing potassium. Magnesium had no effect on diluted fowl semen. Wales and White found that the best protection to spermatozoa was given by the addition of seminal plasma (2.5%) to semen. Bovine albumin, bovine globulin, casein and acacia gum gave some protection against the harmful effects of dilution but none proved as beneficial as fowl seminal plasma. It is not known what component of the seminal plasma acted as the beneficial agent.

Bajpai and Brown (1964a) studied the effect of adding potassium and/or magnesium to an isosmotic, monosodium glutamate solution used for storing diluted turkey semen. It was found that the solution containing magnesium gave higher fertility than either undiluted semen, semen diluted with monosodium glutamate alone, or semen diluted with monosodium glutamate containing potassium.

Under certain circumstances the addition of chloride ions to a diluent for fowl semen tended to increase the incidence of abnormal spermatozoa which resulted in a remarkable reduction of fertility (Lake, 1960; Wilcox and Wilson, 1961; El Zayat and Van Tienhoven, 1961a).

Wilcox (1959b) stored fowl semen in a sodium phosphate buffer containing antibiotics and before use centrifuged the diluted semen and resuspended the spermatozoa in buffer

containing various levels of fructose or other carbohydrates. There was little difference in the fertility obtained with fructose levels varying from 0.25 to 8 mg/ml but there was a slight reduction in fertility at a level of 16 mg/ml. Motility of the resuspended spermatozoa was markedly enhanced by the addition of glucose, fructose, mannose and galactose but was not improved by the addition of any pentoses and disaccharides tested. The addition of large amounts of adenosine triphosphate (ATP) slightly reduced motility. However, it is difficult to interpret this result because it is not even certain that this compound can enter cells.

Wales, White and Lamond (1959) studied the effect of hydrogen peroxide on the motility of fowl, bull, dog, ram, mouse, rabbit and human spermatozoa *in vitro*. Fowl and bull spermatozoa were most susceptible to hydrogen peroxide. The spermatozoa of other species were rendered susceptible when seminal plasma was removed and thus, in these cases, the seminal plasma must have been capable of decomposing hydrogen peroxide. Catalase enzyme was found to be present in the latter and was absent from the fowl.

Lorenz and Tyler (1951) showed that glycine when added to saline diluents at concentrations between 0.003 and 0.133 M prolonged the life span of cock spermatozoa. It was

suggested that amino acids acted by binding certain highly toxic, heavy metals in trace amounts in the diluents.

Page, Smith, Gale, Polin and Folkers (1963) recently tested the addition of compounds related to coenzyme Q, vitamins K and E, several water soluble vitamins and a few antioxidants to diluents for the maintenance of motility of fowl spermatozoa. It was found that 2,3 dimethoxy-5-methyl-6-substituted benzoquinones and corresponding 6-chromenols and 6-chromanols and the chemically related α-tocopherols strikingly prolonged the motility of fowl spermatozoa <u>in vitro</u>. Spermatozoa stored at 10°C in phosphate buffer, containing egg white, fructose and antibiotics alone, showed over 50% motility after 11 to 14 days. However, motility persisted after 20 days when the abovementioned compounds were included in the diluent; the control spermatozoa were all totally immotile after four days of storage. The mode of action of these compounds is not yet known but they were found to be ineffective when the diluent was deprived of egg white. It must be stressed that no information was obtained on the fertilizing capacity of the motile, stored spermatozoa and such information would have been invaluable to workers in this field of research.

Schindler and Nevo (1962) showed that fowl spermatozoa

in thin, flat, glass cells on the microscope stage, became inactive after various time intervals correlated with decreasing oxygen and sugar concentrations in the medium. Aeration restored full activity immediately. Aerobic conditions were required for the maintenance of the motility of spermatozoa in the absence of a reducing sugar in the medium. Under anaerobic conditions motility depended upon the availability of a reducing sugar. Fowl spermatozoa agglutinated in the shape of plait-like "ropes" when they became inactive; a network formed throughout the suspension. The agglutinates dispersed on reactivation and mixing of the spermatozoa. Nevo (1964, 1965) studied the motility and respiration of fowl spermatozoa and those of other species as a function of oxygen concentration. The respiratory rate of all spermatozoa was constant above a critical oxygen partial pressure of 4 mm Hg; below this point there was a sharp decrease, and below 1 mm Hg the oxygen consumption was zero. Motility changes followed exactly those of the respiratory rate. The presence of glucose or fructose did not have an appreciable effect on the critical oxygen concentration or the respiratory rate. Lorenz and Tyler (1951) earlier found that the total oxygen-uptake by fowl spermatozoa in the presence of glycine considerably

exceeded that of controls and it was correlated with an increased duration of motility.

Harris and Hobbs (1964) studied the effects of fluid to gas ratio in tubes, dilution ratio, insemination volume and the level of carbon dioxide on the fertility of chicken spermatozoa stored in hypertonic, carbonated extenders. The fertility of semen stored for 48 hours at 2°C was higher after it had been stored in tubes with a fluid to gas ratio of 1:6.78 than 1:3.33, but the differences were not statistically significant. Semen diluted in media with the highest concentration of carbon dioxide (1.8 gm per 100 ml) gave slightly higher fertility than with the lowest amount (1.2 gm). In addition a fluid to gas ratio in the tube of 1:6.78 was necessary.

The osmotic pressure of fowl body fluids is higher than that of mammals, i.e. is equivalent to a 1% sodium chloride solution (Δ -0.6°C). In this context Wales and White (1958a, 1961) reported that fowl spermatozoa were tolerant of hypertonic solutions. Yamane *et al.* (1962) made a puzzling observation when they suggested that seminal plasma was not isotonic with the spermatozoon particularly with the midpiece. The freezing point depression (Δ) of the seminal plasma was -0.6°C, but the midpiece suffered damage from plasmolysis when the

freezing point depression of citrate diluents was less than -0.93°C.

Hobbs and Harris (1963a,b) used sodium citrate and carbonated extenders to investigate the effect of Δ and the concentration of carbon dioxide on the retention of motility and fertility of fowl spermatozoa *in vitro*. Two sets of sodium citrate solutions with Δ ranging from -0.63 to -1.37°C and -0.59 to -1.36°C respectively were tested. Maximum motility was observed in isotonic solutions and it was reduced in hypertonic extenders. However, for 24 hrs storage at 2°C fertility was preserved best when the spermatozoa were in hypertonic, carbonated diluents. It was suggested that tonicity requirements for optimum fertility after storage are dependent upon the composition of the diluent. Saeki (1963) reported that the incidence of "crooked-neck" fowl spermatozoa was high when semen was diluted with hypotonic saline of Δ -.45°C (.71% NaCl) and low when diluted with hypertonic saline of Δ -0.8°C (1.26% NaCl). The survival of spermatozoa *in vitro* was also prolonged in hypertonic diluents and resulted in better fertility than in hypotonic diluents.

It is difficult to determine the true effects of altering the osmotic pressure of a diluent on the

survival of spermatozoa since different experiments have been performed with fluids of widely different total composition. One or more factors could have contributed to the reported results.

Different temperatures of storage have been examined in attempts to prolong the survival of spermatozoa *in vitro*. It is well known that the spermatozoa of certain mammals are adversely affected by rapid falls in temperature after ejaculation. This is the so-called 'temperature-shock' phenomenon. On the other hand, fowl spermatozoa, which are ejaculated from the body with a relatively high temperature (42°C), appear to be far less susceptible to a sudden drop in temperature than those of, for example, the bull or the ram. Excellent fertility results have been obtained with fowl semen without taking any precautions during its collection, provided it was inseminated shortly after collection. Cooper and Rowell (1958), Wales and White (1959) and White and Wales (1960) could not detect a significant reduction in motility or an increase in the number of dead spermatozoa after subjecting fowl semen to 'cold shock' treatment. However, Bajpai and Brown (1964b) studied the effect of collecting semen at different temperatures (ranging from 0° to 25°C) on the metabolic activity, morphology and fertilizing capacity

of turkey semen. The spermatozoa were damaged within 10 minutes of exposure to temperatures below 15°C but it was not revealed until after the spermatozoa had been stored for 2 hours *in vitro*. Temperatures above 15°C reduced fertility substantially which could have been due to increased metabolic activity at higher temperatures resulting in the accumulation of metabolic end-products that ultimately depressed metabolic activity of the spermatozoa.

Recently, the effect of light on the activity of fowl spermatozoa has been studied and the results could have important implications in the techniques of storing them *in vitro*. Hunsaker and Aitken (1960) reported that the exposure of fowl spermatozoa to an intense beam of "visible" light for 2 hours did not have a detrimental effect on their fertilizing capacity and, although the results suggested a slight improvement in fertilizing ability, the differences between treatments were not statistically significant. Hamner and Williams (1961) and Williams and Hamner (1963) studied the effect of light on the respiration of rabbit, fowl and human spermatozoa and found that respiration of both fresh spermatozoa and those incubated at 38°C for 6 hours *in vitro* was markedly stimulated by light of certain

intensities. Williams and Hamner (1963) found that fowl spermatozoa collected and washed without being exposed to light, respired very slowly or not at all. Ubiquinone, seminal plasma, in -utero incubation and dinitrophenol each markedly stimulated respiration of spermatozoa protected from light. Norman, Goldberg and Porterfield (1962) investigated the effect of visible radiation on the functional life-span of avian spermatozoa. They observed a significant decrease in the number of progressively motile cells after 1.5 hours of exposure to light; after 4.5 hours all spermatozoa lost their motility. It was also found that the irradiated cock spermatozoa declined in fertilizing power and the majority of the few eggs fertilized did not develop normally suggesting that light exposure may have indirectly impaired the activity of nucleic acid in the sperm nucleus.

B. Chemistry of Fowl Semen

(1) Composition of Seminal Plasma

Fowl semen as collected ordinarily by the massage technique consists of spermatozoa, seminal plasma and variable amounts of 'transparent' fluid , which is a transudate, indistinguishable from lymph, that exudes through the epithelium of part of the copulatory organ. Lake (1957a)

suggested that 'transparent' fluid was a doubtful constituent of fowl semen of natural copulation but was easily obtained together with semen from the vas deferens by applying force to the engorged copulatory organ. On the other hand, Nishiyama (1955), Nishiyama and Fujishima (1961) and Nishiyama and Ogawa (1961) presented contradictory evidence, but agreed that 'transparent' fluid was detrimental to the longevity of fowl spermatozoa *in vitro*.

Lorenz (1964) questioned how much importance should be attached to the composition of seminal plasma since the presence or absence of any particular component may reflect merely the existence of a metabolic process which has taken place in the vas deferens. Some constituents could be adventitious excretory products. Although one is tempted to agree with these statements the importance of the composition of the seminal plasma, particularly of clean semen from the vas deferens and its significance to the life of the spermatozoon should not be overlooked. In reviewing the information on the composition of fowl seminal plasma it is quite evident that the variable presence of 'transparent' fluid in semen samples contributes greatly to tremendous variations reported for the presence of certain chemical constituents. A knowledge of the environment in which the spermatozoa are living

before, i.e. in the vas deferens, and after ejaculation can be considered important in work on the development of artificial diluents for poultry semen and also in studies of fertility problems.

Lake, Butler, McCallum and MacIntyre (1958) carried out a preliminary study of the chemical composition of uncontaminated seminal plasma of the domestic cock. It was found that citric acid was absent in cock seminal plasma and other differences found in the concentration of certain inorganic ions in the seminal plasmas of the cock and domestic mammals were attributed to the lack of the typical complement of accessory reproductive organs in the former species. Recently Lake and El Jack (1964a,b) reported a more detailed study of the chemical composition of blood and seminal plasmas of the domestic cock endeavouring to discover the anion-cation balance in the uncontaminated seminal plasma. The results for the chief inorganic ions agreed essentially with those of Lake *et al*. (1958) except that a relatively lower amount of calcium was found in the seminal plasma. The difference might be explained by the use of the atomic absorption spectroscope in the more recent work. The amount of magnesium in seminal plasma was found to be more than three times, and potassium more than twice, the

amount in blood plasma. Calcium and chloride concentrations were higher in blood than in seminal plasma. The chief anion in seminal plasma was found to be glutamic acid and not chloride as in blood plasma.

Takeda (1959) studied the concentrations of sodium, potassium and calcium in blood plasma, 'transparent' fluid and seminal plasma from the vas deferens of the domestic cock. The amount of potassium in vas deferens fluid was significantly higher (145.5 mg%) than that in the 'transparent' fluid (19.3 mg%), while the amount of calcium was lower (3.2 mg% compared to 8.8 mg%). It was found generally that the concentration of bulk cations in 'transparent' fluid was similar to that of blood plasma from which it is derived (Nishiyama, 1955). Hammond, Boone and Barnett (1965) found 338 mg sodium, 7.03 mg calcium, 44.8 mg potassium and 497.3 mg chloride per 100 ml in the seminal plasma of the cock. The very high chloride level would indicate that the seminal plasma was grossly contaminated with 'transparent' fluid and possibly some urine.

Fowl semen, freshly ejaculated from the vas deferens contained none or an extremely small amount of reducing sugar (Lake, 1957a; Lake, Lorenz and Reiman, 1962). Mann (1954) found from 7.7 to 81.0 mg glucose and up to 4.0 mg

fructose per 100 ml of cock semen collected by the massage technique, and similar results were reported by De Meulenaere and Quick (1958), Schindler and Scharf (1963), and Hammond et al. (1965). Mann (1954), De Muelenaere and Quick (1958) and Schindler and Scharf (1963) agreed that glucose was the major sugar in cock semen. It has been shown that the 'transparent' fluid, and thus the blood, is the source of the glucose (Lake 1957a, Nishiyama and Fujishima 1961). The reported amounts of reducing sugar in fowl semen have been very variable obviously because its presence depends entirely on the presence, and the amount of, 'transparent' fluid mixed with vas deferens semen during artificial semen collection.

The source of fructose in fowl semen when it is present is not yet known, but part of it could be derived from the conversion of glucose to fructose by the spermatozoa under certain conditions (Lorenz, 1959, Lake et al., 1962).

Lake and McIndoe (1959) found that glutamic acid (1.0 gm/100 ml) constituted the major component of the free amino acids of the uncontaminated seminal plasma of the domestic cock. Small amounts of alanine, aspartic acid, glycine, serine and an unidentified ninhydrin-

reacting substance were also present. Seminal plasma also contained about 100 mg/100 ml creatine. Goldberg, Pence and Norman (1961), Chubb and Cooper (1962) and Graham, Johnson, and Fahning (1964) agreed that glutamic acid was the chief amino acid found in the seminal plasma of the domestic cock; other additional amino acids were found but the amounts of 'transparent' fluid included in their samples was not specified. 'Transparent' fluid could be expected to contain a similar, free amino acid pattern to blood plasma. Schindler and Scharf (1963) found free glutamic acid in suspensions of testicular tissue, the 'transparent' fluid and whole semen. The protein content of the seminal plasma of the domestic cock is relatively low. Wales, Scott and White (1961) reported the total biuret-reactive material in the seminal plasma of the domestic cock to be 0.93 g/100 ml compared to 2.15, 3.51, 4.07 and 7.04 g/100 ml for dog, ram, man and bull respectively. Most of the biuret-reactive substance in the fowl seminal plasma appeared to be protein although that in the mammalian species contained varying amounts of peptides. Nishiyama (1957) reported that the protein concentration of the 'transparent' fluid of the domestic cock was very low (0.4% as compared to 5.1% in blood plasma). The composition of the protein fraction of the 'transparent'

fluid was similar to that of blood plasma, although the albumin fraction was larger in amount in the 'transparent' fluid .

The seminal plasma of the domestic cock contains a very large amount of acid phosphatase(s) but a small amount of alkaline phosphatase(s) (Wilcox 1961; Bell and Lake, 1962a,b; Hammond et al., 1965). According to Lake (1962b) and Bell and Lake (1962a) intense acid-phosphatase activity was found in the distal parts of the cells lining the entire length of the vas deferens and there was evidence that the enzyme(s) is secreted in large quantities into the vas deferens fluid. Some acid-phosphatase(s) was also produced in the vasa efferentia and seminiferous tubules. On the other hand, alkaline phosphatase(s) was found only in certain parts of the vasa efferentia and inter-tubular tissue of the testes. The physiological functions of these enzymes is not yet known and according to Bell and Lake (1962b) the acid-phosphatase(s) of the cock seminal plasma, unlike that of mammals, was unable to hydrolyse choline-O-phosphate and its activity was not inhibited by dextrorotatory tartrate.

Hammond et al. (1965) reported the presence of glutamic-pyruvic transaminase, glutamic-oxalic transaminase, lactic dehydrogenase and leucine amino-peptidase in fowl seminal

plasma but lipases appeared to be absent.

Scott, White and Annison (1961) determined the concentration of volatile fatty acids and other free fatty acids (long-chain) in the semen of the domestic cock and some mammalian species of animals. Cock semen contained 0.08 m Eq./L long chained fatty acids and 0.51 m Eq./L volatile fatty acids and they concluded that the small amounts of free fatty acids in semen were not likely to be important as substrates for the metabolism of spermatozoa after ejaculation.

Lake (1962c) reported that the content of phosphorus was low in the seminal plasma of the domestic cock (7.5 mg%) and most was present in phospholipids. Dawson (1957) reported that cock semen contained a small amount of phosphorylcholine and no glycerylphosphorylcholine.

Cock seminal plasma was found to contain between 10-15 mg% sorbitol (King, Isherwood and Mann, 1958; King and Mann, 1959), 10 mg% inositol and a trace of erythritol (Ahluwahlia, 1963). The significance of the presence of sugar alcohols has not yet been substantiated.

Turkey seminal fluid resembled the vas deferens seminal plasma of the male domestic fowl in regard to sodium concentration (338 mg/100 ml) and freezing point depression (Δ -0.63^{o}C) (Brown, 1959). However, the potassium

concentration (103 mg/100 ml) was extremely high, possibly due to contamination with urine.

Pace, Moravec and Mussehl (1952) reported a relatively high concentration of fructose in freshly collected turkey semen (60 mg per 100 ml). This finding was questioned by Lorenz (1959) who pointed out that the fructose determinations were made using trichloroacetic acid as a deproteinizing agent; it could have resulted in a considerable amount of bound carbohydrate being extracted from the spermatozoa and consequently giving a relatively high and a misleading figure.

It is difficult to obtain a reliable estimate of the pH of fowl semen due to the ease of contamination with cloacal contents. Nishiyama (1952) reported that the pH of clean, uncontaminated fowl semen was 7.2 while that of semen, containing much 'transparent' fluid , few spermatozoa, some granules of unknown origin and blood cells, was 7.9. Bogdonoff and Shaffner (1954) measured the pH of several samples of fowl semen immediately after collection and reported a range between 6.72 and 6.94. Schindler, Volcani and Weinstein (1958) reported a pH of 7.12 for fowl semen. Schindler, et al. (1955) and Schindler et al. (1958) also demonstrated only slight changes in the pH of cock semen after storage whereas bull semen by comparison became rapidly acid. Wilcox and Shaffner (1957) and

Wilcox (1958) found that the pH of undiluted cock semen dropped when it was stored *in vitro* and reached 6.6 after 24 hours storage at 10°C. Lake (1962a) suggested that disagreements about the pH of cock semen could be due to the quality of semen dealt with in each case. Fowl semen, free from 'transparent' fluid , contains very little or no reducing sugars; if such semen is stored at 2°C for 24 hours, the pH becomes more alkaline and not acid. Contaminated semen contains glucose which can be utilized by the spermatozoa (Lorenz, 1958) and in this case it is possible that the pH would become acid during storage, the degree of acidity depending upon temperature and sugar concentration in the semen.

Wilcox (1958b) used sodium phosphate buffers of pH 6.5, 7.2 and 7.8 in diluting chicken semen for storage and for resuspension of centrifuged semen after storage, and found no apparent effect of pH within this range on the activity of spermatozoa. Wales and White (1958a) investigated the motility of fowl spermatozoa in media of different pH and maximum motility was displayed at pH7.

(2) Composition of spermatozoa

There is a relatively vast amount of information in the literature on the metabolism and chemical composition of somatic body cells e.g. muscle cells and red blood

cells of man and various animals. By contrast, relatively little is known about the composition of the spermatozoan cell, particularly in the fowl, as semen studies have in the main been conducted on seminal plasma. As a result of this state of affairs it is difficult to know how far one can apply information on somatic cell activity to solutions of the problems of storing spermatozoa *in vitro*. The spermatozoon differs from other cells in several respects e.g. a spermatozoon is motile, non-dividing and contains little cytoplasm and it would be of interest to find out how it differs chemically from somatic cells both in structure and metabolic requirements.

Steinbach and Dunham (1961) investigated the amounts of sodium, potassium and chloride in centrifuged spermatozoa of some invertebrates (Arbacia, Mytilus and Phascolosoma); trapped extra-cellular fluid in the pellets was determined with the aid of inulin. It was shown that spermatozoa resembled other cells in maintaining, relative to the sea water environment, high internal potassium and low sodium and chloride concentrations. Cragle, Salisbury and Van Demark (1958), without considering trapped seminal plasma in centrifuged cells, reported higher potassium concentrations (241.0 mg%) within bull spermatozoa compared with the seminal plasma (161.0 mg%).

There were greater amounts of sodium, calcium and chloride in the seminal plasma than within the spermatozoa. On the other hand, Keitel and Jones (1956) reported that the extracellular fluid of spermatozoa was very similar in ionic composition to the intracellular fluid in humans, containing a high concentration of sodium chloride and phosphorus and a relatively low concentration of potassium and carbon dioxide. These workers used Evans blue to determine the trapped seminal plasma in spermatozoan pellets and claimed that it was negligible in amount.

Lake (1962c) reported a preliminary investigation of phosphorus compounds in fowl spermatozoa. The distribution of phosphorus compounds in the spermatozoon is qualitatively similar to that found in the ram (Mann, 1964). However, in the fowl, phospholipid phosphorus accounts for the largest proportion of the total phosphorus next to deoxyribonucleoprotein-phosphorus. Perhaps the phospholipids are relatively more important to the activity of fowl spermatozoa. Ahluwahlia (1963) reported some inositol and erythritol in the spermatozoan fraction of fowl semen. However, the spermatozoan fraction also contained precipitated material from the seminal plasma owing to the method of preparation from whole semen. Further work is necessary to identify the part of the semen in

which the polyhydric alcohol was contained.

No information exists on the electrolyte balance of fowl spermatozoa. In this thesis it is intended to study and discuss the electrolytes of spermatozoa in comparison with somatic cells e.g. red blood cells. There is a little, scattered information available on the composition of the blood of the domestic fowl but invariably the age, sex or breed of fowl has not been specified and analyses have been carried out on pooled blood samples. Chicken erythrocytes are nucleated and larger than human cells but the potassium, sodium and water contents are similar (Maizels, 1954). Like in most (man, rat, rabbit, horse), but not all (dog, cat), mammalian red blood cells, much potassium and little sodium is present in the erythrocytes of the domestic fowl (Maizels, 1954; Morgan and Chichester, 1935) and duck (Tosteson and Robertson, 1956); the cells are normally suspended in plasma containing much sodium and little potassium.

(3) Metabolism of Spermatozoa

Fowl spermatozoa both respire and glycolyze in a similar manner to mammalian spermatozoa. Yoshida and Masuda (1962) and Harris and Wilcox (1962) showed that fowl spermatozoa were capable of utilising various monosaccharides and acetate for respiration while for

glycolysis they utilized only glucose, mannose or fructose. Scott, White and Annison (1962b) showed that octonoate stimulated respiration of fowl spermatozoa more than acetate. Yoshida and Masuda (1962) reported that intermediates of the tricarboxylic acid cycle increased respiration, but only succinate stimulated oxygen uptake markedly. Oxaloacetate stimulated oxygen uptake significantly but its effect lasted for a short time only. Scott, White and Annison (1962a) could not find any evidence for a metabolic pathway, involving the pentose shunt, being operative during the activity of fowl spermatozoa. They suggested that, in contrast to various mammalian spermatozoa, endogenous rather than exogenous substrates for respiration were more important to the activity of fowl spermatozoa. Furthermore, fowl spermatozoa had the lowest metabolic rate compared with the mammalian species studied.

Fowl spermatozoa can utilize fructose under aerobic and semi-anaerobic conditions (Lorenz, 1958) and, like those of the bull and ram, utilize aldoses preferentially to fructose. Lorenz (1958) also reported that fowl spermatozoa were capable of converting glucose to fructose and the rate of formation of fructose was found to be twice as high in aerobic as compared to anaerobic conditions.

The conversion of glucose to fructose by fowl spermatozoa was confirmed by Lake, et al. (1962) and Scott et al. (1962a). The precise reaction pathway for the conversion of glucose to fructose has not yet been identified although sorbitol may be an intermediate (Lake et al., 1962).

Goldberg and Norman (1961) reported the presence of the typical complement of cytochromes in fowl spermatozoa but so far have been unable to determine whether the cytochrome system serves as a major terminal oxidative pathway in metabolism.

Nevo, Caplan and Schindler (1963) maintained a constant pH 7.1 and glucose concentration whilst continuously recording the rate and amount of acid production by fowl spermatozoa under anaerobic conditions at 33°C. Results showed that during the first two hours of incubation both motility and metabolic rate declined gradually and then stabilized at a constant value. Motility remained constant for a period of 40-50 hours and then declined to zero within a few hours. Cessation of motility was not due to the accumulation of toxic substances in the medium and it was suggested that it must have been due to metabolic events occurring within the cell. Acid production remained constant for at least 60 hours at 33°C, twenty hours after motility had stopped. Lake (1962c) obtained res-

ults suggesting that fatty acids from lipids of fowl spermatozoa were depleted during storage *in vitro* irrespective of the presence or absence of exogenous carbohydrate and if lipids are associated with motility metabolism this fact could account for the findings of Nevo *et al*. (1963).

Fowl spermatozoa have been shown to catabolize glutamic acid but not glycine although the presence of both amino acids caused a reduction in the rates of respiration and fructolysis (El Zayat and Van Tienhoven, 1961b). Iype, Abraham and Bhargava (1963) reported that cock spermatozoa, in contrast to mammalian spermatozoa, were unable to incorporate radio-active amino acids into their proteins and they attributed it to the poorly developed acrosome in the bird spermatozoon, which was considered to be the part of the spermatozoon involved in protein synthesis.

Wales and Wallace (1964) studied the effects of potassium, magnesium, calcium and phosphate ions on the metabolism of fowl and certain mammalian spermatozoa. Fowl spermatozoa were found to have the lowest rate of oxygen uptake. Potassium ions stimulated metabolism in all species studied, magnesium depressed the respiration of bull and fowl spermatozoa and calcium was without effect

in either case. Phosphate ions had no significant effect on the metabolism of spermatozoa except for stimulating the respiration of unwashed dog spermatozoa. Yoshida and Masuda (1962), on the contrary, were able to demonstrate that the respiration of fowl spermatozoa was stimulated by calcium and slightly inhibited by phosphate ion. Contradictory findings on the effects of extraneous factors on spermatozoa activity *in vitro* are likely to be due to different investigators using suspension media of differing composition.

The interpretation of the results of studies on the respiration of fowl and turkey spermatozoa and the evaluation of diluent media by such means are complicated by the use of different media. Bade, Weigers and Nelson (1956) studied the oxygen uptake and fructolysis of turkey spermatozoa in artificial diluents. At optimum dilutions, spermatozoa respiring in phosphate buffer (pH 6.8) utilised 3 c.mm. O_2 per 10^8 cells per hour at 37°C (ZO_2 3), those in Earle's solution (pH 8.3) had a ZO_2 of 7. Over a range of 0.3 to 3.5 billion cells/ml, respiration of spermatozoa in phosphate buffer was independent of dilution. However, the oxygen-uptake of spermatozoa respiring in Earle's solution during 2 hours incubation was quite sensitive to dilution. Generally, there was a

decline in oxygen-uptake by spermatozoa with time which was independent of the death of the spermatozoa but positively correlated with the number of motile cells present. Fructolysis continued for a time after the cessation of motility of spermatozoa and it was concluded that neither oxygen-uptake nor fructolysis studies are likely to furnish means of assessing the fertilizing capacity of a semen sample.

There is an indication that a difference exists between the metabolism of turkey and fowl spermatozoa under similar conditions; oxygen was consumed faster by turkey spermatozoa (Ogasawara, 1957). Also mammalian spermatozoa appear to have a higher ZO_2 than those of the bird. Scott et al. (1962a), Van Tienhoven (1960) and Goldberg and Norman (1961) using a similar suspending medium found an oxygen-uptake of about 2.5 mm^3 for fowl spermatozoa and between 4 and 8 for the spermatozoa of several mammals studied.

C. Maturation of fowl spermatozoa in the vas deferens

Munro (1936, 1938a) suggested that fowl spermatozoa underwent a maturation or a "ripening" process during passage through the male reproductive tract which corresponded with a progressive increase in the attainment of motility and fertilizing ability. Spermatozoa taken

directly from the testis fertilized only 3 hens out of 69 (4.3%), epididymal spermatozoa fertilized 5 hens out of 39 (12.8%) and those taken from the vas deferens fertilized 57 hens out of 77 (74%). Fowl spermatozoa appeared to pass very quickly from the testes to the distal region of the vas deferens from which they are ejaculated. The passage time in a sexually-active male was estimated as 24 hours and, in a non-active male, as between 72 to 96 hours.

The epididymis in the cock is extremely short and the spermatozoa must pass quickly through it to the vas deferens in which they spend the greater part of their time before ejaculation. Unlike mammalian spermatozoa, a true kinoplasmic droplet, associated with immaturity, has never been described in fowl spermatozoa although Lake and El Jack (1964b), using electron microscopy, reported the presence of fragments of spermatid cytoplasm adhering to the posterior end of the head of spermatozoa obtained from the testes. These droplets seemed to disappear very quickly and were invariably absent in spermatozoa taken from the vasa efferentia-epididymis region. It is not yet known whether this cytoplasmic fragment is analogous to the kinoplasmic droplet of the mammal spermatozoon.

Nothing is known about the absolute life span of the fowl spermatozoon or how soon it begins to degenerate in

the vas deferens if not ejaculated. This knowledge could be important in studies concerned with the chemistry of semen because it is feasible that ions and molecules from degenerating spermatozoa could enter the surrounding fluid and be mistakenly estimated as components of it.

D. The survival of spermatozoa in the Oviduct

The phenomenon of "delayed fertilization", or fertilization accomplished by spermatozoa which have been stored within the oviduct for long periods, is well-established among the invertebrates. Bishop (1920) and Courrier (1921) reported that the Queen bee was capable of maintaining its fertility for as long as seven years after a single mating. Many female invertebrates have spermathecae which act as a storage site and in which viable spermatozoa are stored for a considerable length of time.

Among the vertebrates, prolonged survival of spermatozoa within the female body is comparatively less common. Some fishes which have internal fertilization, are capable of retaining viable spermatozoa within their reproductive tract for long periods. Some bats mate in the Autumn and their follicles do not ripen until the onset of Spring; the spermatozoa are stored within the oviduct during hibernation (90-159 days) and are capable of fertilization and initiation of embryonic development as late as the end

of Spring (Folk, 1940; Wimsatt, 1942, 1944).

"Delayed fertilization" is well-known among reptiles. Ludwig and Rahn (1943) and Fox (1956) have reported that in the Prairie Rattle snakes spermatozoa are stored in the seminal receptacles, just distal to the uterus, throughout Winter and are capable of successful fertilization the following Spring. Hains (1940) observed that a central American snake kept in captivity laid fertile eggs at least five years after the last possible copulation. Woodward (1933) found that a female African night adder laid fertile eggs for at least 5 months in captivity. More recently, Fox (1963) described special tubular outgrowths of the vagina of certain female lizards which acted as a storage site for spermatozoa. Ewing (1943) reported that a mated, female, box-turtle retained her fertility for two years after being isolated from males, another for three years and two for four years.

The domestic hen, recently evolved from reptilian origin, is capable of retaining its fertility for at least a week after separation from males. Tauber (1875) found that a single copulation frequently resulted in fertile eggs for up to 11 days and occasionally 17-18 days after the removal of the male. Crew (1926) and Dunn (1927) recorded a fertile egg laid by a hen 32 days and 30 days

after isolation from males respectively. All investigators are in complete agreement that after 11 or 14 days following a single insemination in the domestic fowl or withdrawal of the males, fertility tends to fall and, consequently, poultry breeders who include artificial insemination (A.I.) in their breeding programme, inseminate once and sometimes twice a week to maintain high fertility figures in their flocks.

It is generally accepted that the effective life-span of spermatozoa within the oviduct of the majority of mammals is extremely short. However, it would appear that it is not known whether this is a function of the spermatozoa themselves or the conditions in the female reproductive tract.

Several investigations have been made to discover how spermatozoa survive in the oviduct of domestic fowl for unusually long periods. Iwanow (1924) suggested that active spermatozoa fertilized mature as well as immature ova immediately after mating because he failed to terminate fertility by irrigating the oviduct and the peritoneal cavity with spermicidal fluids. Walton and Whetham (1933) confirmed Iwanow's findings, and suggested that the spermatozoa might reside in crevices of the oviduct where they were protected from the spermicidal fluids. Tauber

(1875) described the existence of a "receptaculum seminis" in the hen, consisting of folds close to the anterior margin of the infundibulum, which acted as a storage site for spermatozoa. Van Drimmelen (1946) discovered sperm-containing glands in the chalaziferous region of the infundibulum of the oviduct and called them 'sperm-nests'. It was believed that these glands were the residence sites for spermatozoa after examining one hen in which 'sperm-nests' were found 8 days after intraperitoneal insemination. Recently, Bobr, Lorenz and Ogasawara (1962, 1964a,b), Fujii (1963) and Fujii and Tamura (1963) described a glandular region in the utero-vaginal junction of the domestic hen which they believed acted as a residence site for spermatozoa; spermatozoa invaded these glands rapidly and in large numbers after natural or artificial insemination. Accumulations of spermatozoa, with heads orientated towards the wall of the gland and tails towards the lumen, were found in progressively decreasing numbers for as long as fertile eggs were laid.

Bobr et al. (1962, 1964a,b) found spermatozoa only occasionally in the chalaziferous region of the infundibulum while they were found in profusion in the glands of the utero-vaginal junction in every artificially inseminated or naturally mated hen. Fujii and Tamura (1963) found spermatozoan accumulations in the chalaziferous region of

the infundibulum but they were less concentrated than in the glands of the utero-vaginal junction; they concluded that the utero-vaginal glands possessed some factor(s) that favoured the long survival of spermatozoa and that they acted as a preliminary reservoir for spermatozoa until they reached the glandular region of the infundibulum where they were finally stored to accomplish fertilization of the ovulated ova. More work would appear to be necessary to establish the part played by the glands of the utero-vaginal region and infundibulum in the prolonged survival of the spermatozoa. Secretions in these regions of the oviduct might favour the support of the metabolism of the spermatozoa. If the uterovaginal glands were the residence site of spermatozoa, a control mechanism, causing the periodic release of spermatozoa to travel from this site up to the infundibulum, where fertilization occurs, would have to be investigated.

Lamoreux (1940) reported that the best laying-hens laid fewer infertile eggs and had consistently longer duration of fertility than poor layers. He postulated that the level of oestrogens in circulation in high producers might play a part in maintaining a favourable environment for the spermatozoon in the oviduct.

The duration of the life of spermatozoa in turkey

hens is longer than that of the fowl. Burrows and Marsden (1938) obtained 83.1% fertility from 31 hybrid turkey hens (Bronze ♂ x White Holland ♀) during periods of 30 days between inseminations. Lorenz (1950) found that the duration of fertility in Broad-Breasted Bronze turkey hens could be as long as 6 weeks and McCartney (1950) reported periods of 51.9 days in broody and 47.2 in non-broody White Holland turkey pullets. Van Tienhoven and Steel (1957) reported the duration of fertility in Empire White hens to be 45-50 days. Verma and Cherms (1964) studied the anatomy of the oviduct of Broad Breasted Bronze turkey hens, and found scattered tubular glands in the utero-vaginal junction; the lumen contained aggregates of spermatozoa and it was suggested that this was the normal storage site for the spermatozoa in the turkey. Verma and Cherms (1965) made a detailed study of the storage areas and duration of fertility in Broad Breasted Bronze turkey hens following a single insemination. The accumulation of spermatozoa in the glands of the utero-vaginal junction was confirmed and no spermatozoa were found in the glands of the chalaziferous region of the infundibulum. The number of tubules containing spermatozoa reached a peak at 5 days following insemination and then gradually declined to zero at the 45th day. The decline

in fertility and the progressive decline in the number of tubules containing spermatozoa were found to be significantly correlated. Verma and Cherms (1965) found that the average duration of fertility for 6 hens to be 33 days which was lower than that reported by other workers, due probably to the insemination of hens late in the breeding season when egg production was dropping.

The functional life of spermatozoa within the female reproductive tract of ducks and geese is comparatively shorter than that of chickens and turkeys. Johnson (1954) observed that after artificial insemination an average duration of fertility of 9.6 days resulted in geese. Occasionally a fertile egg was found after 16 days. Ash (1962) observed a maximum duration of fertility of 12 days in White Pekin ducks after isolation from the drake.

Recently it has been reported that fertility decreases with the advance of the breeding season, both with natural and artificial insemination (Wentworth and Mellen, 1964). It was suggested that hens became immunised against spermatozoa after a period of continuous breeding. If substantiated this observation is the first to demonstrate that natural immunity to spermatozoa can occur in poultry with a detrimental effect on fertility.

E. Secretions of the Oviduct and their effect on Spermatozoa

The general histological structure of the oviduct of the domestic hen has been investigated by Surface (1912) and Bradley (1928). The secretory glands of the oviduct have been studied by Richardson (1935), Fujii (1963), Johnston, Aitken and Wyburn (1963), and Aitken and Johnston (1963). The oviduct is complex and generally, the following distinct parts are recognised reading from the anterior end:-

1. Infundibulum or funnel; 2. Chalaziferous region; 3. magnum or albumen-secreting region; 4. the isthmus in two or three parts (where egg membranes are formed); 5. uterus or shell gland; 6. Utero-vaginal junction; 7. vagina.

The composition of oviduct secretions in relation to the activity of spermatozoa has received little attention.

Buckner and Martin (1929) measured the pH of small segments of the oviduct of recently-killed hens and reported that the oviduct secretions were acidic. The albumen-secreting region of an actively laying bird had a pH ranging between 6.3 - 6.6 while the mucosa of the isthmus and uterus was markedly more acid with a pH ranging between 5.6 - 5.9.

Recently Ogasawara, Van Krey and Lorenz (1964) obtained better results when they carried out determinations

of pH using an electrode-probe assembly on the oviducts of anaesthetized laying hens. The fluids on the mucosal surface appeared to be alkaline throughout the oviduct, except in the extreme distal vagina which was acid. The highest pH was recorded in the infundibulum region (pH 7.74) and in the utero-vaginal junction (7.87). Between these two regions, the pH declined steadily to 7.17 in the posterior magnum. The proximal vagina had an average pH 7.7. It is interesting that the highest pH existed in the regions of the oviduct where spermatozoa are stored i.e. utero-vaginal area and infundibulum. Ogasawara et al. (1964) reported that the pH dropped an average of 0.7 units when measurements were carried out 30 to 45 minutes after death of the hen and it was more pronounced at the ends of the oviduct. The authors suggested that this rapid shift in pH after death could account for the low figures reported previously by Buckner and Martin (1929). Recently, Winget, Mepham and Averkin (1965) reported that the pH in the shell gland (uterus) fluctuated between 7.25 and 7.56 according to the stage of development of the egg in the oviduct.

In view of the fact that spermatozoa appear to reside in the utero-vaginal area during the fertility period of a hen the uterus secretions and their changing composition in relation to egg formation may play a role in controlling

sperm movement upwards from this site. From the 5th to about the 24th hour after an oviposition an egg is present in the shell gland, and for about the first 5 hours it is rapidly increasing in volume due to a watery fluid ('plumping' fluid) from the shell gland passing into it through the membranes surrounding the albumen (Burmester, 1940). Beadle, Conrad and Scott (1938) attempted to collect 'plumping' fluid by inserting a rubber tube into the shell gland per vaginam. The tip of the tube was put ventral to the egg and the fluid was drained into a small vial; up to 3.0 ml was collected. The fluid was invariably contaminated by cloacal contents. Hoover and Smith (1952) found that the collection of 'plumping' fluid with rubber, clinical rectal catheters was unsatisfactory. Thus, they used glass catheters, with a perforated, egg-shaped bulb on one end, which was pushed into the shell-gland per vaginam. However, trauma developed occasionally when the catheters were introduced but lubrication with petroleum jelly minimized the damage. The largest catheter (50 ml capacity) caused considerable haemorrhage when inserted into the lumen of the shell gland. In spite of the technical difficulties of collecting shell-gland fluid Hoover and Smith obtained results which suggested that the physiological state of the shell-gland appeared to be more important than the

mechanical stimulus in determining the rate of secretion of the 'plumping' fluid.

Lake (unpublished observations) attempted to collect natural, fluid secretions from the region of the utero-vaginal junction by putting absorbent wool material into the lumen for a variable length of time. However, only a minute amount of fluid was obtained if the wool was left for a short time, while if it was left for a longer period oedema developed and it was not certain whether a normal secretion was obtained. Data on the chemical composition of 'plumping' fluid is sparse; Beadle et al. (1938) estimated the contents of sodium and potassium to be 255 mg and 98 mg per 100 ml of water respectively. This concentration of potassium is very high and its significance in oviduct activity has yet to be determined.

Some attention has been paid to the effects of the secretions of the hen's oviduct on the metabolic activity of fowl spermatozoa in vitro. Ogasawara and Lorenz (1964) reported that when an extract of the fluid from the magnum of laying hens was added to a diluent, the respiratory rate of fowl spermatozoa was raised more than twofold. Fluids from the infundibulum also caused a moderate increase in oxygen consumption, but fluids from the isthmus and uterus had no stimulatory effect. The authors suggested that the active material from the

magnum was an organic substance of high molecular weight, probably protein in nature.

Hamner and Williams (1963) incubated cock spermatozoa in an isolated segment of the hen's magnum and observed that the respiratory rate of subsequently recovered and washed spermatozoa was more than twice that of freshly-washed spermatozoa. Similar results with rabbit spermatozoa were found; the addition of oviduct fluid *in vitro* similarly increased the respiratory rate of spermatozoa. The authors suggested that the enhanced respiration was part of a 'capacitation' process. Williams, Weinman and Hamner (1964) reported that bicarbonate appeared to be the major stimulant of respiration of rabbit spermatozoa in the presence of oviduct fluid. The respiration of human, bull and cock spermatozoa was markedly stimulated by appropriate bicarbonate concentration. With rabbit spermatozoa, the addition of succinate gave similar results to those of bicarbonate and it was suggested that bicarbonate increased respiration by more efficiently coupling glycolysis with the tricarboxylic acid cycle.

THE EXAMINATION OF THE PRINCIPAL INORGANIC IONS OF FOWL SEMEN, BLOOD AND UTERINE FLUID.

A. Material and Methods

(1) Chemical Methods

THE EXAMINATION OF THE PRINCIPAL INORGANIC IONS OF FOWL SEMEN, BLOOD AND UTERINE FLUID

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(1) Chemical Methods

(a) The Determination of Calcium, Magnesium, Sodium and Potassium by Atomic Absorption Spectroscopy

The concentrations of calcium, magnesium, potassium and sodium were determined by atomic absorption spectroscopy (Gatehouse and Willis, 1961) in extracts of materials which were appropriately diluted with doubly glass-distilled water. The purity of the water was always checked by running samples as blanks in the atomic absorption spectrophotometer.

Since Walsh (1955) first suggested the use of atomic absorption spectra for analytical purposes much interest has been aroused in the technique. The application of atomic absorption to chemical analysis has been reviewed by David (1960) and Allan (1962) and it has been used with success for the analysis of several biological fluids e.g. for calcium and magnesium determinations in blood serum (Willis, 1960a,b) and calcium and magnesium in urine (Willis, 1961; Stewart, Hutchinson and Fleming, 1963).

In view of Willis's finding (1960a, 1961) that the

presence of appreciable concentrations of phosphorus in some biological material tended to interfere with the estimation of calcium, a preliminary test was carried out on seminal and blood plasmas to determine the extent of any phosphorus interference. Two sub-samples of each plasma were prepared and to one was added strontium chloride, which prevents the interference by phosphorus. No difference was found between the values obtained and thus samples directly diluted were considered suitable for analysis.

Before finally adopting direct dilution methods for analysis by atomic absorption spectrophotometry an experiment was performed to find out whether wet ashing of the material was a necessary preliminary for extracting all the ions. 0.5 ml of whole semen and seminal plasma were pipetted into platinum crucibles with lids and put in a furnace at 450°C for 16 hours. Afterwards they were cooled, five drops of 6N.HNO_3 were added and the samples heated for 30 minutes at 200°C to get rid of nitric acid. A few drops of hydrogen peroxide were added to complete the ashing. After cooling 0.1 ml 6N HCl and 4.9 ml doubly glass-distilled water were added to dissolve the ash; after thorough mixing the crucible contents were transferred to a 10 ml stoppered pyrex tube. This extract of semen,

which was diluted tenfold, was used for calcium determination and suitable dilutions were made from it for magnesium, sodium and potassium. It was found that the simpler, direct acid dilution method for these ions gave the same results as after the ashing procedure and was preferred to the latter.

Additional aliquots of 0.1 ml semen or seminal plasma were ashed with 0.5 ml 6N HNO_3 and a few drops of hydrogen peroxide for chloride determinations. A blank containing 0.5 ml 6 N HNO_3 and hydrogen peroxide was treated simultaneously. This method of preparing material for the estimation of chloride was abandoned since the element disappeared during ashing.

(b) The Determination of Chloride by Electrometric Titration

Chloride was determined by electrometric titration with silver ions(Cotlove, Trantham and Bowman, 1958). For whole semen, seminal plasma, blood plasma, 'plumping' fluid and 'oviposition' fluid an 0.10 ml aliquot was accurately measured with a 0.10 ml pipette and run slowly into a plastic pot. 10.0 ml of a solution of 0.1 N nitric acid in 10% acetic acid and five drops of gelatin-indicator solution were added and titration carried out whilst continuously stirring the mixture.

Erythrocytes could not be titrated directly in the

above manner as an unknown constituent of water extracts of erythrocytes interfered and elevated the values obtained for chloride content. According to Cotlove (1963), the presence of high concentrations of proteins or sulphhydryl compounds in biological material augmented chloride estimations and to prevent this interference he recommended protein precipitation before titration. Thus, protein precipitation was carried out on water extracts of erythrocytes using the following method. Venous blood was collected under liquid paraffin from the wing vein of males in heparinised, plastic syringes. It was transferred immediately to small, straight-sided pyrex tubes, containing paraffin, and kept in cold water to minimize chloride-shift. The blood was then centrifuged for 30 minutes (3,200 g; 5°C) and the plasma transferred to a clean pyrex tube. The 'buffy' coat of leucocytes was sucked off and 0.50 ml erythrocytes were delivered into a 10 ml stoppered pyrex tube, using a 1.0 ml grade A pipette, and then 7.5 ml double glass-distilled water was added. Protein precipitation was carried out according to Cotlove (1963) using 1.0 ml 10.0 N Zn $SO_4.7H_2O$ and 1.0 ml of 0.5 N NaOH. The contents of the tube were shaken vigorously and left standing at room temperature for 2-3 hours. Afterwards the tube was centrifuged and the supernatant carefully pipetted into a clean pyrex tube.

Aliquots of 3 ml were used for chloride analysis of cell extract and it was diluted as usual with 10.0 ml of nitric acid-acetic acid mixture and five drops of gelatin-indicator solution. Titration was carried out whilst continuously stirring. Blanks and standards were treated identically.

(c) The Determination of Nitrogen

Nitrogen was determined by the method of Chibnall, Rees and Williams (1943) after digesting appropriate aliquots of material in microkjeldahl flasks using copper-selenate catalyst.

(d) The Determination of Carbon Dioxide

Carbon dioxide was estimated by gas chromatography (McKay, Seligson and Taylor, 1959) after being liberated from the body fluids in a reaction chamber of a Blood Gas Accessory apparatus attached to a Beckman GC-2A Gas Chromatograph (Johns and Thompson, 1963). Helium was used as a carrier gas and a silica gel column separated the carbon dioxide from oxygen and nitrogen. The distinct peak of carbon dioxide was recorded on a Honeywell Chart Recorder, the area under the curve was used to estimate the quantity of CO_2 in the sample after specially-prepared sodium carbonate solutions had been used to calibrate the

instrument. Hamilton microliter syringes were used to introduce the fluids into the reaction chamber of the gas chromatograph and care was taken that sufficient lactic acid was used to liberate the carbon dioxide.

(e) The Measurement of the Water Content of Semen and Seminal Plasma

An appropriate volume of semen was centrifuged in a tared tube and the seminal plasma removed; a measured portion of the latter was placed in another tared vessel. The part of the tube above the packed spermatozoa was carefully cleaned and dried and together with the tube containing seminal plasma, was dried at 105°C overnight. Afterwards both samples were cooled in a dessicator and weighed on a micro-balance (Mettler) to constant weight. The air in the weighing chamber of the balance was kept as dry as possible by placing a number of small containers of silica gel about the pan of the balance. Under these conditions the gain in weight due to water addition to the dried container was minimal.

THE EXAMINATION OF THE PRINCIPAL INORGANIC IONS OF FOWL SEMEN, BLOOD AND UTERINE FLUID.

A. Material and Methods

(2) Biological Procedures

(2) Biological Procedures

(a) Experimental Birds. Fifty six male birds of the same age and randomly selected from the control line of Brown Leghorns, which has been developed over several years at the Poultry Research Centre, Edinburgh (Blyth) 1954), were used for semen and blood analysis. They were kept in large, single cages.

The hens used for analysis of uterine fluids were a Breeding-Intensity cross of Brown Leghorns (Blyth, 1954) and were kept in single battery cages so that individual egg laying records could be accurately made.

All the birds were fed a commercial breeders ration and given 14 hours of artificial light daily.

(b) The Collection of Semen, including a Consideration of the Method for the Collection of Consistent Samples of Uncontaminated Semen over a Long Period

Semen was collected by the one-man massage method of Lake (1957a). The cock was removed quietly from the cage and its legs were placed between the knees of the operator who sat on a low stool. A firm but gentle grip was maintained with the knees so that both hands were free. The lumbar region of the back of the cock was stroked tailwards gently with the right hand a few times depending upon the sensitivity of the bird. The hand stroke ended with the thumb and forefinger making a slight upward movement at the base of the tail. As soon as a reflex response occurred, as recognised by the variable degree of tail raising and tension of the legs of the cock, the operator moved his right hand over the tail and gently squeezed on either side of the cloaca using the thumb and

forefinger. Semen was thus expelled from the erected copulatory organ into a 2"-diameter glass funnel held in the operator's right hand.

'Transparent' fluid, obtained from the lymph folds and vascular tissue of the copulatory organ of the cloaca during erection is an extremely variable component of the seminal plasma collected by massage. It is doubtful whether it is part of the normal seminal plasma of copulation (Lake, 1957a) and in any case is harmful to the longevity of spermatozoa *in vitro* (Lake, 1957a; Nishiyama, 1955). In the present work it was intended to study the composition of spermatozoa in a natural physiological *milieu* in which they are known to survive and maintain their activity for a relatively long period *in vivo* i.e. fluid of the distal vas deferens (Munro, 1938b). Spermatozoa in this fluid are obtained when semen is collected by massage as described above and the product is designated as uncontaminated semen (Lake,1957a) for the purpose of this study. Cocks which tended to urinate or defaecate easily during semen collection were not used. Furthermore, the males were milked regularly i.e. at least once a week, to ensure that the minimum number of degenerating spermatozoa were present in the semen sample. This procedure was adopted in the light of the results obtained in the following

experiment.

Material and Methods. Twenty related males of the Breeding line of Brown Leghorns at the Poultry Research Centre (Blyth, 1954) were selected at random from cocks trained for artificial insemination. Semen had been collected, at least once weekly, from all males for a month before the start of the experiment. The males were subdivided randomly into five groups; one group was kept as a control and the other four were rested for two, four, six and eight weeks respectively between ejaculations. The males in the control group were milked for semen continuously at intervals of two, three or seven days over the ten week experimental period.

After the various groups of cocks had been ejaculated at the end of their allotted rest periods, they were subsequently milked as frequently as the controls and once again their semen was investigated, after some weeks of the procedure, to establish that season or age per se had not caused any marked deterioration of semen quality.

A nigrosine-eosin stain technique was used to examine the quality of spermatozoa in ejaculates. 0.60 g nigrosine was added, with constant stirring at room temperature, to 10 ml isotonic glutamate solution (Lake, 1960) containing

0.16 g eosin. Aliquots of 0.5 ml of this stain mixture were put into small test tubes kept in ice-cold water. Uncontaminated semen was collected and 0.02 ml added to the stain and mixed thoroughly. After ten minutes one drop of the stain-semen mixture was placed on a clean slide and a smear made. The slide was dried quickly and thoroughly in warm air and the smear mounted with DPX (Gurr's Ltd) mounting fluid and a coverslip. Duplicate smears were made. A total of 300 spermatozoa was counted on each slide and duplicate counts were made on each specimen. The proportion of unstained, normally-shaped intact spermatozoa (Lake, 1954) was determined in each semen sample and an assessment of viable spermatozoa was made on this basis.

Results. The results in Table 1 and Fig.1 showed that there was a tendency for the number of degenerating spermatozoa in an ejaculate to rise after cocks have been rested between ejaculations for more than two weeks. There were individual variations in the extent of deterioration of ejaculates and some cocks appeared not to be appreciably affected.

However, over the 8-weeks experimental period an analysis of variance (Table 2) revealed that the overall

TABLE I.

THE MEAN NUMBER OF NORMAL SPERMATOZOA IN NIGROSINE-EOSIN SMEARS OF SEMEN TAKEN FROM BROWN LEGHORN COCKS AFTER VARIOUS PERIODS OF REST BETWEEN EJACULATIONS. (THE TOTAL NUMBER OF SPERMATOZOA COUNTED ON EACH SLIDE WAS 300. THE CONTROL MALES WERE MILKED FOR SEMEN AT INTERVALS OF NOT MORE THAN ONE WEEK).

BIRD NO.	WEEKS AFTER COMMENCEMENT OF EXPERIMENT						
	0	2	4	6	8	9**	10**
Control Group							
1.	277.0	281.5	282.0	283.0	284.2	285.0	285.5
7.	270.0	285.0	271.0	269.0	278.2	272.0	283.5
8.	277.0	282.5	276.0	277.0	270.0	281.0	274.0
20.	272.3	275.0	276.0	268.5	278.7	276.0	273.5
	274.1	281.0	276.2	274.4	277.8	278.5	279.1
2-weeks Rest Group							
2.	275.6	273.0				283.0	282.0
5.	234.6	243.5				212.6	249.0
12.	277.3	212.0				279.5	284.5
18.	278.6	271.1				261.0	269.5
	266.5	249.9				259.1	271.2
4-weeks Rest Group*							
3.	274.0		246.0			269.3	261.0
6.	266.0		256.5			260.5	273.5
9.	279.0		253.0			273.5	281.5
	273.0		251.8			267.7	272.0
6-weeks Rest Group							
4.	275.0			274.2		276.5	283.0
10.	282.6			271.0		273.5	270.5
15.	270.0			236.5		276.0	283.0
19.	270.0			147.0		253.6	233.5
	274.4			232.2		269.9	267.5
8-weeks Rest Group							
11.	258.0				184.0	232.0	248.5
14.	276.0				272.6	279.5	280.5
16.	279.0				184.0	272.0	286.0
17.	285.0				195.5	258.0	267.0
	274.5				209.0	260.4	270.5

* One cock in this group died during the experiment for reasons unconnected with the experiment.

** The experimental males were milked at weekly intervals after their selected rest period and test samples were taken twice from all males during weeks 9 and 10.

TABLE 2.

ANALYSIS OF VARIANCE FOR THE DATA OF TABLE I TO TEST WHETHER THERE WAS A SIGNIFICANT TREND IN THE INCREASE OF DEGENERATE SPERMATOZOA IN SEMEN SAMPLES WITH INCREASING INTERVALS BETWEEN EJACULATIONS.

Source of Variation		D.F.	S.S.	M.S.	F(Variance Ratio)
Between groups	Linear	1	8673.02	8673.02	6.5301*
	Residual	3	618.62	206.21	
Between groups		4	9291.62		
Within groups		14	18594.17	1328.15	
		18	27885.79		

* $p < 0.05$

FIG. 1

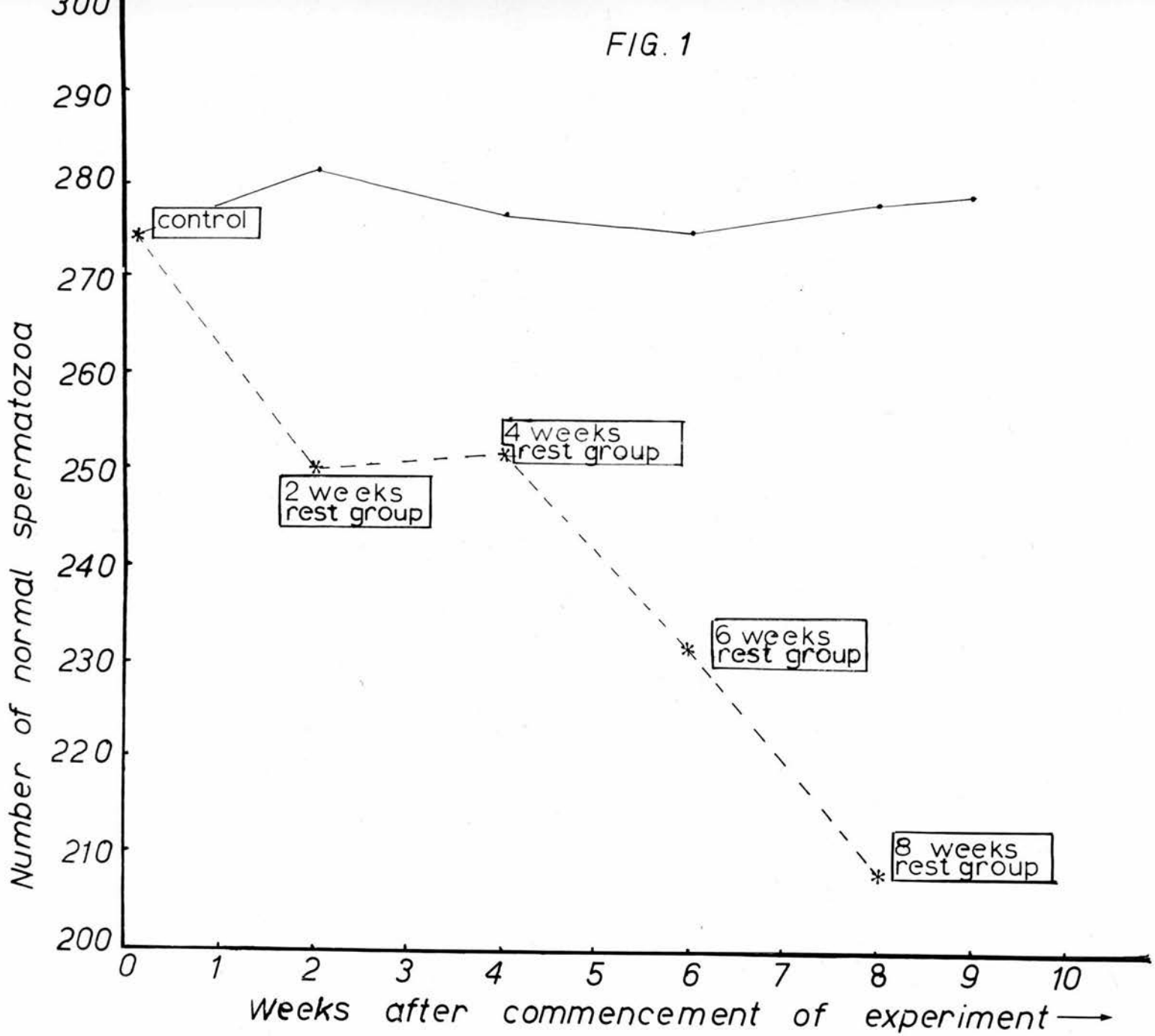
300
290
280
270
260
250
240
230
220
210
200
Number of normal spermatozoa
control
2 weeks rest group
4 weeks rest group
6 weeks rest group
8 weeks rest group
0
1
2
3
4
5
6
7
8
9
10
Weeks after commencement of experiment

FIG. 1. Graph showing the mean number of normal spermatozoa in nigrosine-eosin smears of ejaculates collected from Brown Leghorn Cocks after various periods of rest from ejaculation. A total of 300 spermatozoa were counted on each slide and the number of normal, unstained spermatozoa noted.

trend of an increase in degenerating spermatozoa was just significant ($p < 0.05$).

When the experimental males were ejaculated frequently after their respective rest periods, the semen samples again revealed a tendency to return to the pre-rest level of normal spermatozoa..

Discussion. The observations made in this experiment showed that the number of degenerating spermatozoa in semen samples was liable to increase in some cocks when rest-periods between ejaculations were extended for more than two weeks. It would seem desirable that for work on the chemical composition of semen, cocks should have semen collected regularly from them. Until further evidence is available, it is possible that the presence of large numbers of degenerating spermatozoa might vitiate attempts to analyse seminal plasma and spermatozoa separately, because unusual amounts of intracellular contents may leak out of the spermatozoa under these circumstances and appear in the surrounding seminal plasma.

(c) The Preparation of Whole Semen, the Separation of Spermatozoa from Seminal Plasma and the Estimation of the Volume of Spermatozoa in Semen Samples

It was necessary to use a number of males to collect a sufficiently large pool of semen for all analyses to be carried out simultaneously because the average ejaculate of uncontaminated semen from each cock is only about 0.15 ml.

The semen of 29 males in 1963-64 and 27 males in 1964-65 was pooled on many separate occasions and each time about 20 minutes was required to accomplish this task. Each sample of semen was transferred immediately after collection into a centrifuge tube kept in a bath of cold water. It was hoped in this way that any possible metabolic changes and alterations of ion fluxes in the semen would be minimised.

The pooled semen was mixed thoroughly but gently with a Pasteur pipette by drawing semen in and out, and finally by rolling the tube between the hands. Precautions were taken to avoid frothing during transference and mixing. All aliquots of whole semen for subsequent use were taken immediately after mixing and before spermatozoa began to settle out ... STAGE 1.

For part of the present work it was desired to determine the content of inorganic ions per unit volume of spermatozoa and thus it was necessary to estimate the total volume of spermatozoa in semen samples. For this purpose packed spermatozoa were obtained and the amount of trapped seminal plasma had to be determined. The following

formulae were used to calculate the trapped plasma and hence the total volume of spermatozoa in each semen sample.

$$V = V_a - V_s \quad \ldots \quad (1)$$

$$V_s = \frac{V_D}{(A/B - 1)} \quad \ldots \quad (2)$$

V = Total volume (ml) of spermatozoa.

V_a = Apparent volume (ml) of packed spermatozoa after centrifugation.

V_s = Volume (ml) of trapped seminal plasma in packed spermatozoa.

V_D = Volume (ml) of diluting fluid added to cells after centrifugation to resuspend the trapped plasma.

A = gm N_2 per ml seminal plasma.

B = gm N_2 per ml diluted, trapped seminal plasma.

The following procedures were carried out to find the components of the formulae -

About 3.5 - 4 ml whole semen from STAGE I (page 69) was transferred to a small, straight-sided pyrex tube using a 5 ml-capacity Grade A pipette; it was run out slowly from

the pipette to minimize the amount left behind on the inner wall. The semen was next centrifuged in an MSE (Model 551) centrifuge for 30 min. at 1500 g (4^{o}C).

The speed of the centrifuge was constantly tested throughout the two-year-period during which analyses were performed. The conditions of centrifugation were chosen after preliminary experimentation showed that all spermatozoa were separated from the supernatant seminal plasma and no detectable damage was sustained by the spermatozoa when they were subsequently resuspended. If the semen was spun at more than 1500 g spermatozoa were likely to be damaged; deformed, as well as an increased number of stained heads, were observed in nigrosine-eosin smears of the resuspended semen.

After centrifugation the upper meniscus, i.e. the total semen volume, and the level of the apparent mass of spermatozoa (V_a for formula (1) were marked carefully on the tube with a diamond pencil. Afterwards the tube was thoroughly cleaned and the marks calibrated with Grade A pipettes. The apparent volume of spermatozoa (spermatocrit) was determined by this method because the amount of semen available for all the analyses in this study was limited and there was insufficient to fill a

separate Wintrobe haematocrit tube which could have been centrifuged under the same conditions.

Great care was taken not to disturb the spermatozoa after marking the tube whilst the supernatant was taken off with a pyrex Pasteur pipette and transferred, for safe keeping, to another pyrex tube for use in subsequent analyses of seminal plasma ... STAGE 2.

It was next necessary to resuspend the trapped seminal plasma in a diluting fluid and so the inner wall of the tube containing the spermatozoa was wiped carefully and the small amount of seminal plasma left behind was absorbed using a clean filter paper. The wall was washed by introducing cold, isotonic sodium citrate (3.55%) solution drop by drop down the side of the tube till it was almost full; great care was again taken not to disturb the spermatozoa. The sodium citrate solution was taken off and the inside of the tube again dried with a filter paper making sure that all the citrate was removed from just above the surface of the mass of spermatozoa. 2.5 ml (V_D for formula (2)) of cold sodium citrate (3.55%) solution was added to the tube and the packed spermatozoa resuspended thoroughly using a wide-mouthed Pasteur pipette, making sure that no large aggregates of spermatozoa

remained. Thus a homogeneous suspension of spermatozoa in citrate solution was made. It was centrifuged for 30 minutes (1500 g, 4°C) and the supernatant removed ... STAGE 3..

Isotonic, 3.55% sodium citrate was used for resuspending spermatozoa for two reasons, (a) because Yamane et al. (1962) found that it was a favourable medium for fowl spermatozoa for a few hours in vitro, and (b) because isotonic sodium chloride solution proved detrimental to spermatozoan morphology as judged from an examination of nigrosine-eosin semen smears after its use.

To complete Formula (2) it was necessary to determine the nitrogen of neat seminal plasma (from STAGE 2) and that diluted with a known amount of citrate, i.e. that which had been trapped between cells (from STAGE 3). All the parameters were then available to solve equation (2) and calculate from equation (1) the space (V_s) occupied by trapped seminal plasma in the original centrifuged semen samples. Duplicate 0.1 ml aliquots of neat plasma (from STAGE 2) and 0.4 ml of supernatant from STAGE 3 were taken for the estimation of nitrogen.

Nitrogen was chosen as the most suitable component to estimate since there was no evidence of the leakage of nitrogeneous material from spermatozoa under the conditions

of the experiment. Also the nitrogen estimation was used satisfactorily by Bell (1957) to determine trapped blood plasma in a study of the contents of fowl erythrocytes. On the other hand, if sodium was estimated, using isotonic fructose solution as a resuspension fluid, very erratic results were obtained.

(d) Estimation of the Density of Spermatozoa in Semen Samples

The density of spermatozoa in semen was obtained by using an electronic Fisher Autocytometer. Freshly-collected semen was diluted 60,000 to 90,000-fold with a blood diluting fluid (Fisher Chemicals Ltd.) and put into the apparatus within 10 mins. of collection and dilution. A direct count of spermatozoan density (cells per cmm) was obtained in 20 secs. Initially the results obtained by the conventional haemacytometer technique were compared with the results obtained with the Autocytometer. The density was 8% higher when estimated by the latter method which is consistent with the findings of other workers who have used electronic counters for bull spermatozoa (Glover and Phipps, 1962; Iversen, 1964).

(e) The Preparation of Whole Semen, and Seminal Plasma for Analysis of Calcium, Magnesium, Sodium, Potassium and Chloride

(1) Whole Semen. Two aliquots (0.5 ml) of thoroughly mixed semen (STAGE 1 preparation described on p 69) were accurately measured, with a grade A pipette into a 10 ml stoppered, pyrex tube. 4.5 ml 0.1N HCl was added and after mixing thoroughly was left to stand overnight at room temperature. It was then centrifuged and the supernatant transferred to a clean pyrex tube. This extract was used for sodium, potassium, calcium and magnesium determinations after aliquots had been diluted tenfold (calcium), fiftyfold (magnesium), four hundredfold (potassium) and 1500-fold (sodium).

Duplicate aliquots of 0.1 ml whole semen were taken for chloride estimation.

(2) Seminal Plasma. 0.5 ml aliquots of neat seminal plasma from the centrifuged sample of semen (STAGE 2) were pipetted into a 10 ml glass-stoppered pyrex tube and 4.50 ml of 0.10 N HCl added. Aliquots of this extract were diluted, after thorough mixing and standing overnight, with distilled water tenfold for calcium, fiftyfold for magnesium, 200-fold for potassium and 1500-fold for sodium determinations.

Duplicate aliquots of 0.1 ml neat seminal plasma were used for chloride determinations.

(3) Spermatozoa. The amounts of the various ions in the spermatozoa were usually calculated from a knowledge of the amounts of ions in seminal plasma and whole semen, and knowing the amount of trapped plasma in the pellets of spermatozoa. However to check this another method of preparing spermatozoa for analysis was used occasionally. This made a larger amount of spermatozoa available for analysis. The two methods gave essentially the same results. The alternative method involved pipetting 0.5 ml packed spermatozoa (preparation of STAGE 2 described page 72) with a 1 ml grade 'A' pipette drawn off at the 1 ml delivery mark. The spermatozoan mass was allowed to run out very slowly into a 10 ml glass-stoppered pyrex tube. 9.50 ml 0.1 N HCl was added to the aliquots of packed cells and after thorough mixing was left to stand overnight. It was then centrifuged and the supernatant removed into another pyrex tube. Aliquots of the supernatant were directly diluted 20-fold for calcium, 100-fold for magnesium, 500-fold for potassium and 2000-fold for sodium determinations.

(f) The preparation of Blood Plasma and Erythrocytes for Analysis of Calcium, Magnesium, Sodium, Potassium and Chloride

Twelve males were randomly selected, from the birds used

for the semen analyses in the 1964/65 season. 5.0 ml plastic syringes were used for blood collection. The wall of the syringe was wetted with diluted Heparin (Pularin, Evans Medical Ltd.) and about 3.0 ml blood was collected from the wing vein of each male. It was transferred immediately to a straight-sided pyrex tube kept in cold water and centrifuged in an MSE centrifuge (Model 551) for 30 minutes (3200 g, 5°C). The average figure for trapped plasma between cells under these conditions was established as 6% of the apparent volume of cells by Bell (1957) and this figure was adopted for calculations in the present experiment.

The blood plasma was transferred to another pyrex tube immediately after centrifugation and the 'buffy' coat of white blood cells was sucked carefully away using a Pasteur pipette connected to a vacuum pump.

To determine the ionic composition (except chloride) of the erythrocytes a 1.0 ml grade A pipette with its tip sawn off was used to measure out a 0.5 ml aliquot of the packed cells. They were allowed to run out slowly into a 10 ml, glass-stoppered pyrex tube. 9.50 ml of 6.0 N HCl (Micro-analytical, B.D.H.) was added, the whole mixed and left standing at room temperature for 4-5 hours. It was then centrifuged and the supernatant transferred

to a clean pyrex tube. Corrections in volume were made gravimetrically for the material which stuck to the inner wall of the pipette under these conditions. The extract was diluted 10-fold for calcium, 200-fold for magnesium and 1000-fold for potassium and sodium determinations. Separate aliquots of cells were treated with zinc sulphate-sodium hyroxide solution to precipitate proteins prior to the estimation of chloride (Page 58).

To determine the ionic content of blood plasma (except chloride) 0.50 ml aliquots were pipetted into a 10 ml stoppered pyrex tube and 9.50 ml 6 N HCl (Micro-analytical, B.D.H.) was added. The following dilutions were made for estimation by atomic absorption spectroscopy: calcium (x 20), magnesium (x 20), potassium (x 100) and sodium (x 1500). Separate duplicate 0.1 ml aliquots of blood plasma were taken for chloride estimations.

A Heparin blank was appropriately tested for the presence of the various ions and found to contain negligible amounts.

(g) <u>The Preparation of Blood and Seminal Plasmas for the Estimation of their Carbon Dioxide Content</u>

Blood and semen were collected from individual males into heparinised syringes, sealed with liquid paraffin, and wide-mouthed tubes, containing liquid paraffin, respectively.

Within a few minutes both types of samples were centrifuged (3000g) at 5°C for 15 mins and the respective plasmas extracted and kept under liquid paraffin. The respective samples were analysed within 30 mins of collection from the bird.

(h) The Collection of Fluid from the Uterus of the Hen

The fluid in the oviduct which is likely to be of importance to spermatozoa, particularly as they are stored in the utero-vaginal junction, is that produced in the uterus and surrounding regions.

(1) Collection of 'Plumping' Fluid. 'Plumping' fluid is added to the egg during about the first 5 hours that it is present in the uterus and when it consists of yolk and albumen covered with a soft membraneous covering. (Burmester, 1940).

Laying Brown Leghorn hens were housed in single cages and the times of ovipositions were recorded to the nearest hour. Hens were selected at the appropriate time and if the presence of a membraneous egg in the uterus was confirmed, by palpation per cloaca, they were anaesthetized by giving 0.6 ml Nembutal (Pentobarbitone Sodium, B.P. Abbott Laboratories) intravenously. The hen was then laid on its back, feathers were removed from the left side of the abdomen and a neat, small incision was made

which allowed the introduction of one or two fingers of the operator's left hand. The cloaca was thoroughly cleaned and pressure was applied to the anterior end of the egg in the uterus (shell-gland). A plastic funnel was placed in the mouth of a graduated, pyrex centrifuge tube held in the right hand. Continuous pressure was applied to the uterus until the egg began to appear through the cloaca. A second person removed the egg as soon as it was forced from the vagina and the 'plumping' fluid was ejected into the graduated, pyrex tube applied to the everted vagina. By gently massaging the uterus all the fluid was extracted without contamination from cloacal material. Liquid paraffin was placed in the tube to prevent the loss of carbon dioxide from the fluid. Volumes of fluid ranging from 0.50 ml to 1.70 ml were obtained by this method.

After the operation, which lasted about five minutes, the everted vagina was gently pushed into its normal position and the abdominal incision closed by suture. Frequent observations were made subsequently on the general health and laying record of the operated birds and all continued to lay normally.

(2) Collection of 'Oviposition' Fluid. 'Oviposition' fluid is defined as that fluid which is obtained from the

uterus when a normal hard-shelled egg is present and the hen expected to lay within 2 hrs.

Hens were selected at the appropriate time and the presence of the egg confirmed by palpation per cloaca. Laparotomy, the method of expulsion of the egg and the collection of fluid were the same as described above for 'plumping' fluid.

(i) Preparation of Uterine Fluids for all Analyses

'Plumping' fluid was collected separately from 15 hens for the estimation of the ion contents, and from 8 hens for the estimation of the content of carbon dioxide. 'Oviposition' fluid was obtained from 15 hens for all the analyses. The fluids used for the determination of carbon dioxide content were collected under liquid paraffin and aliquots were taken for analysis within 10 mins.

Duplicate aliquots of 0.1 ml and 0.05 ml fluid were used directly for estimations of chloride and carbon dioxide respectively. The remaining fluids in the stoppered tubes were accurately recorded and a known volume of 0.10 N HCl added. This solution was thoroughly mixed and left standing overnight. Afterwards the following dilutions were made directly for analysis: Calcium (x 100), Magnesium (x 20), potassium (x 200) and sodium (x 1500).

RESULTS

B. RESULTS

General Data.

The average volume of uncontaminated semen obtained from a Brown Leghorn cock was 0.15 ml and the density of spermatozoa was 5.72×10^6 per c.mm. The packed spermatozoan volume (spermatocrit) under the centrifugation conditions of the present work (1500 g, 30 min., 5°C) formed 20.7% of the total semen volume. The mean amount of plasma trapped in the spermatozoan pellet was 72.2% of this volume (S.E. of the mean $\pm$ 1.46; 21 observations), giving an absolute volume of 5.6% spermatozoa in semen samples.

It is not unreasonable to find such a large volume of trapped plasma between spermatozoa in semen centrifuged under the mild conditions of the present work, considering the filamentous shape of the motile spermatozoa. They are unlikely to be compressed as tightly as oval-shaped erythrocytes. It is not known how large the volume of trapped blood plasma between erythrocytes would be under the mild conditions of centrifugation but at over double the speed, i.e. 3200 g (5°C, 30 mins), the volume was found to be 6% of the cell pellet (Bell, 1957). A check was carried out on the magnitude of the estimate of trapped seminal plasma obtained in the present study when Dr. S. Iverson, Beatty Memorial Cancer Hospital, Glasgow, calculated the volume of a fowl spermatozoon as 9.2 μ^3 with

a model of the Coulter electronic counter which also estimated size. From this information and the abovementioned figure for density of spermatozoa in semen samples, it was shown that the trapped plasma could be expected to occupy 73% of the packed spermatozoan volume which was in excellent agreement with the mean figure of 72.2% estimated routinely by the nitrogen method.

The water content of uncontaminated seminal plasma and spermatozoa was 96.4% and 61.1% respectively. The content of water in seminal plasma was higher than that found in cock blood plasma (95%; Gilbert, private communication) which is consistent with the lower protein content of seminal plasma (0.8%) compared with blood plasma (5%). The amount of water in spermatozoa was lower than that in cock erythrocytes (70%; Gilbert, private communication). The specific gravity of the spermatozoon of the Brown Leghorn cock (1.29) exceeded that of erythrocytes (1.09; Gilbert, private communication) and in this respect it resembled the spermatozoon of the mammal (Mann, 1964). There is extremely little cytoplasm in the spermatozoon and the high specific gravity is due mainly to the highly condensed nuclear material, the mitochondria of the midpiece and the tail.

The Content of the Principal Inorganic Ions and Carbon Dioxide in Uncontaminated Seminal plasma and Blood Plasma.

The composition of uncontaminated seminal plasma with regard to the content of the principal inorganic ions was very consistent. The variation in the content of the inorganic ions under study was small (Table I of the Appendix) throughout a period of two years. This is probably not surprising since unlike mammals, there are no accessory reproductive organs to contribute variable amounts of fluid to semen, and 'transparent' fluid from the cloacal glands of birds was not included in the seminal plasma. In addition, the cocks were massaged regularly for semen collection throughout the entire period of study.

Only a limited number of analyses for the carbon dioxide content of seminal plasma were performed and the results are given in Table 2 of the Appendix.

The full data of the analyses of blood plasma from Brown Leghorn cocks are given in Tables 2 and 3 of the Appendix.

A summary of the amounts of sodium, potassium, calcium, magnesium, chloride and carbon dioxide present in seminal and blood plasmas are given in Table 3. In common with most extracellular fluids, sodium was the chief cation in seminal plasma (159.32 mEq./L) and was

TABLE 3.

A SUMMARY OF THE AMOUNTS (MEAN ± S.E. OF THE MEAN) OF THE PRINCIPAL INORGANIC IONS AND CARBON DIOXIDE IN SEMINAL AND BLOOD PLASMAS OF THE BROWN LEGHORN COCK

ELECTROLYTE	SEMINAL PLASMA	BLOOD PLASMA
	mEq./L	mEq./L
SODIUM	159.32 ± 1.33 (34)	162.65 ± 1.65 (15)
POTASSIUM	13.32 ± 0.23 (32)	6.01 ± 0.16*** (15)
CALCIUM	2.53 ± 0.07 (29)	6.40 ± 0.12*** (15)
MAGNESIUM	5.37 ± 0.14 (35)	2.03 ± 0.04*** (15)
CHLORIDE	40.22 ± 1.12 (29)	116.13 ± 0.54*** (21)
CARBON DIOXIDE	21.86 ± 1.76 (4)	27.95 ± 0.72*** (6)

*** $P < 0.001$

Numbers in parentheses refer to the number of pooled samples of seminal plasma, and individual samples of blood plasma, analysed.

not significantly different in amount from that in blood plasma (162.65 mEq./L). The amounts of potassium (13.32 mEq./L) and magnesium (5.37 mEq./L) were high in seminal plasma and significantly greater than in blood plasma (potassium, 6.0 mEq./L; magnesium, 2.0 mEq./L). By contrast, the amount of calcium in seminal plasma was small (2.53 compared to 6.4 mEq./L in blood). The amount of chloride in seminal plasma (40.2 mEq./L) was low and about one-third of that in blood plasma (116.1 mEq./L). The carbon dioxide content of seminal plasma (21.86 mEq./L) was less than that in blood plasma (27.95 mEq./L).

The Content of the Principle Inorganic Ions in Whole Semen, Uncontaminated Seminal Plasma and Spermatozoa in Comparison with Blood.

Full data for the analysis of whole semen are given in Table 4 of the Appendix.

A summary of the contents of the principal inorganic ions in whole semen, uncontaminated seminal plasma and spermatozoa are given in Table 4. The contents of ions in the spermatozoa were calculated from the amounts in whole semen and seminal plasma knowing the volume of trapped seminal plasma in packed spermatozoa and the spermatocrit of samples of semen. The amount of sodium

TABLE 4

A SUMMARY OF THE AMOUNTS (MEAN ± S.E. OF THE MEAN) OF VARIOUS INORGANIC IONS IN UNCONTAMINATED SEMINAL PLASMA, WHOLE SEMEN AND SPERMATOZOA OF THE BROWN LEGHORN COCK.

ELECTROLYTE	SEMINAL PLASMA	WHOLE SEMEN	SPERMATOZOA ‡
	mEq./L	mEq./L	mEq./L cells
SODIUM	158.76 ± 2.05 (21)	152.99 ± 1.67** (21)	53.58
POTASSIUM	12.93 ± 0.26 (21)	15.60 ± 0.28*** (22)	61.38
CALCIUM	2.55 ± 0.08 (17)	2.46 ± 0.12 (18)	0.72
MAGNESIUM	5.11 ± 0.15 (21)	5.80 ± 0.10*** (23)	17.09
CHLORIDE	41.89 ± 1.31 (16)	41.64 ± 1.46 (16)	37.22

In comparison between whole semen and seminal plasma:

** = $p < 0.01$; *** = $p < 0.001$

‡ The amount of ions in spermatozoa was calculated from the amounts in seminal plasma and whole semen knowing the volume of trapped plasma (72.2%) in packed spermatozoa and the spermatocrit.

Numbers in parentheses refer to the number of the pooled samples of seminal plasma and whole semen analysed.

in the spermatozoa (53.58 mEq./L) was much lower, and the amount of potassium (61.38 mEq./L) higher, than that in the seminal plasma (158.76 and 12.93 mEq./L respectively) which is consistent with the situation that exists in the ionic relationships between somatic cells and extracellular fluid. There was a high magnesium content in the spermatozoa (17.09 mEq./L) which is probably associated with the accumulation of mitochondria in the midpiece. There was a negligible amount of calcium in the spermatozoon (0.72 mEq./L). The chloride concentration in the seminal plasma (41.89 mEq./L) appeared to be the same as that inside the spermatozoon (37.22 mEq./L).

The differences in ionic content between spermatozoa and erythrocytes (Table 5), when the difference in the respective water contents is considered, are that the sodium, and magnesium concentrations in spermatozoa are higher, and the potassium lower, than those in the erythrocytes. The difference in magnesium content may be accounted for by the accumulation of mitochondria in the spermatozoon. The potassium content of erythrocytes was slightly higher than that in spermatozoa which may be due to the greater amount of cytoplasm present in the erythrocyte. It is felt that the mean amounts of sodium, calcium and chloride in spermatozoa (Tables 4 and 5) may

TABLE 5.

A SUMMARY OF THE AMOUNTS OF THE PRINCIPAL INORGANIC IONS IN SPERMATOZOA AND ERYTHROCYTES OF THE BROWN LEGHORN COCK.

ELECTROLYTE	SPERMATOZOA*		ERYTHROCYTES*	
	mEq./L cells	mEq./L cell water	mEq./L cells	mEq./L cell water
SODIUM	53.58	87.66	9.58	13.68
POTASSIUM	61.38	100.42	105.87	151.24
CALCIUM	0.72	1.18	0.52	0.74
MAGNESIUM	17.09	27.96	8.31	11.87
CHLORIDE	37.22	60.90	52.60	75.14

* The water content of spermatozoa and erythrocytes is 61% and 70% of their total content respectively.

not represent the true values, and thus the difference between erythrocytes and spermatozoa in these respects may only be apparent. In a few of the calculations of the content of sodium, calcium and chloride in spermatozoa derived from the amounts found in the corresponding whole semen and seminal plasma fractions (Tables 1 and 4 of the Appendix), a small negative amount of these ions was obtained for the spermatozoa. These few discrepancies in themselves would indicate that the amounts of sodium, calcium and chloride are small inside the spermatozoon. However, particularly in the case of sodium, the amount in seminal plasma is very high and the proportion of cells to plasma in semen is much lower than that in blood. These factors would contribute to the difficulty of estimating the intracellular content of sodium if it is assumed that it is much lower in amount than that outside the cell as is generally the case with somatic cells. The same argument could be applied in the cases of chloride and calcium although not to the same extent.

The figures for blood plasma in Table 3 and for erythrocytes in Table 5 (the Summary of Table 5 is in the Appendix) were from the same males and the sodium and potassium contents of erythrocytes were almost identical with those

reported by Maizels (1954). The partition of ions between cells and plasma are as expected from general knowledge of cell physiology.

The Content of Inorganic Ions and Carbon Dioxide in Uterine Fluids.

The complete data for the composition of 'plumping' and 'oviposition' fluids are given in Tables 6 and 7 in the Appendix. A summary of the composition of these fluids is given in Table 6.

'Plumping' fluid is an aqueous solution which passes through the membranes of the egg to become part of the albumen during a period of about five hours after the egg arrives in the uterus. Sodium was the chief cation and its concentration (139.1 mEq./L) was lower than that in blood plasma (162.65 mEq./L) or seminal plasma (159.32 mEq./L). The amount of potassium (15.91) was more than double that in blood plasma (6.0 mEq./L) but only slightly higher than that in seminal plasma (13.32 mEq./L). The amount of calcium (28.3 mEq./L) in 'plumping' fluid was very high. The amount of carbon dioxide present in 'plumping' fluid (82.47 mEq./L) was more than three-times its concentration in seminal (21.86 mEq./L) and blood plasmas (27.95 mEq./L) but the amount of chloride (79.9 mEq./L) was lower than in cock blood plasma (116.13 mEq./L).

TABLE 6.

SUMMARY OF THE COMPOSITIONS OF 'PLUMPING' FLUID AND 'OVIPOSITION' FLUID (mM/Litre).

Electrolyte.	'Plumping' Fluid.	'Oviposition' Fluid.
Cations.		
Sodium	139.10	42.80
Potassium	15.91	75.03
Calcium	14.10	25.80
Magnesium	0.14	10.30
	169.25	153.93
Anions.		
Carbon Dioxide	82.47	91.26
Chloride	79.90	63.42.
	162.37	154.68

However, this chloride level was still double the concentration in seminal plasma (40.22 mEq./L).

'Oviposition' fluid was obtained from hens with a hard-shelled egg in the uterus, that was expected to be oviposited within two hours of the collection of the fluid. The content of potassium (75.0 mEq./L) was even higher than in 'plumping' fluid (15.91 mEq./L) and the magnesium (20.35 mEq./L) and calcium (51.61 mEq./L) concentrations were also raised. On the other hand, the amount of sodium (42.84 mEq./L) was much reduced so that the total concentration of cations was not much different in 'plumping' and 'oviposition' fluids (Table 6).

The amount of chloride in 'plumping' fluid was almost equal to that of carbon dioxide but the latter was the dominant anion in 'oviposition' fluid. Judging from Table 6, the analyses performed on the uterine fluids appeared to account for almost all the constituents present. It is unlikely that phosphate and sulphate contribute much to the fluid since Beadle *et al.* (1938) found little or none in uterus fluid.

DISCUSSION

C. DISCUSSION

Progress in work designed to store fowl spermatozoa *in vitro* or to investigate infertility depends partly upon obtaining information about the chemical composition of spermatozoa in body fluids favourable to supporting their life, as well as information about the body fluids themselves. It was the intention of the present study to investigate the principal inorganic ions and electrolyte balance in fowl spermatozoa in relation to conditions existing in the environments in the vas deferens and oviduct under special circumstances. It was also desired to find out some of the ways in which spermatozoa differed from a somatic cell, if any, and for this purpose a definitive cell, the erythrocyte, was chosen for comparative study.

It is well-established that the ionic composition of a physiological medium is important for enzymes to function properly in it. It is known that sodium stimulates enzymes that catalyse the breakdown of adenosine triphosphate (ATP) whilst potassium helps to promote reactions necessary for the maintenance of ATP. Magnesium is an activating ion in many reactions of carbohydrate metabolism and calcium activates lecithinases. Certain enzymes, for instance those responsible for glycolysis,

are present mainly in solution in cytoplasm but others are confined almost exclusively to the insoluble fraction of cells. Mitochondria contain most of the enzymes responsible for oxidative metabolism. Thus cytochrome oxidase, succinoxidase, some enzymes involved in the tricarboxylic-acid-cycle and the enzyme system responsible for the oxidation of fatty acids to acetoacetate are reported to be present in the mitochondria. The function of mitochondria is very sensitive to the osmotic pressure of the suspension medium and the mitochondria behave towards osmotic changes as if they were surrounded by a semi-permeable membrane, a fact which has been substantiated by electron microscopy. The midpiece of a spermatozoon contains an accumulation of the mitochondria of the original spermatid, from which it metamorphosed, and is very vulnerable to storage <u>in vitro</u> (Lake, 1954). A thorough knowledge of the electrolyte balance of the fluid in the vas deferens, which is known to support the spermatozoa for a much longer time than they can be stored successfully <u>in vitro</u>, might be expected, amongst other things, to yield useful information on the basic ionic requirements of the spermatozoa for maintenance of their continued function. Similar studies of the uterine fluid and its changing ionic composition might be

expected to provide information on the factors involved in regulating the prolonged survival of spermatozoa in the oviduct and their periodic movement up to the site of fertilization.

Unlike the majority of somatic cells there is very little cytoplasm surrounding the spermatozoon. The mitochondria can be considered to be more exposed to the external environment in which they function. The exchange of both sodium and potassium across the membrane of a mitochondrion is believed to be rapid; a minor fraction of the potassium may be confined in an unexchangeable form within the cell. The monovalent cations, together with calcium and magnesium, are essential in the preservation of the integrity of cell membranes as well as being important in various enzyme functions. Potassium is found generally as the principal intracellular cation whereas sodium is the chief extracellular cation in tissues of animals. The nature of forces maintaining the intracellular potassium at a high and relatively constant level has been the subject of much speculation and experimentation. Ussing (1960) has reviewed the hypotheses advanced to explain this phenomenon and active transport is considered to play an important role. The continuation of oxidative metabolism by the cell is essential for active transport

mechanisms to maintain the constancy of the internal environment (Fruton and Simmonds, 1953). This may be an important consideration in the practise of storing spermatozoa at low temperatures; there may be an optimum temperature for storage which just allows the continuation of active transport but at the same time reduces metabolism to a minimal level. Therefore, a low temperature may not be the most important factor *per se* for storing spermatozoa *in vitro* above zero. All the abovementioned points concerning cell function and inorganic ions could be applicable in explaining spermatozoan function. However, at present there is insufficient information available about the balance of inorganic ions in semen and the present study was designed to remedy this situation.

Several interesting features on ionic composition of spermatozoa in relation to their environment in the male and female reproductive tracts arise from the present work. Semen, uncontaminated with cloacal gland fluid ('transparent' fluid) was collected and studied. The seminal plasma of such semen, which was essentially vas deferens fluid, was considered to be a natural *milieu* for the spermatozoa. The composition of uncontaminated fowl seminal plasma agreed essentially with the preliminary findings of Lake *et al.* (1958) and Lake and El Jack (1964a).

The amount of calcium in seminal plasma was much lower than that reported by Takeda (1959) and Hammond et al. (1965) which could be due to breed differences, methodology or both. However, it is more likely that the latter workers used seminal plasma grossly contaminated with 'transparent' fluid in which case the amount of calcium would be similar to that found in blood plasma. There may be a correlation between the small amounts of calcium found normally in uncontaminated seminal plasma and the findings of Wales and White (1958b) that calcium in excess of 0.3 mM was harmful to the survival of fowl spermatozoa in vitro. Both Lake (1956) and Nishiyama (1955) showed that fowl semen containing 'transparent' fluid was difficult to maintain in vitro; in these cases the calcium concentration of the semen would have been high and similar to that of blood plasma and may have been one of the factors which led to the incapacitation of the spermatozoa.

Sodium was found to be the major cation in uncontaminated seminal plasma and the amount agreed closely with previous findings (Lake et al., 1958; Takeda, 1959; Lake and El Jack, 1964a; Hammond et al., 1965). The amount of sodium in seminal plasma was not different from that in blood plasma but the amount of potassium was higher.

Takeda (1959), however, reported a rather lower figure (8.6 mEq./L) for potassium in seminal plasma indicating that the semen was diluted grossly with 'transparent' fluid during collection.

It is interesting to consider the origins of the potassium in uncontaminated seminal plasma. The epithelium lining of the male tract displayed much holocrine and apocrine secretory activity (Lake, 1957b) which could indicate that intracellular material, including potassium ions, is secreted into the lumen of the vas deferens and contributes to the composition of the seminal plasma. Under certain circumstances the number of disintegrating spermatozoa increases in the vas deferens and it is feasible that some potassium could escape into the extracellular fluid from this source. In this context the following pertinent observations have recently been made. Work was begun (Lake, unpublished observations) to investigate changes occurring in spermatozoa during storage <u>in vitro</u>. Clean semen was collected and divided into three equal parts. One part was spun immediately and an analysis carried out on seminal plasma. The second part was stored at room temperature (25°C) for 16 hours and the third stored for the samestime at 2°C. The semen samples were shaken gently

during storage. After 16 hours, the semen samples were centrifuged and the ionic composition of the seminal plasmas determined. The data have so far shown that seminal plasma from the stored semen contained more potassium. In one experiment the potassium concentration was 71.0 mg% compared to that in fresh seminal plasma which was 54.0 mg%. A decrease in chloride concentration (111.4 mg% compared to 128.0 mg% in freshly collected seminal plasma) was also found. The significance of these findings are currently being investigated. The rise in potassium concentration in stored seminal plasma could indicate that there was a progressive leakage of potassium from the spermatozoa during storage. It may be significant in this respect that Wales and White (1958b) found that the addition of potassium ions to diluents for washed fowl spermatozoa restored their motility substantially.

The low amount of chloride found in uncontaminated seminal plasma agreed essentially with previous findings of Lake et al. (1958) and Lake and El Jack (1964a). There was complete disagreement between the present results for chloride and the high amount reported by Hammond et al. (1965). The latter workers obtained very high volumes of semen from cocks (2.07 ml) and it is most

likely that there was gross contamination with 'transparent' fluid. The chloride concentration they found in seminal plasma equalled that of blood plasma. The small amount of chloride anion found in uncontaminated seminal plasma is interesting from the point of view of the ionic balance in this medium, in which spermatozoa mature and survive for at least two weeks. It was found that glutamate assumed the role of principal anion in place of chloride (Lake and El Jack, 1964b). The fact that fowl spermatozoa mature in an environment in which the level of chloride ions is low may be a partial explanation for the detrimental effect of diluents containing approximately blood levels of chloride (Lake, 1960; El Zayat and van Tienhoven, 1961a) on the survival of fowl spermatozoa *in vitro*.

It is certain that the spermatozoon, in spite of having little cytoplasm, contained a relatively large amount of potassium in common with what is known about the general composition of tissue cells and erythrocytes. Magnesium was present in greater amounts in spermatozoa than in seminal plasma and is most likely associated with the mitochondria in the midpiece of the spermatozoon. Further more elaborate techniques will be required to substantiate the true contents of sodium, calcium and chloride within the spermatozoan cell.

The amounts of sodium, calcium and chloride calculated as being present in spermatozoa are regarded as inconclusive. There was no significant difference in the levels of calcium or chloride between seminal plasma and whole semen. The content of sodium in seminal plasma was significantly higher than in whole semen ($P < 0.01$). On the present evidence, therefore, it is considered that the amounts of sodium, calcium and chloride in spermatozoa are small compared to the extracellular seminal plasma but that the true amounts have yet to be conclusively demonstrated. No previous data on the ionic composition of fowl spermatozoa are available to compare with the present findings. Difficulties are encountered in techniques of estimating intraspermatozoan contents especially of sodium and chloride. One reason for this difficulty is the relatively large amount of these ions in the extracellular medium. It is inadvisable to use washed cells for investigation since it would lead to a leakage of unknown quantities of ions. In view of the present findings it is questionable whether the observations of Keitel and Jones (1956) can represent the true picture of the ionic balance in human spermatozoa; it was found that the intra- and extra-cellular contents of sodium and chloride were the same. The

amount of chloride was more than double and the potassium much lower, than was found in fowl spermatozoa. The same technical difficulties to those encountered in the present work would have been experienced in the work of Keitel and Jones. In addition they described the presence of prostatic calculi of unknown composition in the packed cells used for analysis. Cragle et al. (1958) showed a larger amount of potassium in bull spermatozoa than in seminal plasma in spite of taking no account of the trapped plasma in the packed cells used for analysis. On the other hand, Carbo (1964) found a smaller amount of potassium in bull spermatozoa compared to seminal plasma. It is likely that the technical difficulties alluded to above accounted for these discrepancies.

The findings on the composition of blood plasma compared favourably with previous work regarding the ionic composition of blood plasma (Morgan and Chichester, 1935; Maizels, 1954; Takeda, 1959; Lake and El Jack, 1964a). Also the amounts of sodium and potassium found in erythrocytes agreed with those of Maizels (1954). However, Morgan and Chichester (1935) reported more than twice the amount of sodium in fowl erythrocytes but it is most likely to be due to the failure to account for trapped plasma in their analysis of packed cells. Like the

spermatozoa the magnesium concentration was high in erythrocytes compared to blood plasma but the amount of calcium was very small.

Several interesting features were revealed in the examination of the composition of uterine fluids which could serve as a basis for future work on the effects of oviduct fluids on spermatozoan activity and infertility problems. 'Plumping' fluid contributes substantially to the total volume of egg white (albumen) and consists essentially of an aqueous solution of inorganic salts containing a small amount of protein (0.06gm. per 100 ml). (Beadle, et al., 1938). The fluid is produced in the uterus and, in the vicinity, spermatozoa reside in epithelial glands of the uterus-vagina junction during the extended fertility period typical of a hen (Verma and Cherms 1964, 1965; Bobr et al., 1964b). Spermatozoa almost certainly come into contact with fluids of the uterus during their passage up the oviduct and yet nothing is known of possible effects of the changing composition of the fluid on the activity of spermatozoa during the passage of a succession of eggs down the oviduct.

Beadle et al. (1938) reported slight differences in the composition of 'plumping' fluid to those found in the present experiments. However, they experienced trouble

in catheterising the uterus for the collection of the fluid and used less reliable methods of estimating the chemical composition. The sodium concentration in 'plumping' fluid was lower than that in blood and seminal plasmas, and the potassium concentration was high. The amounts of magnesium and calcium found in the 'plumping' fluid in the present study were much higher than those reported previously by Beadle et al. (1938) which is most likely due to the fact that the atomic absorption spectrophotometer was used in the present work and is more sensitive than chemical precipitation methods. However, there was general agreement in that the level of calcium was elevated considerably over that of blood plasma. A most interesting fact about the composition of 'plumping' fluid was the appreciable amount of carbon dioxide and calcium present and it is undoubtedly associated with the shell deposition mechanism. A large amount of calcium (2g) is required to form an egg shell in a relatively short time (Romanoff and Romanoff, 1949).

The composition of the fluid found in the uterus (shell gland) within 2 hours of an expected oviposition ('oviposition' fluid) was changed from the composition of the 'plumping' fluid which is added to the egg during the earlier parts of its stay in the uterus.

The sodium concentration was markedly reduced and it is very difficult to speculate on the reasons for this dramatic fall. Sodium does not appear to enter the egg because the amount inside the egg remains fairly constant through to oviposition (Draper, 1966). The amount of potassium in 'oviposition' fluid was extremely high and may be correlated with recent observations (Draper, 1966). showing that the concentration of potassium in the egg white (albumen) remains low until an appreciable degree of calcification of the egg has occurred, when it begins to rise.

The magnesium concentration of the 'oviposition' fluid was found to be high and more than seven times that in 'plumping' fluid. This may be associated with the fact that the egg shell of the domestic hen accumulates about 21.0 mg of magnesium (Romanoff and Romanoff, 1949; Draper, 1966) in its structure.

The amounts of chloride and carbon dioxide in 'oviposition' fluid were approximately similar to those in the 'plumping' fluid. Carbon dioxide and potassium concentrations in both the uterine fluids were remarkably higher than those in blood and seminal plasmas. If spermatozoa are stored in tubular glands in the utero-vaginal junction (Bobr et al., 1964a,b) then periodically

they must travel up to the infundibulum where fertilisation occurs. The region where the spermatozoa are stored secretes fluids of a high pH which fluctuate with the position of the egg in the oviduct (Ogasawara et al., 1964; Winget et al., 1965) and it has to be asked which of the constituents is responsible and whether any are likely to periodically activate spermatozoa. Vishwakarma (1962) found that the total carbon dioxide and bicarbonate contents of the uterine and tubal fluids of rabbits was very high and was associated with a high pH. Hamner and Williams (1964) showed that fluid from the fallopian tube of rabbits markedly stimulated the respiration of rabbit spermatozoa. Hamner and Williams (1964) and Williams et al. (1964) also found that the presence of pure sodium bicarbonate (1.2 to 4.8 x 10^{-3}M) increased sperm respiration; the stimulatory effects of tubal fluid could be duplicated with bicarbonate - CO_2 mixtures and the authors suggested that bicarbonate might be the sole stimulatory agent in such a fluid. It was also suggested that bicarbonate in human seminal plasma was the effective agent in stimulating motility of spermatozoa in vitro.

In the light of the information obtained on the ionic composition of semen and uterine fluid it is interesting,

and may have some bearing on the problem of storage and natural activation of fowl spermatozoa in the oviduct, that under certain circumstances the inclusion of carbon dioxide in diluents for fowl semen led to considerable improvement in prolonging the viability of the spermatozoa *in vitro* (Hobbs and Harris, 1963b). Also, the motility of spermatozoa can be inhibited by carbon dioxide (Schindler and Nevo, 1962) and potassium, in certain high concentrations, can stimulate motility of spermatozoa (Wales and White, 1958b). Observations such as these would indicate that the uterus fluids of the fowl contain constituents which are capable of influencing activity in spermatozoa and it remains to be investigated whether the changing composition of uterine fluids play any role in the activation or preservation of spermatozoa in the oviduct of the hen.

Apart from direct effects of ions on spermatozoan activity, another possible mechanism for controlling the periodic transport of spermatozoa up to the site of fertilization might be considered for future investigation in the light of the results of the analysis of uterine fluids. It is well known that potassium and calcium ions play a part in regulating smooth muscle contractility (Burnstock, Holman and Prosser, 1963) and thus the great

increase in potassium concentration in uterine fluid around the time of oviposition might be investigated for possible stimulatory effects on the muscular activity of the vagina and uterus and the expulsion of spermatozoa from their residence sites to begin their upward movements in the oviduct.

In conclusion a comment should be made on the tendency to use motility as a criterion of the assessment of the suitability of diluents for the storage of fowl semen. Enhanced motility of spermatozoa in the presence of specific chemical ions or compounds may indicate under certain circumstances that the diluent is unsuitable for the prolonged storage of semen owing to the rapid exhaustion of metabolic substrates for the spermatozoa. However, media supporting active motility are sometimes recommended as possible diluents. Media containing ions and compounds that reduce motility may be more suitable as diluents provided it is recognised that some degree of active transport of materials into and out of the spermatozoa may have to be maintained to preserve the integrity of the membranes.

SUMMARY AND CONCLUSIONS

A study has been made of the composition of the principal inorganic ions in fowl spermatozoa, seminal plasma, blood plasma, erythrocytes and in uterine (shell-gland) secretions. The latter play an important role in the formation of the egg and could also influence the activity of spermatozoa residing in the uterovaginal junction and during the initial stages of their travel up to the infundibulum for fertilization.

Information has been obtained on the electrolyte compodition of fowl semen and uterine secretions which will serve as useful information for the furtherance of work on the problems of infertility in poultry and the compilation of diluent media for the storage of fowl spermatozoa *in vitro* for prolonged periods.

1. A method was developed for the determination of trapped seminal plasma in packed spermatozoa after centrifugation in order to estimate the volume of spermatozoa in semen samples.
2. Atomic absorption spectroscopy was successfully adapted for the estimation of sodium, potassium, calcium and magnesium in semen, blood and uterine secretions.
3. Chloride in semen, blood and uterine secretions was determined by electrometric titration with silver ions. This method was found to be rapid, accurate and especially

useful for analysis of fowl semen as only 0.1 ml of the fluid was required for analysis.

4. Carbon dioxide in the abovementioned fluids was determined by gas chromatography which proved to be a reliable, rapid method, with small volumes of fluid, compared to the chemical methods.

5. Sodium was the principal extracellular cation in seminal plasma and the concentration (159.32 mEq./Litre) was similar to that in blood plasma (162.65 mEq./L). The amount of potassium (13.32 mEq./L) and magnesium (5.37 mEq./L) in seminal plasma was higher than that in blood plasma (6.01 and 2.03 mEq./L respectively). Calcium was found in low concentration (2.53 mEq./L) in seminal plasma.

6. Fowl erythrocytes resembled other somatic cells in containing higher amounts of potassium (105.87 mEq./L) and lower amounts of sodium (9.58 mEq./L) than in blood plasma.

7. Fowl spermatozoa were calculated to contain higher amounts of potassium (61.38 mEq./L) and magnesium (17.09 mEq./L) than seminal plasma. Thus the spermatozoon cell was able to maintain a high intracellular potassium concentration as in somatic cells. The high magnesium content was considered to be associated with the

accumulation of mitochondria in the midpiece of the spermatozoon. The estimation of sodium, calcium and chloride amounts within spermatozoa revealed certain technical difficulties for their accurate analysis and the results for these ions were considered tentative. However, their concentrations within the spermatozoon appeared to be low which is consistent with existing knowledge on somatic cells.

8. Chloride accounted for the bulk of anionic material in blood plasma but was very low in amount in the seminal plasma. This finding has been discussed in relation to ionic balance in this fluid.

9. A method was devised for obtaining appreciable volumes of uterine fluid. It was collected during two separate phases of the development of the egg in the uterus. Fluid from the first phase is called 'plumping' fluid and that from the second phase 'oviposition' fluid. Sodium (139.10 mEq./L) was the principal cation in 'plumping' fluid and potassium (75.03 mEq./L) the principal one in the 'oviposition' fluid. There was evidence of a change in the composition of uterine fluid during egg formation and the possibility of this influence on the activity of spermatozoa was discussed.

10. Both 'plumping' and 'oviposition' fluids contained

appreciable amounts of carbon dioxide and potassium and the possible significance of this finding on the activity of fowl spermatozoa, which reside in the vicinity of the uterus for long periods, has been discussed.

ACKNOWLEDGMENTS

It is with great pleasure that I record my gratitude to the following:-

The Research Committee, University of Khartoum, Sudan, for their generous scholarship; The Agricultural Research Council of Great Britain, for providing the facilities; Professor S.J. Watson of the Department of Agriculture, University of Edinburgh and Dr. M.H. Draper of the A.R.C. Poultry Research Centre for their cordial interest and supervision; Dr. P.E. Lake of the A.R.C. Poultry Research Centre for his unfailing interest and guidance throughout the course of the investigation.

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APPENDIX

TABLE 1.

THE AMOUNTS OF VARIOUS INORGANIC IONS IN UNCONTAMINATED SEMINAL PLASMA OF THE BROWN LEGHORN COCK. SAMPLES WERE TAKEN DURING THE PERIOD JAN.1964 - MAY 1965. EACH SAMPLE IS A POOL FROM 27 OR 29 COCKS OF THE SAME AGE.

SAMPLE	SODIUM		POTASSIUM		CALCIUM		MAGNESIUM		CHLORIDE	
	mg/100ml	mEq./L	mg/100ml	mEq./l	mg/100ml	mEq./L	mg/100ml	mEq./l	mg/100ml	mEq./L
1.	330.0	143.48	48.0	12.31	4.5	2.25	7.8	6.50	141.0	39.72
2.	334.5	145.43	48.5	12.44	3.5	1.75	5.9	4.92	-	-
3.	363.0	157.83	52.0	13.46	4.5	2.25	6.2	5.21	-	-
4.	-	-	-	-	-	-	-	-	146.0	41.13
5.	-	-	-	-	-	-	-	-	132.0	37.18
6.	-	-	-	-	-	-	-	-	143.0	40.28
7.	372.0	161.74	46.0	11.79	5.2	2.60	6.2	5.21	-	-
8.	390.0	169.56	50.0	12.82	5.0	2.50	6.7	5.62	143.6	40.45
9.	390.0	169.56	45.5	11.67	6.1	3.05	5.6	4.67	141.5	39.86
10.	-	-	-	-	4.1	2.05	6.3	5.25	-	-
11.	-	-	-	-	4.0	2.00	-	-	-	-
12.	384.0	166.95	50.0	12.82	5.3	2.65	6.3	5.25	-	-
13.	360.5	156.74	54.1	13.87	5.4	2.70	6.6	5.50	-	-
14.	364.0	158.26	53.6	13.74	5.0	2.50	6.3	5.25	173.2	48.79
15.	350.0	152.17	56.6	14.53	4.7	2.35	6.1	5.08	158.7	44.70
16.	367.5	159.78	52.0	13.33	5.9	2.95	6.4	5.37	111.7	31.46
17.	390.0	169.59	53.0	13.59	5.1	2.55	6.0	5.04	147.7	41.60
18.	-	-	55.0	14.10	4.0	2.00	5.2	4.33	-	-
19.	351.0	152.61	53.5	13.72	4.0	2.00	5.5	4.58	169.3	47.69
20.	371.2	161.39	54.5	13.97	5.9	2.95	4.9	4.12	147.4	41.52
21.	375.0	163.04	53.0	13.59	5.1	2.55	4.7	3.95	164.6	46.37
22.	367.5	159.78	58.0	14.87	6.0	3.00	5.1	4.27	183.9	51.79
23.	330.0	143.48	48.0	12.31	5.5.	2.75	7.1	5.92	133.0	37.46
24.	406.8	176.87	45.0	11.54	-	-	7.0	5.83	141.0	39.72
25.	377.0	163.91	45.0	11.54	-	-	6.1	5.08	126.0	35.49
26.	364.0	158.26	38.0	9.74	-	-	6.5	5.42	137.0	38.59
27.	330.0	143.48	54.0	13.85	-	-	5.4	4.53	160.0	45.07
28.	371.2	161.41	60.0	15.38	-	-	5.6	4.67	160.0	45.07
29.	386.2	167.93	50.0	12.82	-	-	4.1	3.42	192.3	54.17
30.	360.0	156.52	60.0	15.38	-	-	6.5	5.42	150.1	42.28
31.	375.0	163.04	46.0	11.79	5.5	2.75	7.7	6.42	-	-
32.	367.5	159.78	56.0	14.36	4.4	2.20	7.8	6.50	-	-
33.	375.0	163.04	54.0	13.85	7.0	3.50	7.9	6.58	128.0	36.06
34.	371.0	161.30	-	-	5.2	2.60	7.5	6.25	111.0	31.27
35.	371.0	161.30	-	-	4.7	2.35	7.5	6.25	111.8	31.49
36.	375.0	163.04	59.0	15.13	5.4	2.70	7.7	6.42	-	-
37.	351.0	152.61	-	-	-	-	-	-	117.0	32.96
38.	352.5	153.26	53.0	13.59	5.7	2.85	8.5	7.12	109.7	30.90
39.	367.5	159.78	54.4	13.95	4.7	2.35	7.1	5.92	129.9	36.60
40.	367.5	159.78	55.9	14.35	4.7	2.35	7.4	6.20	130.4	36.73
Mean	366.4	159.32	51.9	13.32	5.0	2.52	6.4	5.37	142.8	40.22
S.E.	± 3.1	± 1.33	± 0.88	± 0.23	±0.17	±0.07	±0.17	±0.14	± 3.99	±1.12

TABLE 2.

THE AMOUNTS OF CARBON DIOXIDE IN THE BLOOD AND SEMINAL PLASMAS OF BROWN LEGHORN COCKS.

Sample.	Blood Plasma.		Seminal Plasma.	
	mg/100 ml	mEq./L	mg/100 ml	mEq./L
1.	118.7	26.97	80.3	18.24
2.	137.4	31.22	104.9	23.83
3.	125.6	28.54	113.3	25.74
4.	121.4	27.58	86.3	19.61
5.	119.0	27.04		
6.	116.0	26.36		
Mean	123.0	27.95	96.2	21.86
S.E.	± 3.16	±0.72	± 7.74	± 1.76

TABLE 3.

THE AMOUNTS OF VARIOUS INORGANIC IONS IN THE BLOOD PLASMA OF ADULT BROWN LEGHORN COCKS. EACH SAMPLE IS FROM AN INDIVIDUAL COCK.

COCK.	SODIUM		POTASSIUM		CALCIUM		MAGNESIUM		CHLORIDE	
	mg/100ml	mEq./L	mg/100ml	mEq./L	mg/100ml	mEq./L	mg/100ml	mEq./L	mg/100ml	mEq./L
1.	405.0	176.09	28.5	7.31	13.4	6.70	2.7	2.25	405.0	114.08
1.									416.1	117.21
2.	352.0	153.04	22.5	5.77	14.0	7.00	2.3	1.92	411.0	115.77
2.									398.0	112.11
3.	389.0	156.52	21.0	5.39	14.5	7.25	2.7	2.25	408.8	115.15
3.									399.0	112.39
4.	375.0	163.04	25.2	6.46	13.2	6.60	2.3	1.92	420.0	118.31
4.									415.6	117.07
5.	370.5	161.09	18.5	4.74	13.1	6.55	2.2	1.83	419.6	118.20
5.									416.7	117.38
6.	397.5	172.83	22.5	5.77	13.6	6.80	2.7	2.25	430.7	121.32
6.									437.5	123.24
7.	371.0	161.30	24.0	6.15	12.3	6.15	2.5	2.08	402.1	113.27
8.	370.0	160.87	21.0	5.38	12.3	6.15	2.2	1.83	395.0	111.27
9.	360.0	156.52	21.0	5.38	13.4	6.70	2.4	2.00	408.5	115.07
10.	375.0	163.04	25.8	6.61	12.0	6.00	2.4	2.00	401.0	112.96
10.									408.8	115.15
11.	367.5	159.78	25.0	6.41	11.8	5.90	2.4	2.00	402.0	113.24
11.									415.6	117.07
12.	363.0	157.83	24.0	6.15	11.6	5.80	2.4	2.00	414.2	116.68
12.									432.4	121.80
1.	375.5	163.26	25.5	6.54	12.0	6.00	2.5	2.08	-	-
2.	375.0	163.04	23.5	6.03	11.8	5.90	2.4	2.00	-	-
3.	394.5	171.52	23.5	6.03	11.9	5.95	2.3	1.92	-	-
Mean	374.1	162.65	23.4	6.01	12.7	6.40	2.4	2.02	412.3	116.13
S.E.	± 3.79	± 1.65	±0.20	±0.16	±0.24	±0.12	±0.04	±0.04	± 2.52	± 0.54

TABLE 4.

THE AMOUNTS OF VARIOUS INORGANIC IONS IN UNCONTAMINATED WHOLE SEMEN OF THE BROWN LEGHORN COCK. EACH SAMPLE WAS A POOL FROM 27 or 29 COCKS OF THE SAME AGE. SAMPLES WERE TAKEN DURING THE PERIOD JAN. 1964 - FEB. 1965

SAMPLE	SODIUM		POTASSIUM		CALCIUM		MAGNESIUM		CHLORIDE	
	mg/100ml	mEq./L	mg/100ml	mEq./L	mg/100ml	mEq./L	mg/100ml	mEq./L	mg/100ml	mEq./L
1.	322.5	140.22	57.0	14.61	4.7	2.35	7.2	6.00	141.0	39.72
2.	315.0	136.95	58.0	14.87	3.5	1.75	6.7	5.58	-	-
3.	357.0	155.22	62.0	15.90	4.1	2.05	7.3	6.08	-	-
4.	357.0	155.22	58.0	14.87	3.8	1.90	7.2	6.00	170.0	47.89
5.	371.0	161.30	60.0	15.38	3.8	1.90	7.2	6.00	154.0	43.38
6.	386.4	168.00	58.0	14.87	6.9	3.45	6.6	5.50	168.0	47.32
7.	-	-	-	-	3.8	1.90	7.2	6.00	-	-
8.	-	-	-	-	3.8	1.90	-	-	-	-
9.	375.0	163.04	60.0	15.38	5.4	2.72	7.4	6.17	-	-
10.	357.0	155.22	61.0	15.64	5.3	2.65	7.4	6.21	-	-
11.	350.0	152.17	63.0	16.15	-	-	7.6	6.33	-	-
12.	357.0	155.22	60.0	15.38	4.9	2.46	7.0	5.83	182.3	51.35
13.	348.0	151.30	58.0	14.87	6.0	3.00	6.9	5.79	108.6	30.59
14.	367.5	159.78	67.0	17.18	5.2	2.61	6.8	5.71	144.0	40.56
15.	-	-	60.0	15.38	-	-	7.5	6.25	-	-
16.	351.0	152.61	56.0	14.36	4.4	2.20	6.5	5.42	165.0	46.47
17.	356.2	154.89	63.0	16.15	6.0	3.00	5.9	4.98	135.2	38.08
18.	367.5	159.78	65.0	16.67	5.3	2.67	5.7	4.75	155.9	43.91
19.	356.2	154.89	70.0	17.95	6.0	3.00	5.8	4.85	170.1	47.92
20.	330.0	143.48	62.0	15.90	5.6	2.80	8.1	6.75	129.9	36.61
21.	345.0	150.00	59.2	15.18	-	-	7.5	6.25	136.4	38.41
22.	352.5	153.26	59.2	15.18	-	-	7.0	5.87	113.5	31.97
23.	337.5	146.74	54.0	13.85	-	-	7.1	5.92	139.6	39.32
24.	330.0	143.48	68.0	17.44	-	-	6.1	5.08	151.5	42.69
Mean	351.9	152.99	60.8	15.60	4.9	2.46	6.9	5.80	147.8	41.64
S.E.	± 3.83	± 1.67	±0.84	± 0.28	±0.23	±0.12	±0.13	±0.10	± 5.18	±1.46

TABLE 5.

THE AMOUNTS OF VARIOUS INORGANIC IONS IN ERYTHROCYTES OF ADULT BROWN LEGHORN COCKS. EACH SAMPLE IS FROM AN INDIVIDUAL COCK.

COCK	SODIUM		POTASSIUM		CALCIUM		MAGNESIUM		CHLORIDE	
	mg/100ml	mEq./L	mg/100ml	mEq./L	mg/100ml	mEq./L	mg/100ml	mEq./L	mg/100ml	mEq./L
1.	30.4	13.22	430.5	110.38	0.98	0.49	11.6	9.67	172.2	48.51
2.	34.3	14.92	403.8	103.54	2.36	1.18	11.3	9.42	173.1	48.76
3.	16.9	7.35	428.6	109.90	3.39	1.69	11.3	9.42	178.3	50.22
4.	26.0	11.30	407.4	104.46	0.40	0.20	9.6	8.04	161.7	45.55
5.	21.3	9.26	403.8	103.54	0.76	0.38	9.3	7.72	160.8	45.30
6.	32.1	13.96	407.5	104.49	0.73	0.36	10.0	8.36	155.80	43.89
7.	7.1	3.09	409.3	104.95	0.16	0.08	8.8	7.33	208.1	58.62
8.	12.8	5.56	387.0	99.23	0.24	0.12	8.7	7.25	196.7	55.41
9.	16.9	7.35	381.4	97.79	0.60	0.30	8.6	7.17	194.1	54.68
10.	20.9	9.09	425.9	109.20	0.42	0.21	10.6	8.83	191.2	53.86
11.	18.6	8.09	424.3	108.80	0.30	0.15	10.7	8.92	206.0	58.03
12.	13.3	5.78	448.7	115.05	0.25	0.12	9.4	7.83	242.6	68.34
1.	34.9	15.17	408.4	104.72	0.63	0.31	9.4	7.83	-	-
2.	22.7	9.87	408.5	104.74	1.80	0.90	9.6	8.00	-	-
3.	22.3	9.70	418.5	107.31	2.60	1.30	-	-	-	-
Mean	22.0	9.58	412.9	105.87	1.04	0.52	10.0	8.31	186.7	52.60
S.E.	±2.1	±0.93	± 4.4	± 1.13	±0.28	±0.13	±0.26	±0.21	± 7.2	±2.03

TABLE 6.

THE CONTENT OF THE PRINCIPAL INORGANIC IONS IN THE 'PLUMPING' FLUID OF BROWN LEGHORN HENS. EACH SAMPLE IS FROM AN INDIVIDUAL HEN.

HEN	SODIUM	POTASSIUM	CALCIUM	MAGNESIUM	CHLORIDE	CARBON DIOXIDE
	mEq./L	mEq./L	mEq./L	mEq./L	mEq./L	mEq./L
1.	146.74	15.38	32.00	2.77	78.87	-
2.	122.35	17.97	29.10	1.92	78.08	-
3.	132.78	14.68	35.90	2.50	74.31	-
4.	114.70	17.79	38.00	1.80	81.06	-
5.	167.39	15.38	30.00	2.50	75.03	-
6.	144.56	11.32	25.00	3.69	75.49	-
7.	176.09	16.12	21.05	2.42	74.59	-
8.	139.75	16.22	30.75	2.58	72.03	83.62
9.	123.13	23.67	26.85	1.80	75.55	80.44
10.	113.17	19.23	21.50	1.77	84.97	89.29
11.	149.13	14.10	26.00	1.92	92.99	90.21
12.	135.43	14.61	26.25	2.65	92.40	80.44
13.	149.13	11.28	22.75	6.42	81.55	81.23
14.	130.19	16.41	31.05	3.42	81.64	77.37
15.	142.03	14.54	-	2.85	-	77.18
Mean	139.10	15.91	28.30	2.74	79.90	82.47
S.E.	± 4.58	±0.79	±1.35	±0.30	±1.73	±1.75

TABLE 7.

THE CONTENT OF THE PRINCIPAL INORGANIC IONS IN THE 'OVIPOSITION' FLUID OF BROWN LEGHORN HENS. EACH SAMPLE IS FROM AN INDIVIDUAL HEN.

HEN	SODIUM mEq./L	POTASSIUM mEq./L	CALCIUM mEq./L	MAGNESIUM mEq./L	CHLORIDE mEq./L	CARBON DIOXIDE mEq./L
1.	-	-	-	-	-	71.91
2.	44.35	97.70	33.95	9.64	48.74	83.57
3.	-	-	-	-	51.68	91.80
4.	29.52	89.23	51.00	13.50	67.06	90.50
5.	43.67	59.02	22.55	19.18	41.20	94.61
6.	50.72	94.00	78.30	20.42	53.88	113.20
7.	29.91	67.03	51.70	17.73	55.93	91.61
8.	37.09	86.54	70.60	23.95	67.97	90.05
9.	49.45	69.23	72.10	20.50	62.81	92.02
10.	35.66	74.52	49.60	26.04	87.28	87.09
11.	38.04	67.95	70.00	17.77	-	88.52
12.	45.65	63.73	41.40	14.04	-	100.18
13.	-	-	-	-	62.48	-
14.	43.83	77.69	119.00	51.33	64.80	-
15.	57.83	71.54	23.00	15.75	88.77	-
16.	53.26	71.54	31.00	19.50	89.71	-
17.	38.04	70.77	34.00	19.25	60.99	-
18.	45.65	65.00	26.00	16.67	48.02	-
Mean	42.84	75.03	51.60	20.35	63.42	91.26
S.E.	±2.11	±3.00	±6.85	±2.45	±3.89	±2.79