THE FUNCTIONAL DEVELOPMENT OF THE RETICULO-ENDOTHELIAL SYSTEM IN THE FOWL

by

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This thesis contains no material previously submitted for a degree in any University, either by the candidate or by any other person, except where due reference is made in the text of the thesis.

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ABSTRACT.

A study of the functional development of the reticulo-endothelial system in the chick embryo has been made. The various extra-embryonic fluids and the serum of embryos at different stages in development were examined for their bactericidal activity against a sensitive strain of bacteria - E. coli Lilly. There was no evidence for this activity, whilst adult chicken serum possessed this property. Further investigation revealed that the failure of embryo fluids to kill E. coli Lilly was due to the relative lack of complement.

A method was devised to study the quantitative aspects of phagocytosis by the cells of the reticulo-endothelial system of the chick embryo, using isotopically labelled bacteria. For rough strains of bacteria such as E. coli Lilly and E. coli K12, the rate of removal increased as the embryo matured from a K value of 0.006 in the 10 day old embryo to K = 0.041 in the 18 day old embryo. This increase was apparently not due to an increasing efficiency of the phagocytic cells, but due to the increased number of phagocytic cells in the older embryos. In contrast, the smooth strains, E. coli 2206, S. gallinarum 98, S.gallinarum 9240, were removed very slowly throughout the development of the embryo.

It was shown that the clearance of E. coli Lilly was due to the presence of opsonins (antibody) for this

bacteria in the circulation of the embryo. When the smooth strains of bacteria were opsonised *in vitro* with an opsonic serum, these bacteria were removed just as efficiently as the rough bacteria.

When the bacteria were phagocytosed, their distribution varied according to the age of the embryo. The phagocytic cells in the membrane played a major role in the younger embryos. As the embryo developed the membrane lost this function, the liver becoming the major phagocytic organ.

The study was extended to examine the intracellular bactericidal property of the phagocytic cells. In the case of *E. coli* Lilly the killing capacity increased as the embryo matured. This was in agreement with virulence studies where the LD50 increased from 9×10^2 for the 11 day old embryo to 6×10^4 by the 18th day. *S. gallinarum* 9S, however, was very lethal even at doses of less than 20 bacteria for all ages. The one day old chicken was resistant to *S. gallinarum* 9S, the LD50 being 2×10^4 bacteria. This change from susceptibility to resistance was later shown to be due to the presence of opsonins for *S. gallinarum* 9S in the one day old chicken.

The chemical nature of the opsonins for *E. coli* Lilly and *S. gallinarum* 9S were studied. The opsonins were isolated by ion exchange column and sucrose gradient centrifugation. In normal hen serum, the opsonins for

E. coli Lilly were found to be globulins which, from their chromatographic and centrifugation properties, were defined as 19S and 7S globulins. For S. gallinarum 9S, the opsonin was a 19S globulin. Further analysis of the opsonins in the yolk fluid, and serum from adult chicks, one day old chicks and embryos, showed that the 7S and 19S globulins were present in all fluids except the embryo serum which only possessed 7S globulins. This suggested that the 19S globulins which were the opsonic for S. gallinarum 9S were not able to cross the yolk sac membrane to the embryo's circulation, though they could be secreted across the follicular epithelium of the ovum. This is similar to the yolk sac transfer of antibodies in the rabbit. The 19S globulins do get across the gut wall post hatching - a situation similar to the ungulates. Under conditions of active immunisation, however, the adult hen produces 7S globulins when vaccinated with S. gallinarum 9S. Embryos developing from immunised hens are able to clear S. gallinarum 9S from their blood.

INTRODUCTION.

At the start of this investigation, it was generally accepted that the foetal or neonatal animal was immunologically incompetent. Proof of this immunological incompetence came from experiments which showed that when foetal or neonatal animals were injected with an antigen these animals in adult life could not synthesise antibody against this particular antigen when challenged. These results supported in part Burnet's "Clonal selection theory" (1958) that postulated an immunologically null period in an animal's embryonic or neonatal life. During this period it was believed that the embryo was populated with clones of mesenchyme cells that would mature soon after birth to cells that were capable of antibody synthesis. Each clone had the capacity to respond to one or more antigen(s). However, if during this null period the clone came into contact with the antigen, as it would with all host "self" antigens, then the clones would be destroyed or changed in such a way that they could not respond to the same antigen in adult life. Induced tolerance was cited as an example of this destruction.

However, it would seem unlikely that immunological competence should be shut off during embryonic life and suddenly turned on soon after birth. A more plausible hypothesis seemed that immunological competence developed in the embryo as do all other physiological functions. It

has been known for some time that some antibody may be passively transferred to the embryo, yet very little was known how this could protect the neonatal animal. Since phagocytosis by the reticulo-endothelial system in the adult animal seems to constitute the best major defence mechanism against infection, the development of this capacity and factors influencing phagocytosis has been studied quantitatively in the chick embryo.

CHAPTER I.

PHAGOCYTOSIS AND CERTAIN FACTORS THAT CONTROL PHAGOCYTOSIS BY THE CELLS OF THE RETICULO-ENDOTHELIAL SYSTEM.

Historical Review.

It is difficult to establish the time when man first realised that recovery from a primary infection protected animals from subsequent infections by the same organism. The application of this observation to inducing artificial immunity in man and animals is obscure. The inoculation of humans against small pox was known to the Chinese and Arabians in the eleventh century, [Williams, (1960)]. In the first half of the 18th century, the Greeks, Circassians and Georgians used to puncture their skins with needles charged with small pox virus, [Metchnikoff, (1905)]. In 1721, Lady Montague introduced to Europe the method of inoculating contents of small pox pustules as a prophylactic to small pox, [Metchnikoff, (1905)].

One of the first applied scientific approaches to acquired immunity came from an English practitioner - Edward Jenner (1798). Jenner had lived in the dairy country of Gloucester in England, where it was a practice by the farmers to allow children with lacerations on their hands to milk cows suffering from cow pox to provide protection against small pox that was rampant in those years. It was common knowledge amongst dairy farmers that those

who had suffered from cow pox would not be affected by small pox. Jenner carried out a series of inoculations in humans, using the contents of the fluid from pustules of people suffering from cow pox. He showed that vaccinated people were immune to small pox, while others who did not receive such treatment contracted the disease during an epidemic. The general success of such trials encouraged the authorities to establish clinics for vaccination against small pox [Williams, (1960)].

In 1880, Pasteur and his two associates, Charles Chamberland and Emil Roux, stimulated by Jenner's work, set out to devise methods for making vaccines. In his experiments with chicken cholera, Pasteur was able to demonstrate that injection of old cultures of chicken cholera protected birds against subsequent infection with a young actively growing culture of the same strain. Controls that had not been previously vaccinated died following challenge with an actively growing culture. From these experiments he developed the concept of using "attenuated" strains of bacteria and viruses to confer immunity. Pasteur's ideas on the use of an attenuated strain to confer immunity against subsequent infection were eagerly accepted and his experimental findings were confirmed by others such as Loeffler (1898).

Pasteur postulated that the mechanism of acquired immunity was the result of the micro-organism being unable to grow in a host which had previously supported its growth.

He proposed that this was due to the exhaustion of nutrients for the bacteria after the primary infection. He gave experimental proof of this by demonstrating that it was not possible to grow bacteria in a medium which had already been used for culture, unless a small amount of fresh nutrients were added.

Chauveau (1880), though accepting this general idea, could not accept the same reasoning for the failure of Algerian sheep to resist large doses of anthrax, though the animals were resistant to smaller doses. This, he explained, on the basis of an inhibitory substance which was present in the blood plasma, and whose action became exhausted when large numbers of bacteria were injected.

Hans Buchner (1877) advanced a further theory to explain acquired immunity. He felt that, since each micro-organism had a localised site of infection, the primary invasion set up an inflammatory action, which reinforced the "living elements" of the organ in question.

During this period pathologists were studying the part played by bacteria in disease. Their investigations were confined to examining sections of various organs from patients who had died as the result of an infection. Many of these workers, Mayer (1870), Birch-Hirschfield (1872), Wlebs (1872) and Rindfleish (1872), wrote of micro-organisms being inside white corpuscles. The generally accepted theory by these pathologists was that the micro-organisms

had such a suitable environment inside these cells that they used the cells as a vehicle for spreading infection through the body. However, in contrast to this idea, Panum, in 1874, studying the viability of the "putrefying bacteria", suggested that these bacteria may be ingested and destroyed by the leucocytes of the animal. Grawitz (1877) felt that the white corpuscles contributed by withdrawing the noxious agent from "assimable fluid". Roser (1881) made the most remarkable statement that immunity may depend "on the property of the contractile cells of ingesting the enemy which enters the animal body". These findings did not, however, catch the imagination of other scientists till Metchnikoff (1884) reported his findings.

Metchnikoff, working independently and quite unaware of earlier suggestions on the role of cells in immunity, was attracted by the phagocytic capacity of cells of mesodermal origin. He showed that these cells were able to seize a variety of particles by their living processes. Following ingestion, many of these particles were digested by the cells. He felt that this was such a fundamental phenomenon that it must be a "vital process of many animals".

While working on this theme, Metchnikoff listened to a lecture by Cohnheim, on inflammation and diapedesis of leucocytes through vessel walls. Metchnikoff was struck by the similarity of the role of leucocytes in inflammation

and the amoeboid cells of the mesoderm in the atrophy of the larval organs of the Synaptae. He felt that these types of cells had a very important function to perform in the animal.

He inserted rose thorns beneath the skin of a transparent star-fish larva, and observed that the thorns were soon surrounded by a mass of amoeboid cells. Metchnikoff concluded that the accumulation of leucocytes must be regarded as a defence mechanism whereby the cells are able to devour and ingest a noxious agent. He coined the word "phagocytosis" to describe this function of the cells.

Metchnikoff's interpretation of these experiments met with severe criticism from some of his colleagues, [Weigert (1887); Baumgarten (1888); Ziegler (1889)]. His adversaries argued that the ingestive and digestive capacities of the leucocyte were negligible in the defence of an animal against infection. These opposing views were not without some foundation, since Fodor in 1886 gave evidence of the in vitro killing of a great number of anthrax bacilli by defibrinated rabbit blood, from which most leucocytes were therefore removed.

This bactericidal property of serum was confirmed by Nuttall in 1888, using sera from a large variety of animals. Metchnikoff was opposed to the theory that phagocytes played only a secondary role of devouring dead bacteria and that the cells were not able to rid the body of

living organisms. Amongst the supporters of the humoral theory of defence were Flugge (1888); Bitter (1888); Charrin and Roger (1889); Bouchard (1890); and Buchner (1890), who showed that the bactericidal action of serum was lost when it was heated at 57°C. He called this heat labile component of serum "alexin".

However, there were others who believed in Metchnikoff's cellular theory of defence. Some of these were Lubarsch (1889), Ribbert (1890) and Behring (1890). These workers opposed the humoral theory on the basis that the *in vitro* experiments did not necessarily simulate events *in vivo*.

They did concede, however, that the experiments of Behring and Kitasato (1890) showed that prior treatment of animals with *B. tetanus* or *C. diphtheriae* protected them against subsequent exposures to the toxin of the same organism. They considered this as a special case of defence rather than a general phenomenon.

The controversy took another turn when Denys and van de Velde [illegible] (1896) and Buchner (1900) suggested from their experiments that alexin was a product of the leucocyte. These workers who supported the humoral theory of defence against infection postulated that the main function of the leucocyte was to secrete alexin and that the phagocytic cell then played a secondary scavenging role after the action of alexin. Pfeiffer (1894) supported this concept

and demonstrated by suitable experiments the occurrence of bacteriolysis when Vibrio cholera was introduced into the peritoneal cavity of guinea pigs. He showed that the substance which was responsible for bacteriolysis was present in serum and other body fluids.

Metchnikoff replied with a demonstration showing that Pfeiffer's phenomenon could only occur when destroyed phagocytes were present. He argued that in a situation where leucocytes were not present, such as in subcutaneous tissue, Vibrio cholera is never destroyed. He writes that "these facts appeared to justify me in the conclusion that destruction of micro-organisms takes place in the animal body by means of soluble ferments, the result of phagocytic digestion. These ferments are found under the normal conditions within these phagocytes and escape from them when they are destroyed or receive some transient injury" [Metchnikoff, (1905)].

Jules Bordet (1895) made an important contribution to this field when he studied the nature of the bactericidal system present in serum. He was able to demonstrate that there were two components in serum necessary for its bactericidal action against Vibrio cholera. One of these was the heat labile alexin (which Ehrlich (1900) later called complement because he believed it to be more than one substance), and the other a heat stable substance.

It was not until 1903 that some attempt was made to unite these two somewhat opposing theories regarding the mechanism of an animal's defence against infection. Wright and Douglas (1903) carried out experiments where they allowed bacteria and leucocytes to come into contact with each other on a glass slide. After 15 minutes incubation at 37°C, the leucocytes were stained and the average number of bacteria present in the phagocytic cells counted. By these semi-quantitative experiments they were able to examine the influence of serum factors on the ingestion of bacteria by these cells. Although in some experiments only a small number of leucocytes were counted, they arrived at some very significant conclusions.

They showed that, when bacteria were acted on by serum, it "renders them a ready prey to the phagocytes". They called the materials in serum which were able to react with bacteria in this manner "opsonins". When bacteria were not opsonised, very little phagocytosis took place [Wright and Douglas, (1903)].

In other experiments they showed that normal serum from different animals varied in its reaction toward strains of bacteria. For instance, serum from one species of animal could be highly opsonic, bactericidal and bacteriolytic for some organisms, (V. cholera, Bacillus. typhosa) but only slightly bactericidal, yet very opsonic for other bacteria, (B. coli, Bacillus. dysenteriae). Finally some sera were

not bactericidal, but highly opsonic for certain species of bacteria (Staph. pyogenes, Pasturella pestis, Micrococcus. melitensis, Diplococcus. pneumoniae and Corynebacterium. diphtheria).

Bulloch and Atkin (1905) characterised the opsonin in normal blood as a thermolabile substance. In a comparative study of blood from different animals, they concluded that various sera differed in their opsonic activity. They also confirmed the finding that the leucocyte was only capable of efficient phagocytosis when opsonins were present.

Since Metchnikoff first defined the cells responsible for phagocytosis, a great deal of work has been done on the various types of phagocytic cells. Metchnikoff described two types in his original thesis. One of these was a relatively large cell, possessing a large oval shaped nucleus which he called a "macrophage", and the other a smaller cell with a multi-lobed nucleus, which he named the "microphage". Today these cells are called the wandering macrophage, or histiocyte and polymorphonuclear leucocyte respectively.

Aschoff and Kiyono (1913), however, gave a clearer and more extensive definition of the cells responsible for phagocytosis. It had been known [Hoffmann and von Recklinghausen (1867); Ponfick, (1869)] that, when animals were injected intravenously with particles like carmine and ver-

milion, they were not eliminated in the urine or faeces, but deposited in various organs such as the liver, spleen, lymph nodes and bone marrow. In 1913, Aschoff and Kiyono coined the phrase "Reticulo-Endothelial System" (R.E.S.) to describe cells that are capable of storing vital dyes after an intravenous injection. Under this heading were the fixed phagocytic cells of the various organs as well as the mobile phagocytes. The fixed cells included the Kupffer cells of the liver, the splenic macrophages, phagocytes lining the lymph sinuses and blood capillaries and the microglia of the central nervous system. The wandering cells were the histiocytes of the connective tissue and the blood monocytes. In the original treatise the lymphocytes were excluded, but later work by Downey (1918) and Dougherty (1944) has shown the phagocytic capacity of these cells and included them in the reticulo-endothelial system.

More recently, since the importance of some non-phagocytic cells (the non-phagocytising lymphocytes and the plasma cells) has been realised, a new name, "The lymphoid-macrophage system" which includes all the potential and actual macrophage cells, including the non-granular leucocytes, has been suggested [Taliaffero and Mulligan (1937); Taliaffero (1949)]. This suggestion has been ignored to a great extent by workers in this field.

The importance of the sessile phagocytes in the defence of an animal was known in the latter part of the

last century. Muscatello (1895) noticed that suspensions of carmine, when injected into the peritoneum, were isolated in the liver and spleen within one to two hours. Durham (1897) and Buxton (1906) reported that when bacteria were injected into the peritoneum they rapidly passed into the blood stream via the diaphragmatic lymphatics, the anterior mediastinal gland and right lymphatic duct. Buxton and Torrey (1906) followed the course of typhoid bacilli when injected intraperitoneally into rabbits. A small percentage of the bacteria passed into the general circulation almost immediately and accumulated in the liver and spleen.

There is also evidence of bacteria entering the blood circulation after being given per os. Nasløe (1948) fed mice with S. typhimurium and other related strains of Salmonella by dropping the suspension of bacteria into the mouth. At various times, the animals were killed and the blood, liver, spleen, mesenteric glands and small intestine examined. His findings indicated that a large number of bacteria, when given by mouth, usually failed to establish themselves in the intestine. Some, however, entered the tissues of the alimentary tract and were carried to the mesenteric lymph glands. In these experiments bacteria were seldom recovered from the blood, but were found in the liver, spleen and mesenteric glands.

Studies concerned with the ingestion of particles by the sessile phagocytic cells of the reticulo-endothelial

system have been, to a great extent, confined to *in vivo* experiments. An attempt was made by Rous and Beard (1934) to isolate the Kupffer cells of the liver after they had phagocytosed iron oxide. The Kupffer cells were isolated from the parenchymal cells by passing the mixture through a strong electro-magnetic field. Those cells which had phagocytosed the iron were retained. Unfortunately the isolated cells became abnormal *in vitro* and hence this method was abandoned.

Semi-*in vivo* techniques involving the perfusion of the liver of an animal with an isotonic solution containing the particles under investigation have yielded considerable information on the serum factors responsible for the recognition of foreign material by these phagocytes.

Manwaring and Coe (1916) found that in the rabbit, phagocytosis by the liver was enhanced when pneumococci were suspended in Ringer's solution containing serum from animals that had been immunised against this bacterium. The perfusion fluid contained antiserum, at a dilution 100 times less than the one agglutination dose. Manwaring and Fritschen (1923) tested phagocytosis of both Gram negative and Gram positive bacteria by the liver cells of the dog. They showed that the sessile phagocytic cells of the liver ingested 80 per cent. more bacteria when small amounts of antibody were present in the perfusing blood than when antibody was absent. Howard and Wardlaw (1958) studied

phagocytosis of E. coli by the normal rat liver. They showed that phagocytosis by the Kupffer cells increased from 11 per cent. in the absence of serum to 41 per cent. in the presence of normal human, rat or mouse serum. Jenkin and Karthigasu (1962) used the same technique to show that when aged rat red blood cells were suspended in normal autologous rat serum, there was increased phagocytosis as compared to normal rat blood cells in normal autologous serum. Such studies have undoubtedly shown the importance of serum factors in enhancing phagocytosis by the sessile cells of the liver.

It has been known for some time that when bacteria, colloidal particles and dyes are injected intravenously, the particles are localised in the liver and spleen [Foot, (1920), Nagao, (1920), Wislocki, (1924), Cappell, (1929), Gordon and Katash, (1949)]. These authors usually studied the distribution of particles in the organs histologically. More recently Halpern, Benacerraf and Biozzi (1953) have devised quantitative methods for studying the kinetics of phagocytosis of a variety of particles by the cells of the reticulo-endothelial system. It is necessary to understand that in such studies, only the phagocytes in contact with the blood are considered. These include the Kupffer cells of the liver, reticular cells of the spleen (splenic macrophages), the reticulo-endothelial cells of the bone marrow and the lymph nodes and the phagocytic cells lining

the blood vessels (endothelial cells). This then excludes all the wandering phagocytic cells of the animal that are not circulatory.

There are conditions which must be observed in such investigations. The particles injected must possess the following characteristics. They must be relatively homogenous in size. The prepared material must form a stable dispersed suspension in body fluids and be non-toxic for the reticulo-endothelial cells. The particles should be accurately measurable in the tissues and in blood. It should also be desirable to identify them histologically in the cells of the reticulo-endothelial system. Finally, there should not be any significant uptake of the material by cells other than those under investigation. The clearance of bacteria, denatured protein, dyes, colloidal carbon, silver, gold, iron saccharide, thorium dioxide and other substances follow the same fundamental kinetic pattern. Hence a brief account concerning the kinetics of phagocytosis of carbon by the fixed macrophages will in general suffice for other particles.

Quantitative Aspects of Phagocytosis by the Reticulo-Endothelial System.

The rate of phagocytosis is studied by measuring the removal of particles from the blood and the accumulation of these particles in the reticulo-endothelial cells, at specific times after intravenous injection of a stand-

ard suspension. When the clearance of these particles is plotted as the log of the blood concentration with respect to time, the removal of the substance follows an exponential curve. This exponential rate can be expressed as the phagocytic index (K), which is a measure of the slope of the curve where $K = \frac{\log a - \log b}{t(b) - t(a)}$. Log a and log b are the concentrations of the particles at time (ta) and time (tb) respectively.

When small doses of particles are injected their rate of removal is constant and independent of dose. The phagocytic index can only be measured when the dose injected is greater than a certain minimal dose where the phagocytic index (K) x Dose = constant. This dose would enable one to study the avidity of the phagocytic cells in the various organs.

The Kupffer cells of the liver phagocytose between 80 to 90 per cent. of all injected colloids. The spleen is responsible for about 5 per cent. and the rest is generally distributed between the lung, bone marrow and kidney. Thus the phagocytic cells of the liver and spleen are able to account for about 95 per cent. of the clearance.

To some extent the distribution of particles in the various organs is dependent on the size of the injected material.

Gofman (1949) prepared a large number of colloids of yttrium, and zirconium oxide or hydroxide which were of

different particle size. These were separated into large, intermediate or small particles on the basis that the large particles showed a Tyndall effect and were sedimented at 2000 g. in one hour, the intermediate showed slight Tyndall effect and were not sedimented at 2000 g. and the small particles showed no Tyndall effect and passed with relative ease through biological membranes. When the large particles were injected intravenously into rabbits, about 90 per cent. of the colloids were found in the liver and spleen and 10 per cent. in the bone marrow. In the case of the intermediate particles, 30 to 50 per cent. was found in the bone marrow.

Since the liver is by far the largest reticulo-endothelial organ, the assumption has been that the bigger the liver, the greater the rate of phagocytosis. As the size of the liver varies not only between individuals, but also between species, a correction has to be made to the phagocytic index to allow for variation in liver size, if one is to compare the efficiency of this organ between individuals of the same species or between different species.

For example, the phagocytic index K for 8 mg. carbon/100 gm. animal in the rat is 0.026, in the mouse 0.047 and in the rabbit 0.008. This would suggest that the mouse shows a total phagocytic rate nearly six times that of a rabbit. When the total phagocytic rate is corrected

for the weight of the liver and spleen in terms of weight of the animal, i.e.

$$\text{Corrected value } (a) = \frac{\text{Weight of the animal}}{\text{Weight of the liver + spleen}} \sqrt[3]{K}$$

then the efficiency of these cells (a) is 5.4 for the mouse, 6.0 for the rat and 5.4 for the rabbit [Benacerraf, (1958)]. However, it should be stressed that the correction is valid only when there are no other limiting factors for phagocytosis - a point which appears to have been overlooked in the general use of this corrected index.

In studies on the clearance of bacteria from the circulation, some very pertinent questions have been answered in relation to defence mechanisms. Benacerraf, Sebestyen and Schlossman (1959) studied the removal of E. coli O.III/B4 and Staph. aureus (girogio strain) in mice, rabbits and guinea pigs. The rate of clearance of bacteria depends directly on the titre of specific agglutinins present in serum of these animals. These in vivo experiments have confirmed what had already been demonstrated in vitro in that phagocytosis of bacteria and indeed many other particles such as carbon, starch, bentonite, takes place only in the presence of certain serum proteins called opsonins [Nelson and Lebrun, (1956); Jenkin and Rowley (1961); Potter and Stollerman (1961)]. These opsonins not only aid phagocytosis but in some instances seem to determine the rate of killing of the bacteria within the phagocytic cells, following their ingestion [Jenkin, (1963a)].

More recently, complement has been shown to enhance the clearance of S. typhi [Biozzi and Stiffel, (1961)]. In these experiments mice were decomplemented by an intravenous injection of an antigen-antibody complex and the clearance of the bacteria followed. When guinea pig complement was injected into complement depleted mice, there was increased rate of phagocytosis.

It is clear from these experiments that bacteria, as well as other colloids, are cleared from the circulation by the phagocytic cells of the reticulo-endothelial system. The rate of clearance of these particles depends on the titre of circulating antibody or opsonin.

Antibodies in Embryos.

From the studies in the adult animal it seems that efficient phagocytosis is only possible when bacteria are opsonised. Therefore in an investigation of the functional development of the defence mechanisms of the foetus or neonatal animal it is necessary to consider not only the phagocytic efficiency of the reticulo-endothelial system but also the development of serum factors on which the expression of this efficiency depends, viz. antibody. The two ways in which antibody may arise in the embryo or neonate are either by passive transfer from the mother or by synthesis on the part of the foetus.

Passive Transfer of Antibodies.

The transport of antibodies from the mother to the foetus or neonatal animal was recognised as early as 1892 by Ehrlich. He reported that young mice born of mothers immunised against tetanus toxoid had a high titre of antibody to this antigen in their circulation. The neonates of non-immunised mothers did not have antibody unless they were suckled by immunised mothers. These experiments convinced Ehrlich that immunity could be transmitted passively to suckling mice via the milk.

Since then a great deal of work has been done on the passive transfer of antibodies. The methods of transfer can be divided into at least two categories:

(a) Ex-utero transfer of antibodies.

In some species of animals, antibodies are passively transferred from mother only after birth. A typical example is the ungulate, where the transfer of antibodies occurs via the gut. The system of passive transfer of antibodies from cow to the neonatal calf will be discussed as a representative of this group.

Dixon, Weigle and Vazquez (1961) studied the various concentrations of protein in the serum of cows during pregnancy and after parturition. When the sera of pregnant cows were studied, the levels of gamma globulin remained fairly constant until two months before delivery. At this period there was a rise in gamma globulins until

about a month before delivery, when the level in the adult was almost halved. This was followed by a gradual rise to slightly above normal one month and a return to normal within two months, post partum. It is interesting that virtually all changes in the serum proteins were confined to the gamma globulin proteins.

A concurrent study of the protein changes in the colostrum and milk during lactation explains these patterns. Two months before parturition the cow has a dry period. At this time, lactation from the previous pregnancy stops and the increase in gamma globulin can be associated with the reduced transport of protein to the udder. The fall in gamma globulin one month before delivery is due to the production of colostrum. At this stage, gamma globulin is being concentrated in the udder of the cow. After birth, the rise in serum gamma globulin is due to normal lactation and reduced protein transfer. It is hard to explain the slight increase of gamma globulin in the first month post delivery unless reduced catabolism or increased synthesis ensue. After the second month of lactation until the next dry period the level of gamma globulin in the udder is fairly constant.

The histological changes of the udder during the dry period is in some ways similar to the epithelial follicular changes in the hen. Early in the dry period the acinar epithelium of the cow is composed of columnar cells. Midway

through the dry period, the acinar epithelium increases in size and there is evidence of secretory vacuoles. At parturition there are more secretory vacuoles in the epithelium, more secretion in the acinar lumen and an inconspicuous stroma. At lactation the acinar lumen is greatly enlarged and the acinar epithelium becomes low cuboidal cells filled with secretory vacuoles. At the dry period, the acinar epithelium contains small amounts of gamma globulin. During the formation of colostrum, the acinar epithelium contains large amounts of gamma globulin and no albumin. It has been suggested that plasma cells in the udder might be responsible for the synthesis of gamma globulin [Petersen and Campbell, (1955)]. Dixon et al., (1961) state that at "no time were there significant accumulations of plasma cells in the udder". They are confident that the sole function of the acinar epithelium of the udder is to concentrate gamma globulins which are then drained away by a network of ducts.

The presence of antibodies in the blood of neonatal calves which suckled immunised mothers was recorded by Orcult and Howe (1922). Since then several people have reported similar observations [Jameson, Alvarez-Tostado and Sorter, (1942); Smith, (1930); Kerr and Robertson, (1943); etc.]. It is now clearly established that the antibodies are concentrated in the colostrum and transferred via the gut of the neonate to the lymphatics which drain into the blood circulation. Comlin, Roberts and Titchen (1951) have

shown that the transfer takes place entirely in the small intestine of the calf. This permeability of the gut lasts for only 24 hours. In other animals like the ox, goat, sheep, pig and horse, however, transfer stops after 36 hours.

In a small proportion of animal species such as the rat and mouse, antibody may be transferred from mother to offspring both in-utero and ex-utero. In contrast to the ungulates, antibody may be absorbed from the gut in these two species for much longer periods of time - 16 days in the mouse and 20 days in the rat.

(b) In-utero transfer of antibodies.

Some of the animals that fall into this class are man, the primates, the rabbit and the guinea pig. Though the study in man and the primates has been scanty, it has been shown that antibodies percolate through the allantoic-chorionic placenta to reach the foetal circulation [Hemmings and Brambell, (1961)]. In the rabbit, however, Brambell, Hemmings, McCarthy and Kekwick (1949) have shown that the yolk sac fluid of the embryo contained the principal proteins of maternal serum. To test the possibility that antibodies would also be found in the yolk fluid, they vaccinated female rabbits with Brucella abortus. The rabbits were mated and eight days later, when the agglutinating titre of the maternal serum was between 1/640 to 1/2560, they tested the yolk fluid of the foetus for specific agglutinins. They found that the antibody titre of the yolk sac fluid of the embryos

was comparable to the titre present in maternal serum. When pregnant females were passively transfused with rabbit anti-Brucella serum, the agglutinins were still transferred to the foetus and could be found in the yolk sac. They concluded that significant quantities of maternal antibodies enter the yolk sac crossing the bilaminar omphalopleur at about the eighth day of gestation.

Morphologically, the yolk sac is an extra embryonic extension of the mid-gut. In 1934, Rodolfo showed that, when pregnant female rabbits were immunised with Brucella abortus, there was a rise in the titre of antibodies in the foetus from the 22nd day of gestation to full term. He did not, however, explain the mechanism of transport of these antibodies from mother to embryo. Brambell, Hemmings and Rowlands (1948) immunised rabbits on the 15th day of gestation. All these rabbits gave birth to normal litters with agglutinating titres in the serum similar to those found in the maternal circulation. Following these observations, Brambell, Hemmings, Henderson, Parry and Rowlands (1949) injected anti-Brucella serum into the lumen of one of the two uterine horns of a pregnant rabbit at the 24th day of gestation. Sera from the embryos implanted in the injected experimental uterine horn had similar agglutinin titres to that found in the serum of the mother (1/160 to 1/320). No agglutinins could be demonstrated in the serum collected from embryos present in the control uterine horn. These

results suggested that agglutinins from the parent were secreted into the uterine lumen, and from here they were passed into the foetal circulation - transport being most probably across the yolk sac splanchnopleur, since this is the largest and most vascular part of the foetal membrane exposed to the uterine lumen. It is certain that no transfer of antibodies takes place via the allanto-chorionic placenta.

The Passive Transfer of Antibodies from the Hen to the Chick Embryo.

It has been known for a long time that an "*in utero*" passive transfer of antibodies also prevails in the *Aves*. In 1893 Klemperer reported the passive transfer of immunity from parent hen to the hatched bird. Since then there have been many confirmations of the early report that antibodies may be passed from the hen to the yolk of the egg [Brandly, Losses and Jungherr, (1946); Fraser, Jukes, Branion and Halpern, (1934); Brierley and Hemmings, (1956); Patterson, Youngner, Weigle and Dixon (1962a)].

More recently an extensive study has been made of the mechanism of transport of antibodies from the maternal circulation to the yolk. When adult chickens were injected with a single dose of Iodine^{131}-labelled bovine serum albumin, the antigen was eliminated from the circulation within four days. Antibodies could be detected in the adult from the fourth day onwards, reaching a maximum titre on

the seventh or eighth day, and then declined to normal by the 24th day. When the yolks of sequentially laid eggs were examined, the antibody appeared four days after the primary response in the adult and the peak yolk levels were reached five to six days after the peak serum levels in the hen. These workers also showed that, when hens were passively immunised with Iodine^{131}-labelled chicken gamma globulin, similar results were obtained, i.e. the Iodine^{131}-labelled gamma globulin was found in the yolk [Patterson *et al.*, (1962a)].

The amount and distribution of antibody in the yolk largely depends on the developmental stage of the ovum at the time of antibody synthesis by the hen. Hens have a single ovary containing hundreds of small ova, of which some are induced to develop and mature. At sexual maturity, the largest ovum is about 0.1 cm. in diameter. There is a period of gradual deposition of yolk material until the ovum is 0.6 cm. in diameter. Then follows a rapid phase of deposition of yolk and development, when the ovum increases in diameter to about 3.5 cm. in six days [Patterson *et al.*, (1962a)].

During this development, the transport of proteins is the function of the follicular epithelium (cells derived from the epithelium covering the gonad of the chickens) which are columnar in shape and at this time undergo a decrease in height. This change initiates an increase in the transport of gamma globulin across the epithelium. At the final stage of development, a non-cellular, vitelline membrane appears

between the follicular epithelium and the edge of the ovum. During the passage of the ovum down the oviduct, the egg white, shell membrane and the shell are deposited around the yolk substance. In normal hens, one egg is laid per day. The largest ovum next in succession is released into the infundibulum of the oviduct within thirty minutes after the preceding egg has been laid. The fully formed egg is usually laid 24 hours after ovulation. [Patterson et al., (1962a and b)].

Patterson et al., (1962a) found that the yolk of the egg laid one day after the injection of labelled gamma globulin was free of labelled protein since this egg was already in the oviduct at the time of injection. However, labelled gamma globulin could be detected in the yolk of eggs laid on the second day after injection. The maximum concentration of antibody was in the yolk of eggs laid on the fifth day. There was a decrease after this time, partly due to catabolism of the injected protein and due to the fact that ova developing in the first five days contained a high proportion of the passively transferred labelled gamma globulin.

The distribution of the antibody in the yolk varied in relation to the time when the eggs were laid. Eggs that were laid shortly after passively administered antibody directed against albumin had the antibody distributed at the periphery of the yolk substance. In later eggs the antibody was more concentrated and centrally distributed in the yolk.

The mechanism of transport of antibody from the yolk to the embryo is not clearly understood. Antibody must pass the endodermal layer and the splanchnic mesoderm before entering the vitelline blood vessels in the mesoderm. It is known that there are phagocytic cells in the yolk sac endoderm, which liberate enzymes that attack constituents of the yolk [Remotti, (1927); Goldstein and Gintsbourg, (1936)]. These products of enzyme degredation are transferred from the endodermal cells to the blood vessels of the yolk sac and thence to the embryo.

At about the time of hatching there is an acceleration in the rate of absorption of the yolk fluid. The residual yolk that is present at the time of hatching is absorbed into the gut cavity, from where it is transferred to the circulation in the first five to seven days post hatching.

The Selective Absorption of Antibodies.

Not all antibodies from the mother are transferred to the foetal or neonatal animal. Brambell, Hemmings and Henderson, (1951) showed that rabbit antibodies to Brucella or Diphtheria toxoid were transferred preferentially to the rabbit foetus in contrast to equine or bovine antibodies to Brucella or Diphtheria toxoid. The antibodies to Brucella abortus or Diphtheria toxoid could not be detected at any time in the foetus after the 24th day of gestation following passive transfer to the pregnant doe. However, if the embryos were at only the eighth day of gestation, equine and

bovine antibodies were transported quite freely.

Dixon et al., (1961) have shown that while there was an increased concentration of gamma globulin by the acinar epithelium of the udder of the cow, albumin, which is a smaller molecule, is selectively precluded from the milk during this period.

In chickens, Brierley and Hemmings, (1956) demonstrated that pigeon antibodies, when injected into the yolk sac of the developing chick, were absorbed much less readily than were fowl antibodies. Rabbit and bovine antibodies were not transmitted in detectable amounts.

In the rat, agglutinins formed in the mouse and passively administered to pregnant females are transferred to the foetus as readily as the homologous rat agglutinins. Antibodies synthesised by the cow, sheep or fowl were not transmitted to the foetus in amounts that were detectable by agglutination techniques.

In humans, the bivalent Rh antibodies, blood group isoagglutinins, and somatic 'O' antibodies, pass the placental barrier poorly as compared to the Rh blocking or incomplete antibodies [Vahlquist, (1958)]. Though no specific reason has been suggested for the selective passage of these antibodies from mother to foetus, both molecular size and the species difference must be considered. It is known that specific antibody may occur in two molecular sizes, characterised by their sedimentation constant as 7S globulins and

19S globulins. It will be shown later that for the chick embryo the selective transfer of homologous antibody in this species may be explained by the difference in the molecular size of the antibody.

The protective effect of the passively transferred antibodies is only transitional. In the human infant, for instance, [Orlandini, Sass-Kortsak and Ebbs, (1955); Oberman, Krikor, Burke, Ross and Rice, (1956)] the gamma globulin level falls exponentially in the first four weeks. The concentration is stabilised at a low level after one month. After three months there is an increase till adult levels are reached by 18 to 24 months. It has been assumed from such data that the infant starts synthesising antibodies after four weeks. In the hatched chicken, active synthesis begins after one month [Patterson, Youngner, Weigle and Dixon, (1962b)]. It is also significant that the antibodies have a longer half-life in the neonate than the adult. Patterson et al., (1962b) found that the half-life of labelled gamma globulin is 35 hours in the adult hen, but 72 hours in the one to seven day old chicken. However, it should be noted that no studies measuring the rate of incorporation of labelled isotopes into the gamma globulin have been made in this species. Hence, from the published data it is almost impossible to determine the time at which individuals start to actively synthesise gamma globulin.

Synthesis of Antibody by the Foetus and Neonatal Animal.

It has been assumed that the foetal or neonatal animal lacked the capacity to synthesise antibodies. This was based on the observation that, when neonatal animals were immunised, antibody could not be detected in the serum on subsequent tests [Grasset, (1929); Burnet, Stone and Edney, (1950); Osborne, Dancis and Julia, (1952); Ebert, (1958)]. Burnet (1958) postulated that antibody producing cells in all animals arose from clones of mesenchyme cells known as stem cells. Each clone was responsible for the formation of one antibody. These cells underwent changes from a null period of immaturity to a mature period, the cells at this time being able to respond to an antigenic stimulus by producing antibodies. During the null period which corresponds in time to the foetal and early neonatal life of the animal, the cells could not respond immunologically to an antigenic stimulus. Billingham, Brent and Medawar, (1956) reported that the injection of foetal or neonatal mice with spleen cells from another inbred strain of mice conferred on the recipient animals the capacity to accept homografts from the donor of the spleen cells. The treated recipient animal was known as the "tolerant" animal. Control mice, which had not been so treated, rejected the graft after a period of ten to twelve days. This experiment has been repeated in a variety of animals with similar results. Burnet, (1958) postulated that the immature mesenchyme cells of an animal were destroyed

or changed if they met the antigen during the foetal period. Dixon and Weigle, (1957) presented evidence to suggest that not only were the antibody forming cells of the neonatal animal not capable of antibody response, but the biochemical environment of the foetus or newborn animal was such that it would not support efficient synthesis of antibody by transferred immunologically competent adult cells. The experimental evidence of these workers has been challenged by Trnka and Říha, (1959) who attribute the inefficiency of the transferred cells to synthesise antibody as due to a homograft reaction by the host.

Experimental evidence has now been reported, indicating that the foetal and neonatal animal may be immunologically active. Simonsen, (1957) found that when spleen cells from adult hens were injected intravenously into 18 day old chick embryos, a marked splenomegaly resulted in the host. Isaacson and Boyer, (1960) injected whole blood from adult hens into 12 to 13 day old embryos, and reported similar results. Biggs and Payne, (1961a and b) using cytological techniques were able to show that the enlargement of the spleen was due to both donor and host cell proliferation. Whilst it is clear that the adult donor cells were immunologically competent, and thus able to react against host antigens, it is apparent from their findings that the host may also react to the presence of foreign antigen.

Several people have reported that the transfer of

maternal antibodies in the pig occurs via the colostrum. The neonatal pig lacks gamma globulin before suckling [Earle, (1935); Barrick, Matrone & Osborne, (1954); Rutqvist, (1958)]. Recently, Šterzl, Kostka, Mandel, Říha and Holub, (1959) immunised piglets, which had been removed by Caesarian operation six days before normal parturition, with Brucella suis antigen. The pigs were fed on artificial diet throughout the experiment. Antibody to Brucella suis was detected on the 20th day of extra uterine life, which is 14 days after normal term. Piglets injected six days after normal birth synthesised antibodies on the 20th day of life and one month old pigs showed detectable antibodies seven days after stimulation.

Similar evidence demonstrating the immunological competence of premature infants was presented by Smith (1960). One hundred and fifty infants weighing between 1000 - 2500 gm. were inoculated intradermally with S. typhi vaccine. The cord blood of these infants did not have antibodies to S. typhi before immunisation. Seven days after injection, 50 per cent. of the babies synthesised agglutinins against the "d" flagellar antigen of S. typhi, agglutinins titres varying between 1/10 and 1/5120. Eighty per cent. of these infants were positive by the fourteenth day. No detectable agglutinins against the 'O' somatic antigen were present in any of these babies. Starch block electrophoresis of the serum showed the antibody to be a gamma - 1 globulin (also known as beta 2m or macroglobulin). Further analysis by analytical

ultracentrifugation methods showed that this component was a globulin with a sedimentation constant of 19S. This component of serum is not usually transferred across the placental barrier in humans [Vahlquist, (1958)].

The most convincing evidence of antibody synthesis in the foetus was obtained by Silverstein, Uhr, Kraner and Lukes, (1963). Sheep have a gestation period of 150 days and there is no transfer of maternal antibodies in utero. These workers anaesthetised pregnant ewes at various stages in their gestation period. The gravid horn of the uterus was manipulated and the posterior end of the foetus lifted through the incision. The antigen was then injected through the uterus and foetal membranes into the muscle of the thigh.

The antigens used for inoculation of the foetus were 10^{10} plaque forming particles of bacteriophage ØX174, 100 µg. of diphtheria toxoid, 2 mg. of viable BCG, 1.25×10^9 heat killed S. typhosa, 1 mg. crystalline ovalbumin and 1 mg. ferritin. The age of embryos varied from 65 days to about 110 days post coitus. The presence of antibodies was tested in the various age groups from seven days post inoculation. Antibody against phage was detected by the phage neutralising test, for S. typhi, by the use of the agglutination or by the tanned red cell haemagglutination technique. Antibodies against ovalbumin and ferritin were also assayed by the tanned erythrocyte haemagglutination technique and diphtheria by the skin sensitisation test in rabbits. Antibodies against

mycobacteria were tested by the modified tanned erythrocyte haemagglutination technique of Boyden, (1951). These techniques vary in their sensitivity to detect antibody. Diphtheria antitoxin can be assayed at a concentration of 0.003 µg antibody per ml., the tanned cell haemagglutination technique is capable of detecting 0.005 to 0.01 µg. antibody per ml. Antibodies against the Salmonella and mycobacteria (BCG) could be detected at 0.001 µg per ml.

The results of these experiments showed that the 60 day old foetus was able to form antibody to phage ΦX174 within six days. Antibody to ferritin was present in a titre of 1/128 ten days after immunisation of the 70 day old foetus. However, antibody to ovalbumin was only produced after a very long period of time. Antibody to this antigen appeared in the circulation 60 days following immunisation of the 65 day old foetus. When this same antigen was injected into the 90 day old foetus, antibody appeared 30 days later. Hence, in both instances, the first appearance of antibody against ovalbumin was detected on the 120th - 125th day of gestation. In general, older embryos synthesised a higher titre of antibody to these antigens. Antibody against diphtheria toxoid, S. typhi or B.C.G. could not be detected in any of the embryos. Indeed, even normal new-born lambs could not synthesise antibodies to S. typhi and B.C.G. In the adult sheep, however, antibodies to ovalbumin, ferritin, bacteriophage, S. typhi and B.C.G. could be readily detected. Only diphtheria toxoid

had a poor antibody response.

The controls in the case of the embryo experiments were the twin foetus which were left uninjected. In no instance were antibodies found in the control embryos. The serum of the pregnant ewes was also examined for the presence of antibodies to these antigens with negative results.

It is clear from these experiments that the foetal lamb may produce antibodies against certain antigens.

Schinkel and Ferguson, (1953) also studied the ability of the foetal lamb to mount an immunological response against transplantation antigens. Foetal lambs at the 80th day of gestation were grafted with autologous and homologous skin. The donor skin was sutured to the foetal skin surrounding the graft bed and the foetus returned to the amniotic cavity.

Both microscopic and histological examination of the grafted foetus showed that the autografts were accepted, whilst the homografts were rejected. In utero, the autografts were vascularised and the epithelium of the graft and the host established continuity. The graft also produced wool. The homografts showed necrotic changes 20 days following transplantation. When the graft bed was examined post-partum, only a scar was left in the place of the graft. When a second graft was transplanted from the same donor to the host it was rejected in nine days, indicating a second set enhanced graft rejection. These experiments showed

quite clearly that the foetal lamb at this age is capable of producing a vigorous immunological reaction against grafted foreign tissue.

Another analogous response between the adult and foetal sheep is the type of antibody synthesised. It has been shown that in the adult, the initial type of antibody produced to an antigen is the 19S type globulin. When a higher dose is injected, the 19S type is followed by the synthesis of the 7S type globulin. While the 19S response is seen two or three days after stimulation, the 7S is only formed after seven days. The 19S globulins have a shorter life than 7S globulins, and continued synthesis of the macroglobulins (19S) seems only to persist in the presence of the antigen. The gamma globulin (7S type) synthesis can continue for several months. A second stimulation leads directly to an anamnestic response of 7S globulins.

The foetal lamb also produces the 19S globulins to phage ØX174. Prolonged stimulation initiates the 7S globulins. Older foetuses were more prone to produce 7S globulins. These results of Silverstein et al., (1963) are in agreement with Uhr, Dancis, Franklin, and Finkelstein and Lewis. (1962) on the premature infant.

The postulate that the foetal animal is immunologically null or incompetent can no longer be accepted. It is possible that the foetal embryo may mature to respond to various antigens as it grows. To verify this, it is first

necessary to try varying doses of antigens, as too high a dose may lead to immune paralysis or too small a dose may not stimulate the synthesis of detectable amounts of antibody. Silverstein et al., (1963) realised that varying doses of antigen must be used before any general conclusions could be reached for all antigens.

However, it is becoming increasingly apparent that antibody synthesis may involve more than one cell type, the macrophage being possibly involved indirectly in this synthesis. It is important that if a better understanding of the development and evolution of the immune response is to be obtained, a more detailed knowledge is required of the fate and distribution of antigens injected into embryos of various animal species.

CHAPTER II.

MATERIALS AND METHODS.

Bacterial Strains.

In the present study the following two species of bacteria were used - Escherichia coli and Salmonella gallinarum.

Escherichia coli. The strains E. coli 8206, belonging to the serotype O111, B4 and E. coli K12 (rough) were received from Prof. D. Rowley. Escherichia coli Lilly (rough) was supplied by Dr. A. C. Wardlaw of the Connaught Medical Research Laboratories, Toronto, Canada. These bacteria have been adequately described by the above authors [Rowley, (1952); Rowley and Wardlaw, (1956)].

Salmonella gallinarum. S. gallinarum 9240 and S. gallinarum 9S were obtained from Dr. William-Smith, Lilystone Hall, Stock, Essex. S. gallinarum N.C.T.C.9240 is virulent for adult chickens, whilst S. gallinarum 9S, a mutant strain derived from the S. gallinarum 9240, is avirulent [William-Smith, (1956)].

Maintenance of Bacterial Strains.

The cultures were received as freeze dried preparations in sealed ampoules. The ampoules were opened aseptically and their contents resuspended in 1 ml. of nutrient broth. The total contents of each of these ampoules was subcultured in 10 ml. of nutrient broth and incubated at 37°C

for 18 hours. A loopful of the respective cultures was then streaked out on to nutrient agar plates and incubated, to verify the purity of each culture.

If the culture appeared pure, several colonies were picked off into 10 ml. of nutrient broth. After overnight incubation samples of 1 ml. were dispensed into sterile ampoules, sealed and snap-frozen by immersion in solid carbon dioxide-ethyl alcohol mixture. The ampoules were then stored at -20°C.

When a culture was required, one ampoule was thawed and the contents placed in 10 ml. of nutrient broth and incubated overnight.

A sample of the culture was then streaked on to ten nutrient agar slopes and incubated at 37°C for 18 hours. The agar slopes, in one ounce screw-capped bottles, were then kept at 4°C. Each slope was used not more than ten times and, at the end of the series, a new ampoule opened and the procedure repeated.

Preparation of P^{32}-labelled Bacteria.

The liquid medium for the growth of bacteria was a supplemented casamino acid medium containing the following ingredients:

Sodium citrate $3H_2O$	0.5 gm.
Magnesium sulphate $7H_2O$	0.1 gm.
Glucose	2.0 gm.
Casamino acids	10.0 gm.
Distilled water	1000 ml.

The pH of this medium was adjusted to pH 7.0 and after dispensing in 50 ml. amounts, sterilised by autoclaving at 15 lb. pressure for 15 minutes [Benacerraf, Sebestyen and Schlossman, (1959)].

To 50 ml. of casamino acid medium in a 500 ml. Erlenmeyer flask 0.5 millicuries of P^{32} as orthophosphate was added. The Erlenmeyer flask was placed in an enamel basin and packed tightly around with cotton wool. The medium was inoculated with 10^7 bacteria in 0.1 ml. of casamino acid medium and shaken at one agitation per second at 37°C for 18 hours.

The isotopically labelled bacteria were harvested by centrifugation at 3000 r.p.m. for 20 minutes and washed three times with 50 ml. of physiological saline in firmly capped polythene centrifuge tubes. The third volume of washing fluid contained negligible amounts of radioactivity in the supernatant. The bacteria were finally suspended in casamino medium. A 1:30 dilution of this suspension in physiological saline was measured on the Shimadzu Photo-Electric spectrophotometer Model Q.R.50, at a wave length of 675 mμ. This diluted bacterial suspension of optical density 0.016 had been shown by viable count to contain 3.3 x 10^7 bacteria per ml. The original suspension was standardised to 10^9 bacteria per ml. and kept at 4°C and not used for longer than five days.

Opsonisation of Bacteria.

Equal volumes of the bacteria and the serum under

investigation were mixed and allowed to remain at 4°C for 30 minutes. During this time there was no visible agglutination or fall in viability. The mixture was then centrifuged at 2000 r.p.m. for 15 minutes.

The serum treated bacterial deposit was washed three times with 10 ml. of 0.15M saline. Failure to wash the bacteria led to death of the embryo, due to the toxic effect of the serum [Lum.A.(1958)]. The bacteria were finally resuspended in casamino acid medium to the original volume and used in the experiments.

Preparation of Carbon for Injection.

Carbon (C 11-1431/a ,Gunther Wagner, Hanover) was obtained as a stabilised colloidal suspension at a concentration of 32 mg./ml. in 2 per cent. gelatin. The carbon particles were fairly homogenous in size and measured about 250 $\mathring{A}$ [Biozzi, Benacerraf and Halpern, (1953)]. The various doses of carbon were prepared by heating the original carbon for 30 minutes in a 56°C water bath, and making appropriate dilutions in 1 per cent. gelatin in saline. The various concentrations of carbon in 1 per cent. gelatin were kept at 37°C before injection and taken up in a prewarmed 1 ml. tuberculin syringe.

Collection and Incubation of Eggs.

Eggs were obtained from a pure strain of White Leghorn fowls. They were collected and kept in the cold room at 5°C for not longer than seven days prior to incubation.

A "Gamble All Electric Model" incubator with an electrically controlled thermostat and forced air circulation was used. The temperature was maintained at 37-38°C and humidity controlled at 65 per cent. [Beveridge and Burnet (1946)]. All eggs were incubated on their side so as to allow the formation of the vascular system on the broad side of the egg. During incubation the racks were turned through 45° twice each day. All eggs used in experiments in the present investigation had an average weight of 50 ± 5 gm.

Determination of Embryo Viability.

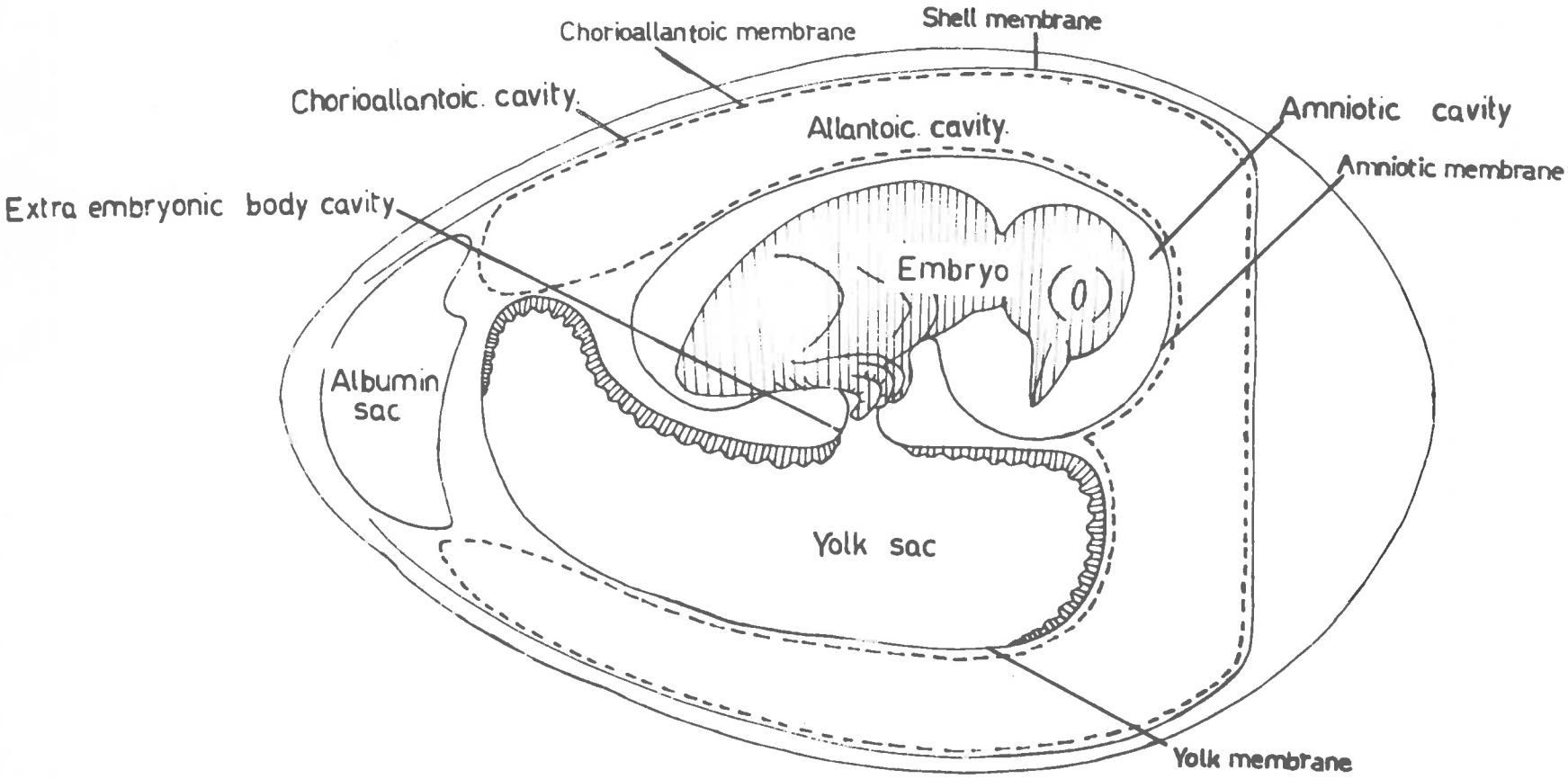
To ascertain whether the embryos were living and to mark out the veins suitable for injection, the eggs were candled in the dark room with the aid of an egg viewer. This was a box with an oval opening on the top side, edged with felt, and illuminated with a 100 watt electric light bulb. The candling procedure did not exceed 5 seconds and the eggs were not kept at room temperature for more than 5 minutes.

Collection of Embryonic Blood and Extra-Embryonic Fluids.

a. Preparation of serum from embryo chickens.

Eggs were candled and the chorio-allantoic vein (which was often the largest vein) marked. A pencilled arrow was also made on the shell to indicate the direction of blood flow, which was towards the floating end of the chorio-allantoic vein. An area of the shell 5 mm. wide and 1 cm. long overlaying the vein was drilled using a dentist drill with a rotating 3-3/4 inch volcarbo No. 16 disc.

Fig.1 Diagrammatic sketch showing full development of membranes and cavities of a 12 to 15 day old chick embryo.


Chorioallantoic membrane
Shell membrane
Chorioallantoic. cavity.
Allantoic. cavity.
Amniotic cavity
Amniotic membrane
Extra embryonic body cavity
Embryo
Albumin sac
Yolk sac
Yolk membrane

The drilled area was swabbed with liquid paraffin (Harrington liquid heavy paraffin 0.865-0.890 g. at 20°C) and three minutes later, the shell carefully picked out with a pair of fine forceps. Care was taken not to damage the shell membrane. The exposed membrane was lightly swabbed with more liquid paraffin to emphasize the blood vessels against the transparent membrane.

A 30 gauge needle fixed to a 1 ml. tuberculin syringe was used to withdraw blood. The needle was inserted into the vein against the flow of the blood and the blood slowly sucked into the syringe. The amount of blood removed varied from 0.2 ml. in the case of the 10 day old embryo, to 1 ml. in the case of the 19 day old embryo. Age of the embryo was defined in terms of days from the start of the incubation. Blood from individual embryos of the same age was pooled (Fig. 2 and 3).

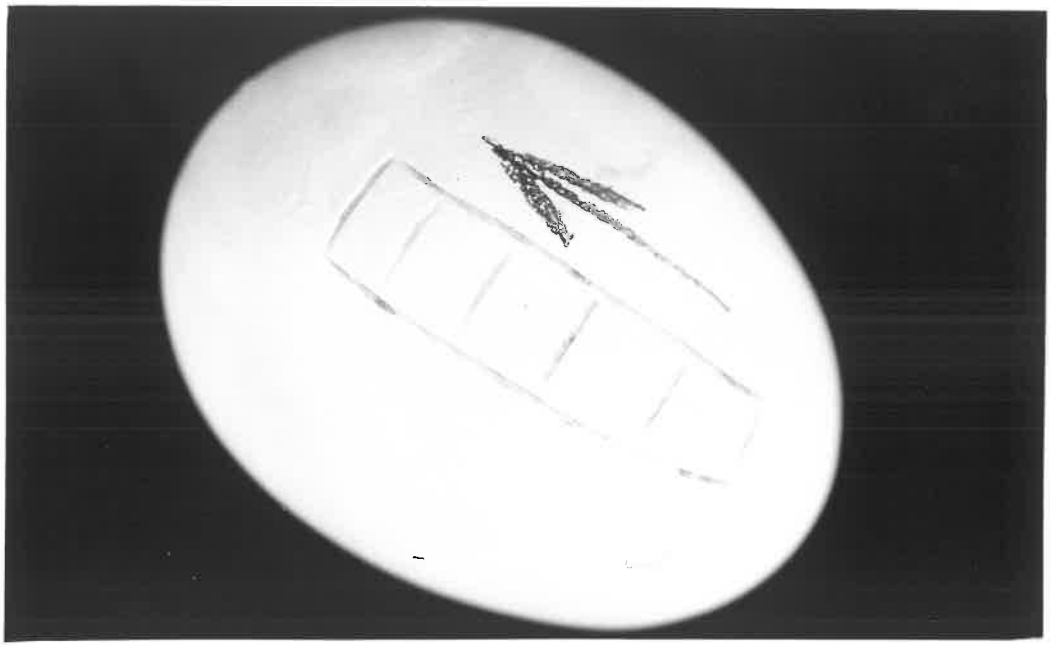
The blood was allowed to clot at 37°C for 30 minutes and then transferred to the refrigerator at 4°C for 30 minutes. Following this, the blood was centrifuged at 1500 r.p.m. for 10 minutes and the serum collected aseptically. Serum samples were stored at -20°C. Most serum samples were thawed only once, or at the most, twice, since repeated freezing and thawing brought about a decrease in biological activity. All samples were used within six months of initial collection of serum.

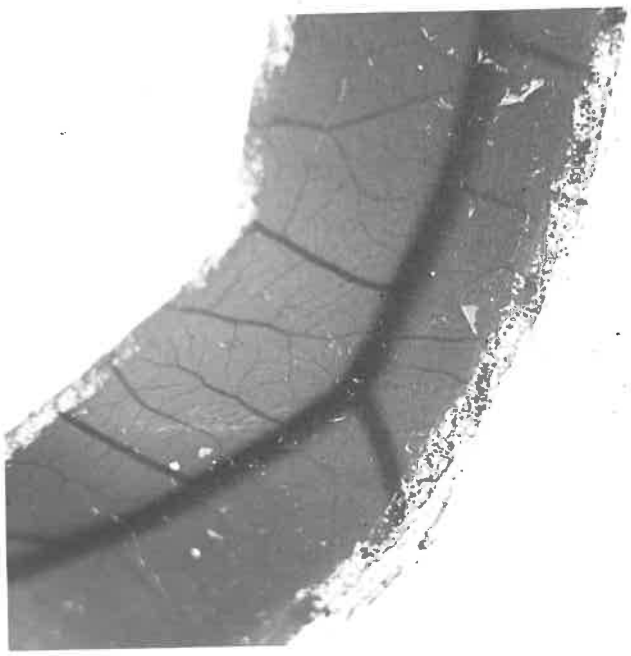
b. Collection of Allantoic Fluid.

The shell around the air sac was removed and the ex-

Fig.2 Egg drilled prior to exposing the chorio-allantoic vein. The arrow points in the direction of blood flow.

Fig.3 The exposed chorio-allantoic vein of a 12 day old chick embryo magnified five times.





posed membrane swabbed with sterile paraffin oil (Fig.1). The membrane was pierced with a sterile Pasteur pipette in an area where there were no blood vessels, and the allantoic fluid withdrawn. The allantoic fluid removed from the embryos of the same age group was pooled and dispensed in 5 ml. amounts into 1 ounce screw-capped bottles and stored at -20°C.

The amount of allantoic fluid that could be removed varied from approximately 5 ml. in the case of the ten day old embryo to 0.5 ml. with the older embryo.

c. Collection of Egg Yolk Fluid.

By carefully teasing away the shell membrane and the chorio-allantoic membrane in the region of the air sac, the yolk sac was exposed (see Fig.1). The yolk sac fluid was then removed by means of a sterile Pasteur pipette, pooled, dispensed and stored as previously described.

d. Collection of Albumin.

The area of shell just above the albumin sac was carefully broken and the albumin fluid exposed (see Fig.1). The albumin was collected by means of a Pasteur pipette, pooled and stored as above.

Preparation of Animal Sera.

Blood from adult hens was collected aseptically, either by cardiac puncture or venous puncture of the wing vein. The blood was treated in the same way as the embryo blood. The pooled serum was dispensed in two to three ml.

amounts in sterile 1 ounce screw-capped bottles and stored at -20°C. Adult pig blood was obtained from the Adelaide Metropolitan Abattoir during the slaughter of healthy pigs. The serum was prepared as described above, and sterilised by Seitz filtration. The serum was pooled and dispensed as previously mentioned.

Design of Experiments to Measure the Bactericidal Properties of Various Fluids from the Chick Embryo.

Small (1 cm. by 7.5 cm.) tubes with aluminium caps were used for these experiments. The tubes were boiled in Calgon and sodium metasilicate solution for 20 minutes, rinsed three times in tap water, three times distilled water and finally three times in deionised water [Hanks, (1955)]. Following rinsing, the tubes were dried and sterilised by autoclaving at 15 lbs. for 10 minutes. The washing procedure was very important as uncleaned tubes yielded erroneous results.

Serial twofold dilutions of the various embryonic fluids were made in minimal medium [Davis and Mingioli, (1950)]. Minimal medium was used as the diluent because the test organism was killed in saline [Ali, (1959)]. Control tubes containing only minimal medium were also included. The tubes were allowed to equilibrate in a 37°C water bath. From an 18 hour broth culture of E. coli Lilly a suitable dilution was made in minimal medium to give approximately 1000-2000 bacteria in 0.02 ml. This diluted sus-

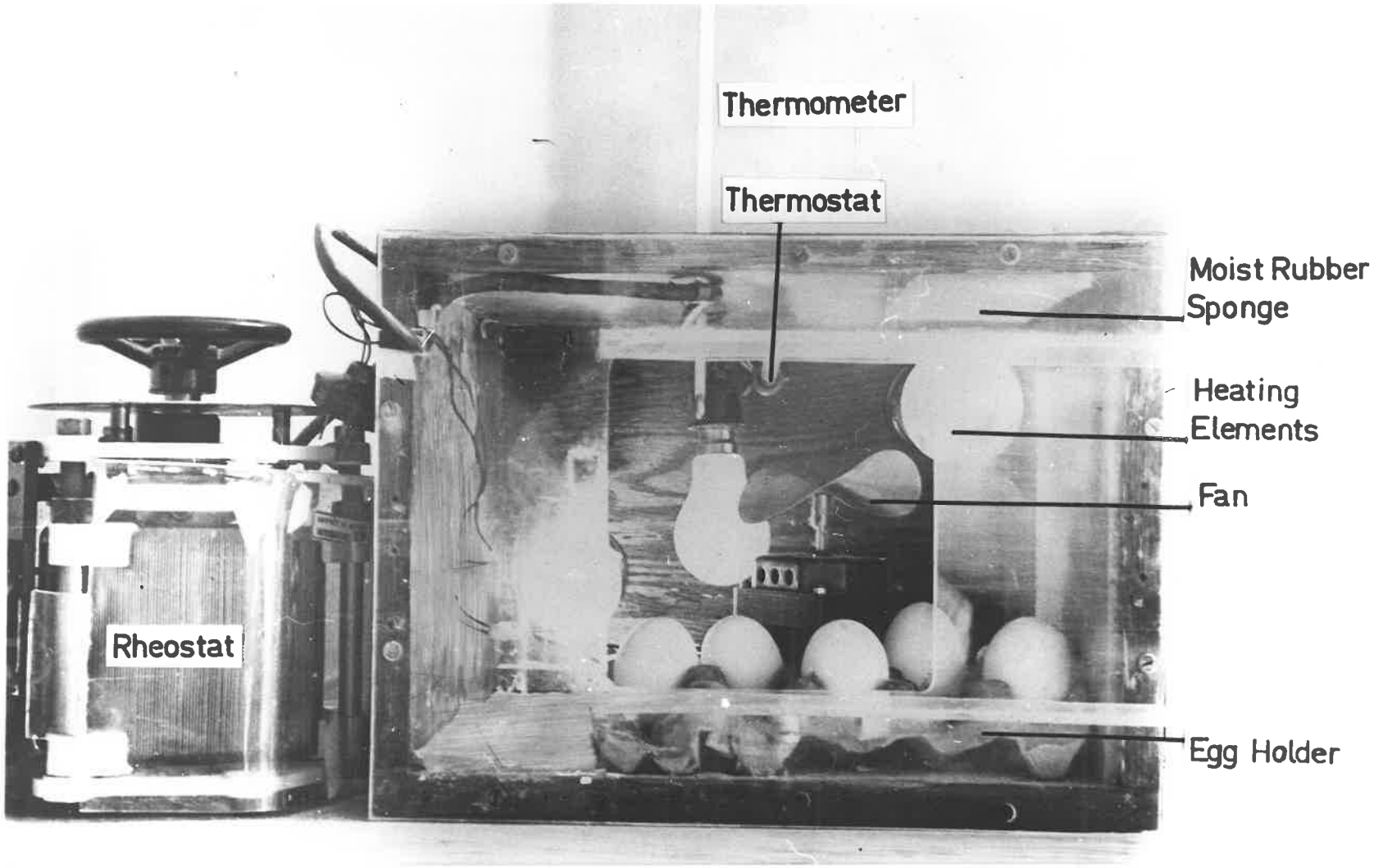
pension was also equilibrated at 37°C before addition to the tubes containing the various fluids. At intervals of 0, 15, 30 and 60 minutes after adding the bacteria, duplicate volumes of 0.1 ml. were plated on to dried nutrient agar plates. The plates were incubated at 37°C for 18 hours and the bacterial population counted.

Methods of Determining the Rate of Elimination of Bacteria from the Circulation of the Chick Embryo.

The eggs were candled and the large allantoic vein pencilled for a length of 2-3 cm. An area of shell of the same length and 5 mm. in breadth was drilled and removed as previously described. Care was taken not to damage the shell membrane. The eggs were then kept in a bench incubator thermostatically controlled at 37°C (see Fig.4), uniform temperature and humidity in the incubator being controlled by a revolving fan.

A sample containing 2×10^8 isotopically labelled bacteria in 0.2 ml. was injected intravenously in the direction of the blood flow. A needle (Mizzy Hypodermic Needle, stainless steel, luer type - length $\frac{1}{2}$ in., gauge 30) attached to a 1 ml. tuberculin syringe was used for removal of blood samples. A similar-sized needle was also used for injection. At intervals of 0, 5, 15, 30, 45 and 60 minutes a sample of 0.04 ml. of blood was removed from the vein. The blood was injected into small Durham tubes (3 cm. by 0.3 cm.) and 0.02 ml. of each sample immediately pipetted

Fig.4 Bench incubator used during the course of the experiment to keep embryos at standard temperature and humidity.


Thermometer
Thermostat
Moist Rubber Sponge
Heating Elements
Fan
Rheostat
Egg Holder

on to filter paper gummed to steel planchettes. The eggs were kept in the bench incubator during the course of the experiment.

The amount of radioactivity present in each sample was assayed using a thin mica end-window Geiger counter installed in a Nuclear Chicago automatic sample changer C110A (Nuclear Chicago, Chicago) with an automatic printing timer C111, coupled to a model 183 scaling unit to record the results. This apparatus was modified by the insertion of a General Electric helium filled thin-window Geiger counter tube which increased the sensitivity of the machine threefold.

Distribution of Injected P^{32}-labelled Bacteria in the Developing Chick Embryo.

Sixty minutes after the injection of P^{32}-labelled bacteria, the embryo was perfused with saline at 37°C. Perfusion was carried out by intravenous injection of the warm saline in the direction of blood flow at the rate of 1 ml. per minute. The effluent fluid flowed out of the severed vein 1 cm. behind the point of injection. About 10 ml. of saline was used per embryo before the effluent fluid was free of blood.

Following perfusion, the spleen, liver, embryonic membranes and the rest of the embryo were each separately digested for 12 hours at 60°C in 10 per cent. sodium hydroxide. The volume of each digest was measured and 0.06

ml. of the respective digests assayed for radioactivity. A sample of the original bacterial inoculum was also assayed giving an estimate of the total amount of radioactivity injected into the embryo.

Method of Blockading the Reticulo-Endothelial System of the Chicken Embryo.

Bacterial suspensions were used to blockade the reticulo-endothelial system. Bacteria were grown in a 500 ml. flask containing 200 ml. of sterile nutrient broth. They were incubated at 37°C for 18 hours under agitation for maximum aeration. The bacteria were harvested, washed twice in saline, and standardised as described for radioactive bacteria.

The standard doses used for blockade were 2×10^8 bacteria or 4×10^8 bacteria injected in 0.1 ml. of casamino medium. The effectiveness of blockade was determined by comparing the rate of clearance of 0.1 ml. of 2×10^8 P^{32}-labelled bacteria between such treated embryos, and embryos that had been injected with 0.1 ml. of casamino medium only.

Method for Determining the Rate of Elimination of Carbon from the Circulation of the Chick Embryo.

The eggs were prepared as described for bacterial clearances and 0.2 ml. of the colloidal carbon suspension was injected intravenously. At the same time intervals as above, 0.04 ml. of blood was withdrawn and 0.02 ml. samples

delivered into 3 ml. of 0.1 per cent. sodium carbonate solution. The concentration of carbon was determined by reading the optical density at 675 mμ. in a spectrophotometer CF_4 ("Optical Milan", Italy). The experimental samples were compared with a solution of 0.02 ml. of normal embryo blood in 3 ml. of 0.1 per cent. sodium carbonate [Biozzi, Benacerraf and Halpern, (1953)].

Haemagglutination Method of Assaying the Titre of Antibody (Opsonin).

Lipopolysaccharides of the various bacteria were prepared by the phenol-water method as described by Westphal, Lüderitz and Bister (1952). Lipopolysaccharide at a concentration of 250 μg./ml. was treated for 18 hours at 37°C with 0.02 N sodium hydroxide. The mixture was then adjusted to pH 7.0 with 0.2N hydrochloric acid.

For the absorption of lipopolysaccharide on to sheep red blood cells, the method of Crumpton, Davies and Hutchinson, (1958) was followed. The erythrocytes were washed in saline and made up as a 2.5 per cent. (v./v.) solution. Equal volumes of cells and lipopolysaccharide solution (final concentration of lipopolysaccharide in the mixture was 50 μg./ml.) were incubated for one hour at 37°C. Following incubation the cells were washed three times with an 0.5 per cent. solution of normal heated rabbit serum in saline. The serum had been heated at 57°C for 30 minutes to destroy haemolytic complement. Finally the sensitised cells were

made up as a 0.5 per cent. (v/v) solution.

Serial twofold dilutions of serum were prepared in haemagglutination trays such that the fluid volume per cup was 0.2 ml. To each dilution an equal volume of sensitised cells was added and the mixture incubated at 37°C for 60 minutes. Controls of lipopolysaccharide-treated red blood cells against saline and unsensitised sheep red blood cells against sera were included. The greatest dilution at which visible agglutination of the erythrocytes could be detected was taken as the end point.

Immunisation of Adult Fowls.

The vaccine was prepared by washing an overnight broth culture of bacteria twice in saline, resuspending in saline and heating at 100°C for two hours. The turbidity of the bacterial suspension was adjusted to the equivalent of 5×10^8 viable organisms by optical density measurements. The birds were then injected according to the following schedule. Two intravenous injections of 1 ml. with a five day interval were followed by three 2 ml. intravenous injections seven days apart. The birds were allowed to rest for ten days and a final intramuscular injection of 1.5 ml. of vaccine was given.

Serum was collected from the birds before the course of injections and also during the subsequent laying period. Eggs were collected from these hens one week after the final intramuscular injection.

Method for Determining the Agglutination Titre of Fowl Serum.

The bacteria were washed off an 18 hour nutrient agar slope with 10 ml. of 0.15 M saline. The organisms were then washed twice with 0.15 M saline and standardised to give approximately 2 x 10^9 bacteria/ml. To this an equal volume of absolute alcohol was added and the mixture heated at 57°C for 30 minutes, washed and standardised in saline to give approximately 2 x 10^9 bacteria/ml.

Twofold dilutions of the serum were made using 0.15 M saline as the diluting fluid. An equal volume of the above standardised alcohol treated bacterial suspension was added to each tube. The tubes were incubated at 37°C for 18 hours. The agglutination titre of the serum was expressed in terms of the greatest dilution of the serum giving visible agglutination. Control tubes of bacteria suspended in saline were included in the test.

Intravenous Inoculation of Chick Embryo and Hatched Chickens in Virulence Tests.

Eggs were candled and the chorio-allantoic vein marked for 5 mm. along its length. The area of shell above the vein was removed as previously described. Various dilutions of the test bacterial suspension in 0.1 ml. volume were injected into the vein in the direction of the blood flow. Any egg which haemorrhaged was discarded. Embryos serving as controls were injected with the same volume of physiological saline. After injection the shell membrane was

resealed by spraying with Nobecutane (Evans Medical Ltd., Liverpool) to prevent dehydration. Eggs were candled at daily intervals and viability assessed by movement of the embryo.

The one day old and one week old chickens were injected via a leg vein. The chickens were housed in warm brooder compartments. Control birds were injected with 0.1 ml. of saline by the same route. Deaths of birds were recorded after every 24 hours.

Fate of Bacteria in the Various Organs of the Embryos and the Hatched Chickens.

A suitable dilution of an overnight broth culture of the bacteria was injected intravenously into a group of chick embryos. At various times after injection the blood was sampled, and the embryos perfused with warm saline as previously described. The liver, spleen and membrane were homogenised in sterile pyrex homogenisers fitted with teflon pestles, [Cat. No. 31-Tri-H; Jamaica Instruments, New York,] and the respective organ homogenates assayed and bacterial population estimated by plating out duplicate 0.1 ml. samples on to nutrient agar plates. Two embryos were tested at each time period. The plates were incubated and the numbers of colonies present counted the next day.

With hatched birds, a similar procedure was followed, and the numbers of bacteria associated with liver, spleen and blood determined at various time intervals after intravenous injection of the test dose of bacteria.

Analysis of Serum Proteins of the Chick Embryo and Adult Hen.

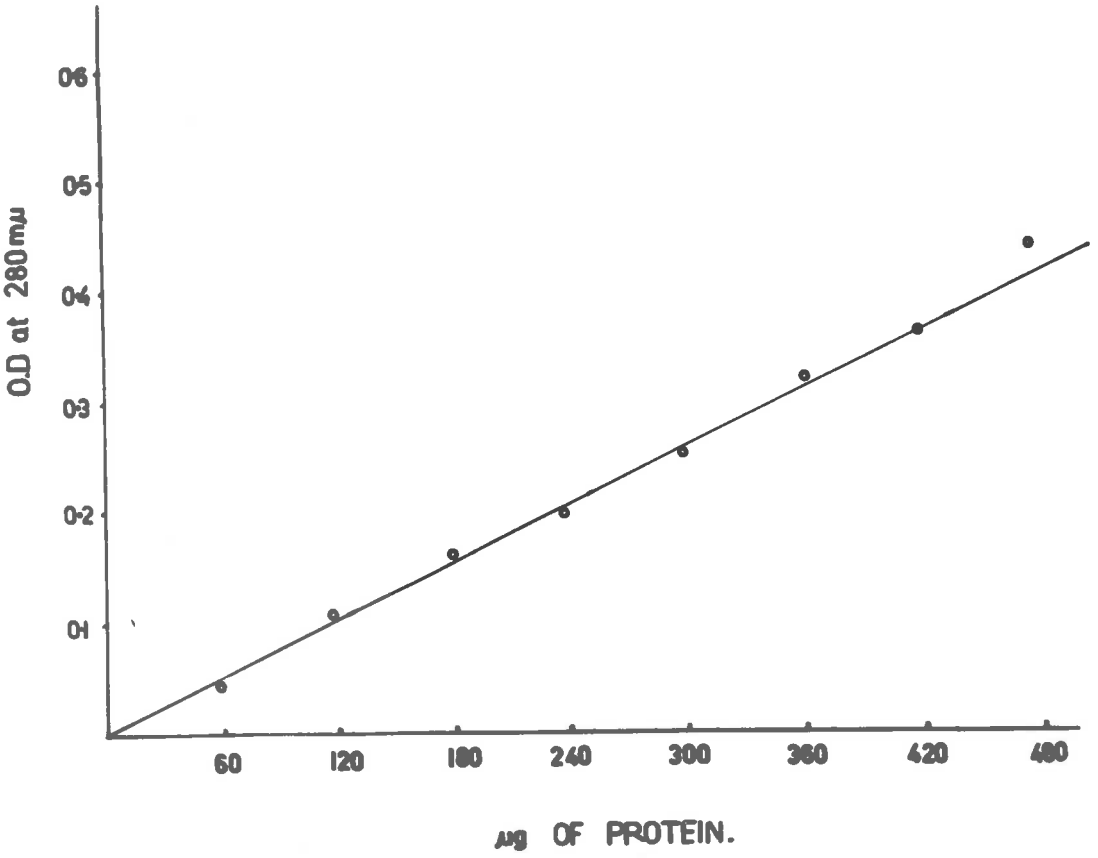
(a) Qualitative analysis - zone electrophoresis of serum proteins.

Samples of serum were analysed for their various serum components by the Spinco model R paper electrophoresis cell, standardised at 150 volts, and 12.5 m.amps. for 18 hours in 0.075M veronal buffer at pH 8.6. The strips were dried in an oven at 120°C for 30 minutes and then stained for 30 minutes in 0.1 per cent. solution of bromphenol blue in methanol. The strips were then washed in 5 per cent. acetic acid until all background staining was removed and finally dried at 120°C for 15 minutes. They were then placed in a closed vessel containing ammonium hydroxide to allow maximum colour development and finally passed through a Spinco Model R.B. analytrol. From the graphs obtained by analysis of the paper strip in the analytrol, the individual serum components were expressed as mg. of protein per ml. of serum.

(b) Quantitative estimation of total serum proteins.

Serum from embryos and adult birds was collected and pooled as previously described. The protein content was determined at 280 mμ. in a Shimadzu Spectrophotometer. Quantitative estimations were made by comparison with a standard protein curve of bovine serum albumin [Commonwealth Serum Lab., Batch No. C-6950000B]. See Fig.5.

Fig.5 The standard protein curve.


O.D at 280mμ
0·6
0·5
0·4
0·3
0·2
0·1
60
120
180
240
300
360
420
480
μg OF PROTEIN.

Fractionation of Adult and Embryo Serum by Ion Exchange Cellulose.

Diethylaminoethyl (DEAE) cellulose obtained from Whatman was pretreated and washed by the method of Levy and Sober (1960). The cellulose, suspended in 0.01M Na_2HPO_4 at pH 8.0 was packed in a column (1 x 15 cm.) with nitrogen at 10 lb./sq. in. It was then washed with 80 ml. of the above buffer, care being taken to prevent the column from drying out. During the course of the experiment the temperature of the column was kept constant at 4°C by circulating cold water through an outer jacket.

Five ml. of serum (adult hen serum and embryo serum contained a total of 172 mg. and 107 mg. protein respectively) that had been dialysed against 500 ml. of the same buffer was then layered on the top of the column. For dialysis, cellulose dialysis tubing type Visking 36, OC pore size 24Å was used at all times. The serum was then eluted off the column by a phosphate buffer gradient utilising a six chambered mixing apparatus as described by Peterson and Sober (1959). In this system the buffers had a decreasing pH from pH 8.0 to pH 5.6 and an increasing molarity from 0.01M to 0.4M. The final traces of protein were eluted with 50 to 80 ml. of 1M or 2M NaH_2PO_4 at pH 5.6. The column eluates were collected in 5 ml. aliquots at a flow-rate of 1 ml. per minute in a fraction collector designed and manufactured by Paton Industries Ltd., South Australia.

Constant volumes were regulated by a photoelectric cell. An air pump exhausted each sample from the collecting cell into acid-cleaned test tubes on a turntable.

The tube contents were assayed for protein by absorption at 280 mμ in the Shimadzu Spectrophotometer. On the basis of the elution pattern, the contents of certain tubes were pooled, dialysed in 0.15 M saline, concentrated by "Carbowax", and redialysed against 0.15 M saline. One ml. of each of the fractions was then assayed for biological activity.

Isolation of High and Low Molecular Weight Opsonins.

One ml. samples of 40, 35, 25 and 10 per cent. sucrose in 1 M NaCl were layered successionally in "Lusteroid" tubes of the S.W./39 Spinco rotor. This discontinuous sucrose gradient, after being allowed to equilibrate for 24 hours at 4°C, was overlayed with 1 ml. of serum. The tubes were centrifuged in a swing-out S.W./39 rotor at 35,000 r.p.m. for 18 hours in a Spinco ultracentrifuge as described by Kunkel, (1960). After centrifugation, each "Lusteroid" tube was capped with a rubber bung bearing a 19 gauge hypodermic needle connected to a piece of rubber tubing. The tube was gently pierced at the bottom with a 25 gauge needle to a distance of about 1 mm. By means of a screw clip attached to the piece of rubber tubing, the flow rate could be regulated to 2 drops/minute. A series of acid cleaned tubes containing 3 ml. of 0.15 M saline

were used to collect the two drop aliquots.

The protein content of each sample was assayed by absorption at 280 mμ. on the Shimadzu Spectrophotometer. On the basis of the sedimentation pattern obtained by plotting the protein content against tube number, the samples were finally pooled to give three main fractions. The samples were concentrated by means of a L.K.B. ultrafilter under vacuum according to the method of Aronsson and Gronwall (1957). After dialysis in 0.15 M saline the concentrates had a final volume of 3 ml. per fraction. The fractions were then assayed for biological activity.

CHAPTER III.

THE BACTERICIDAL PROPERTIES OF SERUM AND EMBRYONIC FLUIDS FROM THE CHICK EMBRYO.

In the study of the host defence mechanisms of the adult animal, there has been a great deal of interest shown in the bactericidal properties of normal serum. Nuttall (1888) showed that the serum from rabbits or dogs was bactericidal for B. anthracis and B. subtilis. Since then there have been numerous reports on the bactericidal properties of serum from a variety of animals against a range of bacteria [Muir, (1909); Mackie and Finkelstein, (1932); Skarnes and Watson, (1957); Wedgwood and Pillemer, (1956); Kent, Stevens and Lawson, (1961); Sterzl, Kostka and Lanc, (1962)].

The bactericidal effect of serum may be attributed in general to the presence of specific antibody and complement. However, under certain conditions such as in the presence of Ethylenediaminetetra-acetic acid (EDTA) and lysozyme (an enzyme present in egg albumin), bacteria may also be lysed [Repaske, (1958)]. In the present study of the functional development of the reticulo-endothelial system of the embryo and its relationship to immunity, it seemed important to investigate at first the bactericidal properties of sera and extra-embryonic fluids at various stages in its development. If such a humoral mechanism as des-

cribed above were present, it could quite well affect the subsequent studies regarding the survival of bacteria following injection into the embryo. Bactericidal tests were carried out using E. coli Lilly, as this strain is extremely sensitive to the bactericidal action of normal adult chicken serum [Ali, (1959)].

The experimental design is given in Chapter II and some of the results obtained are presented in Table I. Neither the serum, nor any of the extra-embryonic fluids showed any bactericidal activity against E. coli Lilly at any of the developmental stages studied, from ten days to almost the time of hatching. Only the data obtained using the highest concentration of the various fluids are presented. The dilutions of fluids assayed were in the range from 1:2 to 1:16.

Although it seemed that the fluids of the embryo were devoid of bactericidal activity, the experiment did not establish whether this lack was due to the absence of antibody, absence of complement, or both. Experiments were designed to test for the presence of these factors in the chick embryo serum.

A serum pool from 20 adult chickens was tested for its titre of specific antibody to E. coli Lilly and the presence of haemolytic complement by the bactericidal test, this being a sensitive method of detecting both these factors. The results in Tables II and III show that the pooled

TABLE I.

THE BACTERICIDAL PROPERTIES OF EMBRYONIC FLUIDS FOR A ROUGH STRAIN OF ESCHERICHIA COLI LILLY.

Test Fluid	Time in Min.	Age of Embryo in Days						
		10	12	14	15	17	19	Control
		Percentage survival of bacteria						
	0	100	100	100	100	100	100	100
Embryo	15	114	97	100	100	105	140	110
Serum	30	128	114	92	105	140	200	120
	60	172	124	110	120	140	240	140
	0	100	100	100	100	100	100	100
Amniotic	15	105	120	110	130	150	140	120
Fluid	30	130	130	100	150	90	150	120
	60	110	120	110	200	120	160	130
	0	100	100	100	100	100	100	100
Allantoic	15	110	120	110	110	90	130	115
Fluid	30	120	170	150	130	140	140	120
	60	140	200	200	130	160	200	150
	0	100	100	100	100	100	100	100
Yolk	15	100	110	110	100	160	100	130
Fluid	30	160	100	100	120	200	110	130
	60	200	140	100	140	200	120	140

TABLE II

BACTERICIDAL ACTIVITY OF SERUM FROM ADULT HENS FOR ESCHERICHIA COLI LILLY.

Time in Min.	Dilution of Serum					
	Neat	1:2	1:4	1:8	1:16	Control
	Percentage survival of bacteria					
0	100	100	100	100	100	100
15	83	100	100	110	120	100
30	10	5	5	105	130	120
60	0	0	0	20	150	125
120	0	0	0	0	550	135
180	0	0	0	0	900	250

TABLE III.

BACTERICIDAL ACTIVITY OF SERUM FROM ONE DAY OLD CHICKENS AND TWENTY DAY OLD EMBRYOS FOR ESCHERICHIA COLI LILLY

Time in Min.	One Day Old Chicken Serum					Embryo Serum	
	Neat	1:2	1:4	1:8	Control	Neat	Control
	Percentage survival of bacteria						
0	100	100	100	100	100	100	100
15	100	110	100	125	100	140	100
30	60	65	65	130	120	200	120
60	20	20	65	135	125	250	125
120	0	30	50	230	135	700	135
180	0	65	150	250	250		250

serum from adult chickens was bactericidal for this organism.

It is interesting to note that serum obtained from one day old chickens possessed a bactericidal system for E. coli Lilly, whereas the serum taken from embryos one day prior to hatching lacked this bactericidal property. The serum of the one day old chicken, however, is not as active as the adult chicken serum.

In view of the results, the presence of complement in the serum from the chick embryo was investigated by the following two methods.

Method of Assay for the Presence of Complement in Embryo Serum.

E. coli Lilly was treated for 60 minutes with adult chicken serum which had been preheated at 57°C for 30 minutes to remove the heat labile component of complement. Following this, the bacteria were washed in 100 ml. of minimal medium, diluted in minimal medium, and finally added in a volume of 0.02 ml. to 1 ml. of embryo serum. This gave between 2×10^3 to 3×10^3 bacteria per ml. Duplicate 0.1 ml. samples were plated out at 15 minute intervals. Control tubes contained 1 ml. of minimal medium instead of embryo serum.

The results presented in Table IV indicate that the bacteria were not killed under these experimental conditions. Since the bacteria were treated with antibody from the adult

TABLE IV.

THE BACTERICIDAL PROPERTIES OF SERUM FROM CHICK EMBRYOS COMPARED WITH IDEAL TREATED SERUM FROM ADULT CHICKENS FOR ESCHERICHIA COLI LILLY.

Experimental Design	Time in Min.				
	0	15	30	45	60
	Percentage survival of bacteria				
Heated Serum plus 13 day Embryo Serum	100	130	140	160	200
Heated Serum plus 19 day Embryo Serum	100	100	150	170	170
Heated Serum plus Minimal Medium	100	100	120	120	150

serum, the failure of the embryo serum to supplement bactericidal activity could not be due to the lack of antibody but to the lack of complement.

Haemolytic Assay for Complement.

In view of the previous results, serum from embryos at different stages in development were examined for their titre of complement. The method followed was essentially that described by Kabat and Mayer (1961). Sheep red cells were washed in saline and sensitised by mixing a 5 per cent. solution of cells with an equal volume containing 10 minimal haemolytic dose of rabbit haemolysin obtained from the Commonwealth Serum Laboratories, Melbourne. The mixture was allowed to incubate for 15 minutes at 37°C and the excess haemolysin removed by centrifugation and washing in saline.

The sensitised cells were mixed with neat serum, incubated at 37°C for 60 minutes and then kept at 4°C for 18 hours. Control tubes included neat adult chicken serum mixed with sensitised cells and saline mixed with sensitised cells. Following incubation in the cold, the tubes were centrifuged at 1500 r.p.m. for 15 minutes and the supernatent read against standards, for percentage lysis. The standards were prepared by mixing varying volumes of 2.5 per cent. normal sheep red cells with distilled water, hence standards representing 10 per cent. to 100 per cent. lysis were obtained. The results are expressed in Table V.

It is clear from these results that whilst under the above experimental conditions embryo serum appeared to lack

haemolytic complement, adult serum possessed this activity. However, the lack of complement may be relative rather than absolute. It was therefore decided to test the presence of complement by the more sensitive test of McGhee (1952).

Accordingly, 14 day old embryo serum was titrated for the presence of complement. Various amounts of serum were mixed with 0.2 ml. of 0.25 per cent. sensitised sheep red cells as described above. The final volume in all tubes was made up to 0.6 ml. by addition of veronal buffer at pH 7.0. The mixtures were incubated at 37°C for one hour and the results scored as positive for lysis and negative for non-lysis. The results in Table Va show that embryo serum does contain small amounts of complement. The amount, however, was not enough for the bactericidal property of embryo serum to be evident.

It is interesting to compare these results with those of Rywooch (1917) who failed to demonstrate the presence of complement before the 21st day of incubation. Sherman (1919) also found that complement activity for sensitised sheep red cells only appeared when the embryo chick was pecking through the shell. Polk, Buddingh and Goodpasteur (1938), testing both serum and extra-embryonic fluids, were unable to demonstrate any complement activity for sensitised sheep red cells. They reported that after hatching, complement is suddenly present and it seemed to increase to a

TABLE V.

THE TITRE OF HAEMOLYTIC COMPLEMENT IN SERUM FROM CHICK EMBRYOS OF DIFFERENT AGES.

Experimental Design	Age of Chick Embryo Serum							
	10	12	14	16	18	19	Adult	Control
	Percentage lysis of red blood cells							
Sensitised Sheep Red Blood Cells Plus Serum	0	0	0	0	0	0	80	0

TABLE Va.

TITRATION OF HAEMOLYTIC COMPLEMENT IN EMBRYO SERUM BY THE METHOD OF McJHEE (1952).

	Tube No.					
	1	2	3	4	5	Control
Vol. of serum/ml.	0.4	0.3	0.2	0.1	0.05	-
Vol. of sensitised cells/ml.	0.2	0.2	0.2	0.2	0.2	0.2
Vol. of buffer/ml.	-	0.1	0.2	0.3	0.35	0.4
14 day old Embryo Serum	+	+	+ -	-	-	-
Adult Chicken Serum	+	+	+	+	+	-

\+ = lysis of red blood cells

\- = no lysis of red blood cells

maximum in the adult fowl. Our present results show that embryo serum does possess small amounts of complement.

Bactericidal Activity of Embryo Serum to E. coli Lilly After Addition of Complement as a Test for Specific Antibody in the Embryo.

Since embryo serum seemed to lack sufficient complement to constitute a bactericidal mechanism, it was decided to supplement this with complement from adult chicken serum. Accordingly, the antibodies present in adult chicken serum to E. coli Lilly were removed by incubating the serum with 10^{10} E. coli Lilly per ml. at $4^{\circ}C$ for 18 hours. This treatment, whilst removing antibody, would retain haemolytic complement. The treated serum was centrifuged at 3000 r.p.m. for 15 minutes to remove the bacteria, and finally sterilised in a Swinny type syringe filter (13 mm. filter with a pore size of 0.45μ, Bedford, Mass., U.S.A. Cat. No. : HAW PO 1300) attached to a Luerloks syringe (Becton, Dikinson and Co., U.S.A., Cat. No. 12505).

The experiment was carried out as presented in Table VI. The inoculation of bacteria into the samples and experimental procedure was the same as described previously. The results are shown in Table VI.

It is obvious from these results that the addition of complement to serum from the embryo constitutes an efficient bactericidal mechanism. The controls did not show any activity except normal serum which retained its bactericidal activity.

TABLE VI.

BACTERICIDAL ACTIVITY OF CHICK EMBRYO SERUM FOR ESCHERICHIA COLI LILLY AFTER THE ADDITION OF COMPLEMENT.

Experimental Design	Time in Minutes				
	0	15	30	60	120
	Percentage survival of bacteria				
0.5 ml. of treated serum + 0.5 ml. of embryo serum	100	85	90	60	50
0.5 ml. of normal serum + 0.5 ml. of embryo serum	100	100	5	0	0
0.5 ml. of treated serum + 0.5 ml. of medium	100	100	115	120	130
0.5 ml. of embryo serum + 0.5 ml. of medium	100	130		190	350
1.0 ml. of medium	100	100	110	140	150

Conclusion.

In this chapter the following conclusions may be reached. The serum and the extra-embryonic fluids are not bactericidal for B. coli Lilly. The failure of embryo serum to kill B. coli Lilly is due to the lack of sufficient complement and not to the lack of antibody. A fully developed bactericidal mechanism is, however, present one day after hatching which suggests that both antibody and sufficient complement are present.

CHAPTER IV.

THE REMOVAL OF BACTERIA BY THE PHAGOCYTIC CELLS OF THE RETICULO-ENDOTHELIAL SYSTEM OF THE CHICK EMBRYO.

It is now well established that the reticulo-endothelial system constitutes one of the most important host defence mechanisms against bacteria. Quantitative studies have shown that any particle injected into the blood stream is selectively removed by the fixed phagocytic cells of such major reticulo-endothelial organs as the liver and spleen [Howard, (1961)].

Whilst a great deal is known regarding the phagocytic capacity of these organs in mature animals, little is known regarding the functional development of this system. Certain evidence would suggest that these phagocytic cells may participate in the events leading to antibody synthesis [Fishmann, (1961)]. Hence, if we are to obtain a better understanding of the ontogeny of the immune response and the animal's ability to differentiate 'self' from 'not-self' it is important that the functional development of the reticulo-endothelial system be explored more fully.

Apart from two early reports [Beard and Beard, (1927); Goodpasture and Anderson, (1937)] little is known about the phagocytic capacity of the reticulo-endothelial system of the chick embryo. These early experiments were of a qualitative, rather than a quantitative, nature, phagocytosis

being measured by the ability of the phagocytic cells to kill bacteria dropped on to the chorio-allantoic membrane.

A study was therefore designed to investigate the phagocytic capacity of the reticulo-endothelial system of the chick embryo and its importance as a host defence mechanism at various stages during development.

Both smooth and rough strains of bacteria and colloidal carbon were employed in blood clearance studies. The dynamics of phagocytosis, the relative phagocytic capacity of the various organs of the chick embryo and the part played by serum factors in phagocytosis have been investigated.

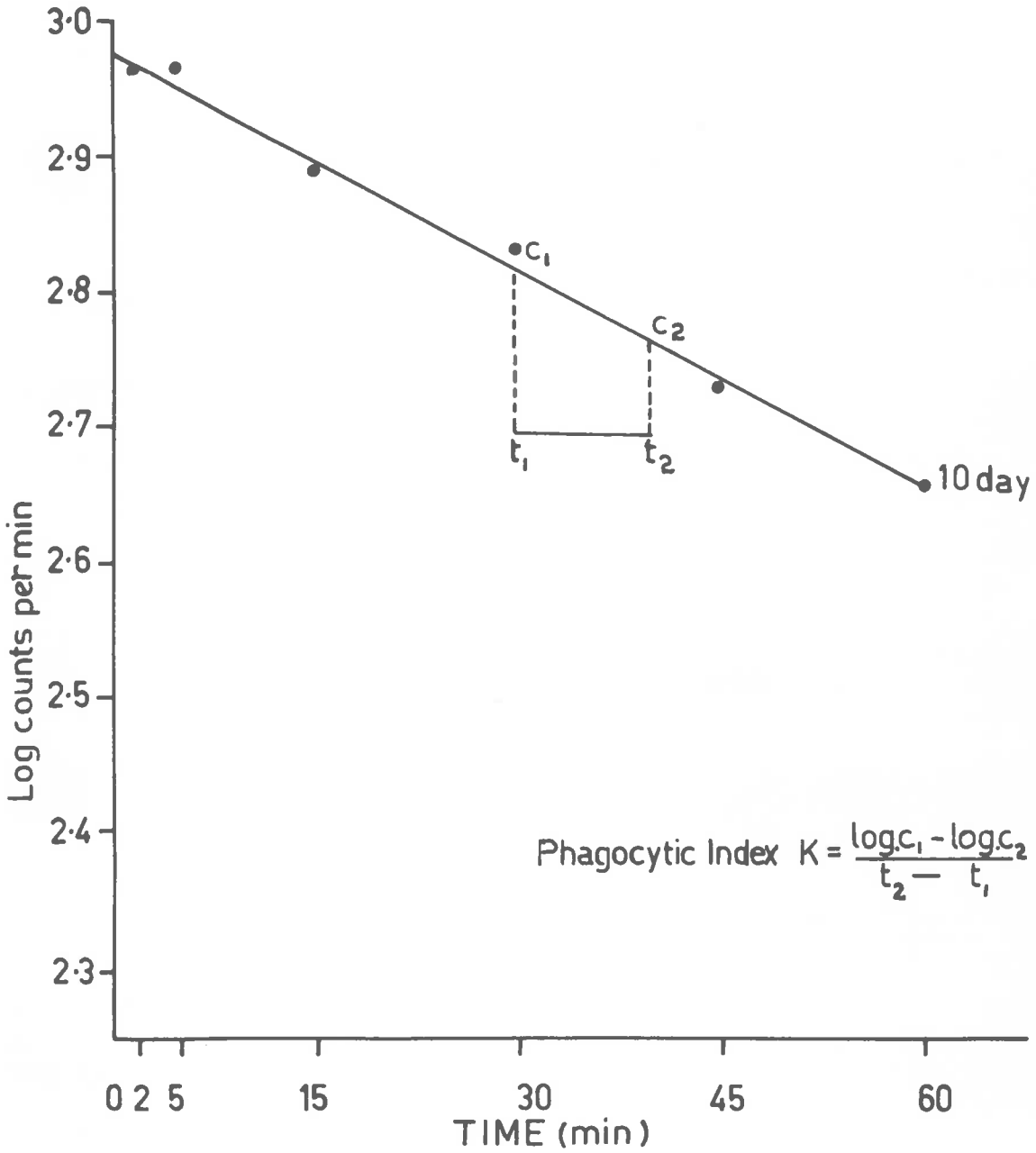
Clearance of P^{32}-labelled E. coli Lilly in the Chick Embryo.

E. coli Lilly was grown in the presence of P^{32} as orthophosphate, washed and standardised. Embryos from 10 to 19 days of development were prepared for clearance studies as previously described. Each embryo was injected with 2×10^8 bacteria suspended in 0.2 ml. of casamino acid medium and 0.02 samples of blood taken from the chorio-allantoic vein were assayed for radioactivity at 2, 5, 15, 30, 45 and 60 minutes following injection.

The results were plotted as the log of the counts per minute against time. A typical curve showing the rate of elimination of bacteria from the circulation is illustrated in Fig.6. It may be seen that the clearance of these particles from the circulation is represented by an exponential curve. The rate of clearance or phagocytic index K may be

Fig.6 Clearance of P^{32}-labelled *E. coli* Lilly in 10 day old embryo.

C1 and C2 = Log concentration of bacteria at time (t_1) and (t_2) respectively.


3·0
2·9
2·8
2·7
2·6
2·5
2·4
2·3
Log counts per min
c_1
c_2
t_1
t_2
10 day
Phagocytic Index $K = \frac{\log c_1 - \log c_2}{t_2 - t_1}$
0 2 5
15
30
45
60
TIME (min)

obtained from the slope of the curve (see Fig.6). It was observed that the clearance from the chick embryo circulation of all particles studied remained exponential until 75 per cent. to 85 per cent. of the particles had been removed. Thus the phagocytic index K is only a measure of the rate of elimination of particles during this initial exponential phase.

The rates of clearance of E. coli Lilly from the circulation of chick embryos at different periods in their development are given in Table VII. Each K value given is the average result obtained from at least 20 embryos. It was found in these experiments that the variation of the phagocytic index K from one egg to another at a particular stage of the incubation was not great and seemed independent of the weight of the egg.

Following each experiment at a certain stage in the development, the embryo was removed from the egg, dried by blotting with filter paper and then weighed. After weighing, the embryo was dissected, the liver and spleen removed and the weights of these organs noted.

The blood volume of the embryo was calculated in the following manner. The amount of radioactivity in terms of counts per minute injected in a constant volume of 0.2 ml. of casamino medium into each embryo was known at the start of the experiment. The exponential clearance curve obtained was extrapolated to zero time and hence one could

determine the extent to which the radioactive material had been diluted. The volume of the injected material multiplied by the dilution factor gave an indication of the blood volume of the chick embryo (Table VII).

In adult animals, where antibody is not the limiting factor in phagocytosis, there is some correlation between the size of the liver and the rate of uptake of the particle by this organ [Benacerraf, (1958)]. Since the size of the liver may be related to the weight of the embryo, a correction can be made to the phagocytic index K, which would allow for this. This corrected phagocytic index a should be constant for individuals of an experimental group, providing that the observed change in the phagocytic index is related entirely to changes in liver size.

It may be seen from the corrected phagocytic index given in Table VII that the observed increase in rates of phagocytosis of E. coli Lilly by chick embryos at different stages in their development might in general be explained by an increase in liver size. However, subsequent results will show that this generalisation is subject to some modification.

Comparison of the Rate of Clearance of E. coli Lilly in 10 Day Old Embryos and 17 Day Old Embryos.

During the course of these experiments, it was noticed that embryos at the 17th day of incubation cleared E. coli Lilly exponentially only during the first 15 minutes

TABLE VII.

CORRECTED PHAGOCYTIC INDEX FOR CHICK EMBRYOS AT VARIOUS STAGES IN THEIR DEVELOPMENT

ESCHERICHIA COLI LILLY USED AS THE TEST ORGANISM.

	Age of Embryo (Days)								
	10	11	12	13	14	15	16	17	18
Weight of embryo (gm)	2	2.2	3.7	4.4	7.7	8.9	10.3	14.5	16
Weight of liver (gm)	0.04	0.05	0.09	0.13	0.18	0.20	0.23	0.40	0.50
Weight of spleen (gm)	0.001	0.001	0.002	0.003	0.004	0.007	0.008	0.012	0.017
Blood volume (ml)	1.5	1.7	2.0	2.7	3.0	3.2	3.5	3.8	4.1
Phagocytic index K	0.006	0.009	0.012	0.014	0.017	0.022	0.031	0.035	0.041
Phagocytic index α	8.8	8.9	9.1	7.9	10.7	11.8	13.5	11.5	10.66

(see Fig.7). Following this exponential phase the amount of radioactivity that could be recovered from the blood showed an increase rather than the expected decline. To explain this, two possibilities were considered. Firstly, the removal of micro-organisms from the blood of the embryo might be due to mechanical trapping of the bacteria in small capillaries, and therefore the rebound observed after 15 minutes of the exponential clearance might be explained by release of bacteria from these sites. A second explanation could be that the increase in radioactivity in the blood after 15 minutes may be due to the labelled products of the digested bacteria being passed back into the circulation from the phagocytic cells of the reticulo-endothelial system.

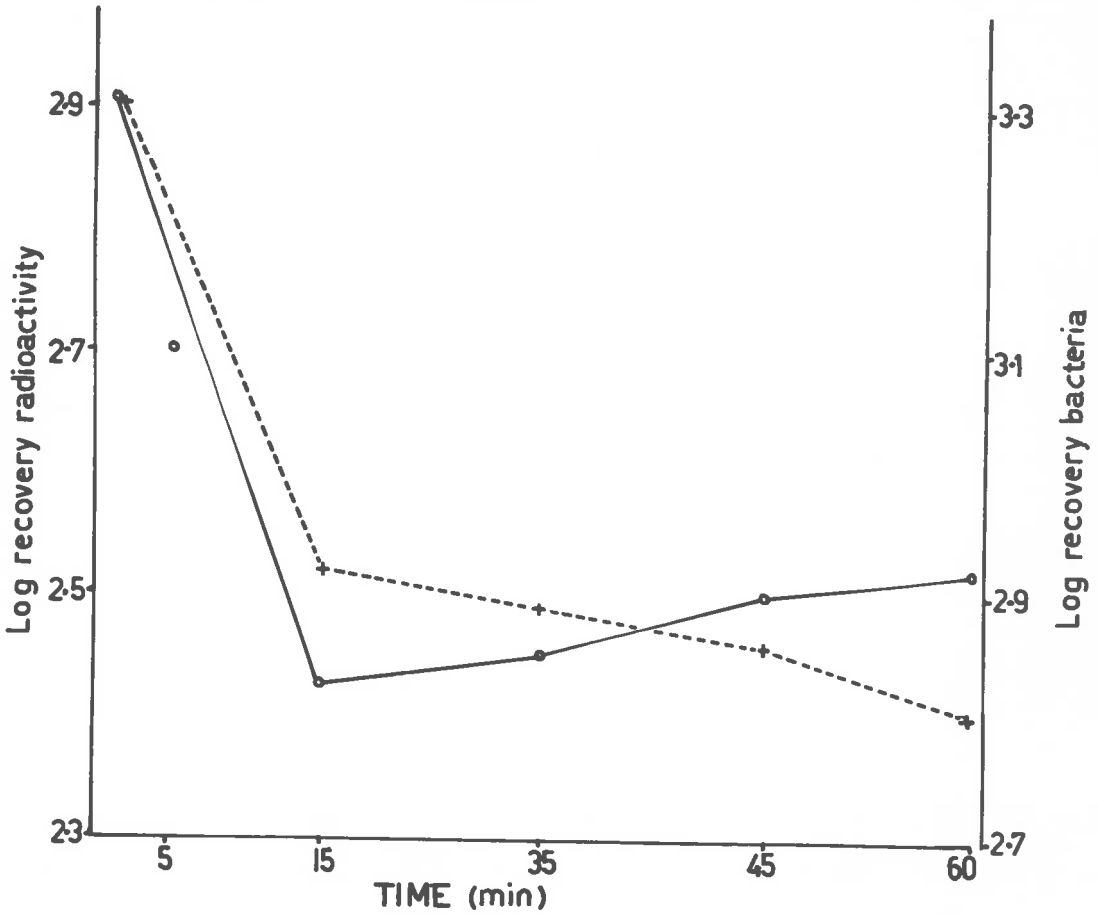
To obtain an answer to these two possibilities, an experiment was designed in which the number of living bacteria and the amount of radioactivity in the circulation was compared at the same times.

Labelled E. coli Lilly (2×10^8 organisms in 0.2 ml. of casamino acid medium) was injected intravenously and at time intervals 0.05 ml. samples of blood were taken for assay. The blood was then treated as follows - 0.02 ml. sample of the blood was pipetted on to a planchette for assay of radioactivity, and a further 0.02 ml. diluted in 100 ml. of medium and duplicate 0.1 ml. samples of the dilution spread out on to nutrient agar to determine the viable count.

Fig.7 The rate of clearance of viable labelled E. coli Lilly as compared with the amount of radioactivity in the circulation of the 17 day old chick embryo.

x---x Recovery of viable bacteria.

o___o Recovery of radioactivity.


Log recovery radioactivity
2·9
2·7
2·5
2·3
Log recovery bacteria
3·3
3·1
2·9
2·7
5
15
35
45
60
TIME (min)

The results illustrated in Fig.7 suggested that the increase in radioactivity following the initial fall is not due to re-entry of viable bacteria into the circulation. Further data to be discussed later would suggest that the material is released from cells of the reticulo-endothelial system following the ingestion of the labelled bacteria.

Clearance of Salmonella gallinarum 98 as Compared with E. coli Lilly.

Since the rate of clearance of E. coli Lilly increased with the age of the embryo (see Table VII), it was interesting to compare this rate with that of another enteric strain of bacterium, namely Salmonella gallinarum 98. Chick embryos at different stages in their incubation were injected as described for E. coli Lilly and blood samples collected at time intervals and assayed for radioactivity.

The results of these experiments are given in Table VIII, and may be compared with the rates of clearance of E. coli Lilly in embryos of the same age group. These results, which are the average results from six embryos at each stage of incubation, show that while E. coli Lilly was cleared progressively faster as the embryo matured (from K = 0.009 in the 11 day old embryo to K = 0.041 in the 18 day old embryo) there was little enhanced clearance of S. gallinarum 98. It was apparent from these observations that different strains of bacteria are treated quite differently by the developing embryo. In view of these findings, a survey of the rates of clearance of a limited number

TABLE VIII.

RATES OF CLEARANCE OF BACTERIA (EXPRESSED AS THE PHAGOCYTIC INDEX K) FROM THE CIRCULATION OF CHICK EMBRYOS.

Test Bacteria	Days of Incubation								
	10	11	12	13	14	15	16	17	18
	Phagocytic Index K								
E. coli Lilly	0.006	0.009	0.012	0.014	0.017	0.022	0.031	0.035	0.041
E. coli K12			0.011		0.018		0.053		
S. gallinarum 98		0.003	0.004	0.003	0.002	0.003	0.003	0.002	0.002
S. gallinarum 9240			0.002		0.004			0.002	
E. coli 2206			0.003		0.001			0.002	

of bacteria was carried out.

The Rate of Phagocytosis of Various Strains of E. coli and S. gallinarum by the Developing Chick Embryo.

Two further strains of E. coli (one rough strain E. coli K12 and one smooth strain, E. coli 2206) and one strain of S. gallinarum (S. gallinarum 9240) were labelled with P^{32} and standardised as above. The rate of elimination of these strains of bacteria from the circulation of the chick embryo was followed. The results of these studies are given in Table VIII and compared with the results observed for E. coli Lilly and S. gallinarum 95. Each phagocytic index given is the average result from six embryos at each stage of incubation.

In cases where the phagocytic index was greater than K = 0.010, the actual variation between individual embryos was not greater than $\pm$ 0.002. In experiments where bacteria were cleared slowly with K values of K = 0.003 the variation was $\pm$ 0.001.

The most interesting feature of these results is the finding that rough strains of bacteria such as E. coli Lilly and E. coli K12 are cleared progressively faster during the development of the chick embryo, whereas the smooth strains are phagocytosed extremely poorly.

CHAPTER V.

THE ROLE OF OPSONINS IN THE CLEARANCE OF BACTERIA FROM THE CIRCULATION OF THE CHICK EMBRYO.

In the preceding chapter, it was found that rough strains of bacteria were removed from the circulation of the chick embryo whilst smooth strains were removed by comparison extremely slowly. From our knowledge of the phagocytosis of bacteria by the reticulo-endothelial cells *in vivo*, and the *in vitro* studies on the uptake of bacteria by cells such as the mouse peritoneal macrophages and polymorphonuclear leucocytes, the above observation could be interpreted to mean that the chick embryo possesses antibody against rough strains of bacteria but not against smooth strains (see Chapter I). Recently, Šterzl (1963) has suggested from liver perfusion studies in the rat, that rough strains of bacteria may be phagocytosed in the absence of antibody. However, initial studies on the bactericidal properties of chick serum had shown that the serum from the chick embryo did possess antibody against *E. coli* Lilly (see Chapter III). It was therefore of some importance to determine whether in the present system, antibody was the main factor in determining the rate of removal of these two strains of *E. coli*.

Several workers have studied the kinetics of phagocytosis of various particles from the circulating blood of

adult animals. If animals are injected intravenously with a large dose of certain particles, then some time later this is followed by a similar injection of a smaller dose of the same particles, this second dose is removed slowly by the phagocytic cells of the reticulo-endothelial system. The term "blockade" has been used to describe the failure of the phagocytic cells to remove the second dose of particles. In the adult rat, the phagocytic capacity of the cells returns to normal after three days [Benacerraf, (1958)]. However, if the second dose is opsonised in vitro, then these treated particles are cleared almost as rapidly as if the animal had not been injected with the first blockading dose [Jenkin and Rowley, (1961); Murray, (1963); Miler, (1963)]. Jenkin and Rowley, (1961) suggested that the initial injection of the large dose of particles had depleted the animal of circulating opsonins for that particle and that a later dose was not removed because of the lack of serum factors. This is in contrast to Biozzi, Benacerraf and Halpern, (1953) who suggested that the phagocytic cells had become saturated and were thus unable to ingest the second dose. Using this technique of reticulo-endothelial blockade, the factors responsible for the uptake of the two rough strains of bacteria were studied.

Experiments Designed to "Blockade" the Reticulo-Endothelial System of the Chick Embryo.

In these experiments blockade was induced by the in-

jection of a suspension of unlabelled bacteria. The effectiveness of the blockade of the reticulo-endothelial system was tested by examining the clearance of the same strain of radioactive P^{32}-labelled bacteria. The degree of inhibition in the clearance rate of the labelled dose of bacteria was a measure of the blockade. In all instances the total volume of both injections was 0.2 ml. All experiments were carried out on 14 day old embryos. Control, unblockaded embryos were also tested in each experiment with isotopically labelled E. coli Lilly to verify that they behaved normally with respect to clearance. Casamino acid medium injected controls were included to verify that prior injection of the diluent fluid had no adverse effect on normal clearance.

Titration to Determine the Dose of Bacteria Required to Induce Blockade.

Doses of 10^8, 2×10^8, and 4×10^8 E. coli Lilly suspended in 0.1 ml. of casamino medium, were injected into 14 day old embryos, six embryos being used for each dose tested. Sixty minutes later, the clearance rate of 2×10^8 labelled E. coli Lilly was followed by the usual method. Embryos injected with 0.1 ml. of casamino acid medium served as controls to ensure that this diluent fluid produced no changes in the normal rates of clearance. As an additional control, embryos injected with P^{32}-labelled bacteria were used to verify that the normal clearance rate was within the limits that previous study had shown (see Chapter IV).

The results in Table IX show that the degree of blockade was dose dependent. As there was no significant difference between 2 x 10^8 and 4 x 10^8 blockading doses, the former was used as the standard amount injected in all subsequent experiments.

Duration of the Period of Blockade.

This experiment was designed to investigate the length of time during which blockade in the embryo persisted. Previous experiments by Jenkin and Rowley, (1961) had shown that in the adult mice the blockade produced by bacteria rapidly returned to normal. It was important for subsequent experiments that blockade was effective during the experiment. Embryos were injected with 2 x 10^8 non-labelled bacteria and, at 30, 60 and 90 minutes following injection, the clearance of 2 x 10^8 labelled bacteria was followed. Controls, as described in previous experiments, were included.

The results in Table X show that from 30 to 150 minutes after the primary injection the phagocytic capacity of the cells of the reticulo-endothelial system in the chick embryo was considerably reduced with regard to E. coli Lilly. The 60 minute period has been used as the standard for subsequent experiments.

The Rate of Clearance of E. coli Lilly in Blockaded Chick Embryos.

If 'blockade' of the reticulo-endothelial system can

TABLE II.

TITRATION TO DETERMINE THE DOSE OF BACTERIA REQUIRED TO INDUCE BLOCKADE.

Blockading Dose	Test Bacteria	Phagocytic Index K
Uninjected Controls	E. coli Lilly	0.017
0.1 ml. Casamino med.	E. coli Lilly	0.017
0.1 ml. 10^9 E.coli Lilly	E. coli Lilly	0.008
0.1 ml. 2 x 10^8 E. coli Lilly	E. coli Lilly	0.004
0.1 ml. 4 x 10^8 E. coli Lilly	E. coli Lilly	0.003

TABLE X.

DURATION OF RETICULO-ENDOTHELIAL BLOCKADE IN THE CHICK EMBRYO FOLLOWING THE PRIMARY INJECTION OF 2×10^8 E. COLI LILLY AS INDICATED BY THE RATE OF CLEARANCE OF A SECOND DOSE OF ISOTOPICALLY LABELLED E. COLI LILLY.

Substance Used for Blockade	Time of Injection of Second Dose of Labelled E. coli Lilly	Phagocytic Index K
No Treatment	0 mins.	0.016
0.1 ml. Casamino med.	30 mins.	0.017
0.1 ml. Casamino med.	60 mins.	0.016
0.1 ml. Casamino med.	90 mins.	0.015
0.1 ml. 2×10^8 E. coli Lilly	30 mins.	0.005
0.1 ml. 2×10^8 E. coli Lilly	60 mins.	0.005
0.1 ml. 2×10^8 E. coli Lilly	90 mins.	0.005

be explained in terms of depletion of opsonins, then a second dose of bacteria that have been opsonised *in vitro* should be cleared more rapidly from the circulation than unopsonised bacteria. However, if the opsonised bacteria are not cleared more rapidly, then blockade and clearance of these rough strains of bacteria have to be explained in terms other than depletion of opsonins. Sixty minutes after the blockading dose of 2×10^8 *E. coli* Lilly, the clearance of opsonised labelled *E. coli* Lilly was followed. The source of opsonins was either adult chicken serum, or 19 day old embryo serum. Controls, as described in the previous experiment, were included.

The results in Table XI show that the opsonised bacteria were cleared in the blockaded embryos at a rate similar to the rate clearance of unopsonised bacteria from the circulation of embryos that had not been blockaded. However, the disappearance of unopsonised bacteria from the blood of the blockaded embryos proceeded much more slowly. These results suggest that the uptake of rough strains of bacteria by the phagocytic cells of the reticulo-endothelial system was dependent on the presence of antibody (opsonin). When antibody is limiting the reaction between phagocytic cell and bacteria, the organisms are phagocytosed extremely poorly. This is in keeping with the observations of most other workers.

TABLE XI.

RATE OF CLEARANCE OF B. COLI LILLY IN BLOCKADED CHICK EMBRYO.

Material Used in Blockade	Treatment of second dose of B. coli Lilly	Phagocytic Index K
None	Unopsonised	0.017
0.1 ml. of Casamino med.	Unopsonised	0.017
B. coli Lilly 2×10^8	Unopsonised	0.005
B. coli Lilly 2×10^8	Opsonised with adult chicken serum	0.018
B. coli Lilly 2×10^8	Opsonised with 19 day old embryo serum	0.015

The Specificity of Opsonins in the Chick Embryo for Rough Strains of Bacteria.

It has been shown that rough strains of E. coli are cleared rapidly from the blood of chick embryo. However, it is not apparent from these studies whether the opsonins for E. coli Lilly and those for E. coli K12 are specific for the respective strains of bacteria or directed against common antigens shared by both.

In the following experiments the 14 day chick embryo was blockaded with E. coli K12 and S. gallinarum 98. It was reasoned that if a depression in the rate of clearance of E. coli Lilly after blockade with E. coli K12 was apparent, then this would suggest that the chick embryo possessed common opsonins for both strains. If, however, the clearance rate of E. coli Lilly was not altered, then it is likely that the major portion of the antibody against these two strains was different. S. gallinarum 98 was injected into the chick embryo as a blockading agent to serve as a control. This strain of bacteria had a very low clearance rate and therefore was unlikely to compete with E. coli Lilly for available opsonins.

In the experiment non-radioactive E. coli K12 and S. gallinarum 98 were prepared as described and 2 x 10^8 suspension of bacteria was injected as a blockading dose. The rate of clearance of labelled E. coli Lilly was then followed. The results in Table XII show that when the

blockade was initiated by E. coli K12, a subsequent dose of E. coli Lilly was cleared more slowly than in control of unblockaded embryos. However, blockade could not be achieved by the primary injection of a smooth strain of bacteria such as S. gallinarum 98. Opsonisation of E. coli Lilly with adult chicken serum resulted in a normal rate of clearance of this organism from the circulation of chick embryo blockaded with E. coli K12. (See Table XII).

These findings indicate that, since it was possible to deplete the embryo of opsonins for E. coli Lilly by prior injection of a blockading dose of E. coli K12, both these strains have common antigens against which the major portion of the antibody found in the chick embryo is directed. The evidence that E. coli Lilly opsonised *in vitro* is cleared after blockading the embryo with E. coli K12 also substantiates the above statement. It is also apparent that S. gallinarum 98 does not compete in any way with the antibody directed against E. coli Lilly or E. coli K12. In this system, reticulo-endothelial blockade cannot be due to the saturation of the phagocytic cells at the dose levels employed in these experiments.

Effect of Treating the Rough Strains of E. coli Lilly with Various Opsonic Sera and its Subsequent Clearance by the Reticulo-Endothelial System of the Chick Embryo.

The previous experiments showed that E. coli Lilly was cleared progressively faster as the age of the embryo increased. It has also been demonstrated that this increase

TABLE XII.

SPECIFICITY OF OPSONINS IN THE CHICK EMBRYO FOR ROUGH STRAINS OF B. COLI EMBRYOS WERE BLOCKADED WITH 2×10^8 BACTERIA.

Material Used for Blockade	Test Organism	Phagocytic Index K
No treatment	B. coli Lilly	0.018
B. coli Lilly	B. coli Lilly	0.004
B. coli K12	B. coli Lilly	0.009
B. gallinarum 95	B. coli Lilly	0.016
B. coli K12	B. coli Lilly opsonised with adult chicken serum	0.018

in the rate of phagocytosis seems to correlate in part with the increase in size of the liver. However, since the rate of phagocytosis in mature animals may be controlled within certain limits by the titre of antibody, it was conceivable that the observed rates of clearance of E. coli Lilly in the chick embryo did not represent the maximum phagocytic efficiency of the reticulo-endothelial system.

E. coli Lilly was opsonised in vitro with serum from 17 day old and 20 day old embryos, and also with pig and adult chicken serum. The latter two adult sera had been shown previously to be highly opsonic for a number of species of bacteria [Jenkin and Rowley, (1961)]. The rates of phagocytosis of the treated bacteria were compared with the rates of phagocytosis observed using unopsonised bacteria. Embryos at various stages in their incubation were tested and since in all age groups the results were essentially similar, i.e. opsonisation did not increase the rate of phagocytosis of this strain, only the results obtained with 13 day old embryo are given in Table XIII. In view of the enhanced clearance in blockaded embryos following opsonisation with adult chicken serum (Table XI), one may conclude that the phagocytosis of this strain of bacteria by the chick embryos is not limited by the titre of opsonins, but by the number of phagocytic cells.

Effects of Opsonising "Smooth" Strains of Bacteria.

In the adult animal, when bacteria are cleared at a

TABLE XIII.

EFFECT OF TREATING THE ROUGH STRAINS OF E. COLI LILLY WITH VARIOUS OPSONIC SERUM AND ITS SUBSEQUENT CLEARANCE BY THE RETICULO-ENDOTHELIAL SYSTEM OF THE CHICK EMBRYO.

Strain of Bacteria	Serum Used for Opsonisation	Phagocytic Index K
E. coli Lilly	None	0.014
E. coli Lilly	17 day old embryo serum	0.016
E. coli Lilly	19 day old embryo serum	0.015
E. coli Lilly	Pig serum	0.014
E. coli Lilly	Adult chicken serum	0.014

very slow rate from the blood circulation, it has been possible to enhance phagocytosis by treating the bacteria *in vitro* with antibody before injection [Jenkin and Rowley, (1961); Biozzi and Stiffel, (1961)]. It has been shown (Chapter IV) that smooth strains of bacteria are eliminated very slowly from the circulation after intravenous injection.

Isotopically labelled smooth strains of bacteria, *S. gallinarum* 98, *S. gallinarum* 9240, and *E. coli* 2206, were opsonised with adult chicken or pig serum and their clearance from the blood of the chick embryo at various stages in development followed. The labelled bacteria were opsonised and injected at a concentration of 2×10^8 organisms in 0.2 ml. of casamino acid medium.

The results given in Table XIV indicate that the rate of phagocytosis of smooth strains of bacteria by the chick embryo was limited by the titre of antibody.

If these opsonins are made available to the bacteria prior to injection, then the rates of phagocytosis of the smooth strains of bacteria are similar to those observed for unopsonised rough strains.

The Phagocytosis of Colloidal Carbon by the Developing Chick Embryo.

It has been suggested recently that the phagocytosis of carbon by the reticulo-endothelial system is dependent on the presence of serum opsonins, [Jenkin and Rowley, (1961)].

TABLE XIV.

THE RATE OF PHAGOCYTOSIS OF SMOOTH STRAINS OF BACTERIA IN CHICK EMBRYO FOLLOWING OPSONISATION WITH VARIOUS ANIMAL SERA.

Strain of Bacteria	Serum Used for Opsonins	Age of Embryos in Days				
		11	12	14	17	18
		Phagocytic Index K				
S. gallinarum 98	None	0.003	0.004	0.002	0.003	0.003
	Pig serum	0.009		0.026	0.046	
	Adult chicken serum		0.014	0.018	0.035	0.045
S. gallinarum 9240	None		0.002	0.004	0.002	
	Pig serum		0.010	0.016	0.038	
	Adult chicken serum		0.014	0.026		
E. coli 2240	None		0.003	0.001	0.002	
	Pig serum		0.006	0.019	0.036	
	Adult chicken serum		0.005	0.017	0.036	

Indeed it would appear that a variety of non-viable particles such as bentonite and starch require the presence of certain serum factors before they are ingested by phagocytic cells, [Potter and Stollerman, (1961)]. In this study, various doses of carbon were injected intravenously into 12 day and 17 day old embryos. The clearance of carbon was measured as described in Chapter II. The results shown in Table XV indicate that, though carbon was phagocytosed by the 12 day old embryo, the rate of clearance was of a low order and was independent of the doses injected. However, in the older 17 day embryos the rate of removal of carbon was more rapid and showed a similar dose relationship to that observed in the adult animal [Biozzi et al., (1953)].

The Effect of the Dose on the Rate of Clearance of Bacteria from the Circulation of the Chick Embryo.

Since it was apparent that the clearance of carbon in the 17 day old embryo was dose dependent, it was of interest to see if the clearance of bacteria was also dependent on the number of organisms injected.

The results given in Table XVI show that the rate of clearance of bacteria, unlike carbon, was independent of the dose of bacteria injected over the range tested.

Distribution of Radioactive Label Following Clearance of Isotopically Labelled Bacteria from the Circulation of the Developing Chick Embryo.

The clearance of isotopically labelled bacteria was

TABLE XV.

RATE OF CLEARANCE OF CARBON FROM THE CIRCULATION OF CHICK EMBRYOS OF DIFFERENT AGES.

Age of Embryo in Days	Wt. of Carbon Injected (mg.)	Phagocytic Index K
12	3.2	0.008
	1.6	0.008
	0.8	0.009
	0.4	0.009
17	3.2	0.007
	1.6	0.017
	0.8	0.025

TABLE XVI.

EFFECT OF DOSE ON THE RATE OF CLEARANCE OF BACTERIA IN THE 15 DAY OLD EMBRYO.

Strain of Bacteria	Dose Injected	Phagocytic Index K
E. coli Lilly	2×10^8	0.022
	2×10^7	0.021
	2×10^6	0.023
S. gallinarum 9S	2×10^8	0.003
	2×10^7	0.003

followed over a period of 60 minutes. The embryos were then perfused as previously described (Chapter II) with warm saline via the chorio-allantoic vein until the effluent fluid appeared free from the blood. The perfused embryo, its liver, spleen and membranes were digested with sodium hydroxide and the radioactivity associated with each expressed as a percentage of the injected dose.

The results illustrated in Fig.8 show that, during the early stages of incubation, the extra-embryonic membranes of the embryo account for a high percentage of the material phagocytosed. The importance of these structures, as a phagocytic system, decreases toward the time of hatching, when the liver is the most important organ of phagocytosis.

Since S. gallinarum 98 is cleared only very slowly under normal conditions, the distribution of the label was determined following opsonisation of this strain with adult chicken serum. A detailed analysis of the distribution of the radioactivity in embryos at various ages is given in Table XVII and Table XVIII. It is clear that when S. gallinarum is opsonised, the sites of phagocytosis are similar to that of E. coli Lilly.

Conclusion.

It was clear from these studies that rough and smooth strains of bacteria were only phagocytosed in the presence of opsonins. The chick embryo possessed opsonins for the

Fig.8 The percentage recovery of radioactivity from various organs of the developing chick embryo.

o___o Total recovery.

o___o Membrane recovery.

+___+ Liver recovery.

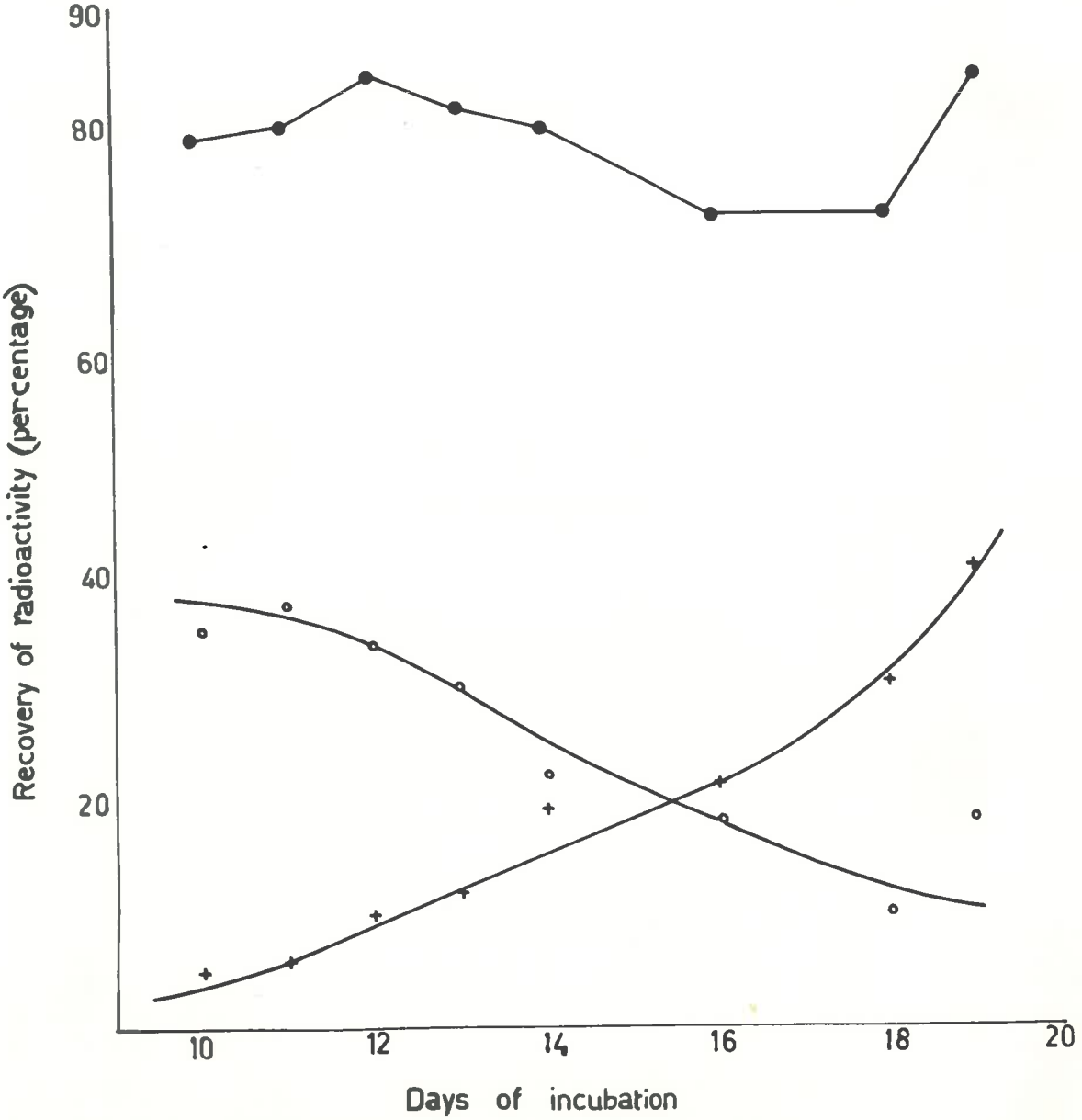

90
80
60
40
20
Recovery of radioactivity (percentage)
10
12
14
16
18
20
Days of incubation

TABLE XVII.

THE RECOVERY OF RADIOACTIVITY FROM VARIOUS ORGANS OF THE CHICK EMBRYO FOLLOWING INJECTION WITH E. COLI LILLY.

Age of Embryo	Distribution of Label					Total Percentage Recovery
	Blood	Liver	Spleen	Membrane	Embryo	
	Percentage Recovery of Radioactivity					
10	30	5	0.5	35	8	78
11	30	6	0.5	36	7	79
12	29	10	0.5	34	11	84
13	26	12	1.0	30	12	81
14	25	19	1.0	22	12	79
16	20	21	2	18	10	71
18	15	30	2	10	15	72
19	15	40	3	18	17	93

TABLE XVIII.

THE RECOVERY OF RADIOACTIVITY FROM VARIOUS ORGANS OF THE CHICK EMBRYO, FOLLOWING INJECTION WITH S. GALLINARUM 9S OPSONISED WITH ADULT CHICKEN SERUM.

Age of Embryo	Distribution of Label					Total Percentage Recovery
	Blood	Liver	Spleen	Membrane	Embryo	
	Percentage Recovery of Radioactivity					
12	44	4	1	26	8	83
14	37	10	-	17	6	70
17	20	35	3	5	10	73

rough strains but not for the smooth strains.

During the early stages of development of the chick embryo, the phagocytic cells localised in the membrane played a major role in engulfing bacteria. As the embryo matured, the liver became a more important organ of phagocytosis.

CHAPTER VI.

DEVELOPMENT OF BACTERICIDAL CAPACITY OF THE PHAGOCYTIC CELLS OF THE EMBRYO.

It seems clear that while opsonins are needed to facilitate the uptake of particles by phagocytic cells, they may also play an important part in determining the rate of killing of bacteria following ingestion [Jenkin, (1963)]. Although it has been shown that phagocytic cells of many adult animals possess the ability to kill a great number of bacteria in the presence of opsonins, little is known regarding the nature of these intracellular bactericidal mechanisms of phagocytic cells nor the development of this potential. Finally, it is not known what changes occur in the resistance of the embryo against bacterial infection as it passes from the embryonic stage to the neonate and adult life.

The Fate of Living E. coli Lilly Following Intravenous Injection Into the Chick Embryo at Different Stages in its Development.

A volume of 0.2 ml. containing 3 x 10^3 log phase bacteria was injected into the chorio-allantoic vein. At various time intervals, two embryos from the experimental series were treated as follows. A sample of 0.3 ml. of blood was withdrawn and duplicate 0.1 ml. samples plated on to nutrient agar. The embryos were then perfused with 10 ml. of warm

saline until the perfusate appeared free of blood. The liver, spleen and membranes were removed by dissection, homogenised and duplicate 0.1 ml. samples plated to determine the number of viable bacteria. The volume of the homogenised organs was measured so that the total number of bacteria in the various organs could be calculated.

Tables XIX and XX show the fate and distribution of the bacteria at different stages in development of the embryo. The importance of the membranes as a phagocytic organ is evident and the increasing phagocytic capacity of the liver as the embryo matures is also apparent. The results in Fig. 9, where the total recovery of viable bacteria is plotted against time, suggest that in the younger embryos (11th and 13th day) very little killing occurs during the first sixty minutes following injection. After this the rate of multiplication of bacteria exceeds the rate of killing. In the older embryos (15th and 17th day) ingested bacteria continue to be killed over the time period studied.

This experiment was repeated using S. gallinarum 98. Since this organism is removed very slowly from the circulation of embryos at all ages, phagocytosis being limited by the absence of specific antibody, it was apparent that unopsonised bacteria would not measure the bactericidal potential of the cells of the reticulo-endothelial system for this particular strain of organisms. Thus the fate of S. gallinarum 98 in the chick embryo was followed after

TABLE XIX.

THE RATE OF LIVING E. COLI LILLY FOLLOWING INTRAVENOUS INJECTION IN THE CHICK EMBRYO AT DIFFERENT STAGES IN ITS DEVELOPMENT.

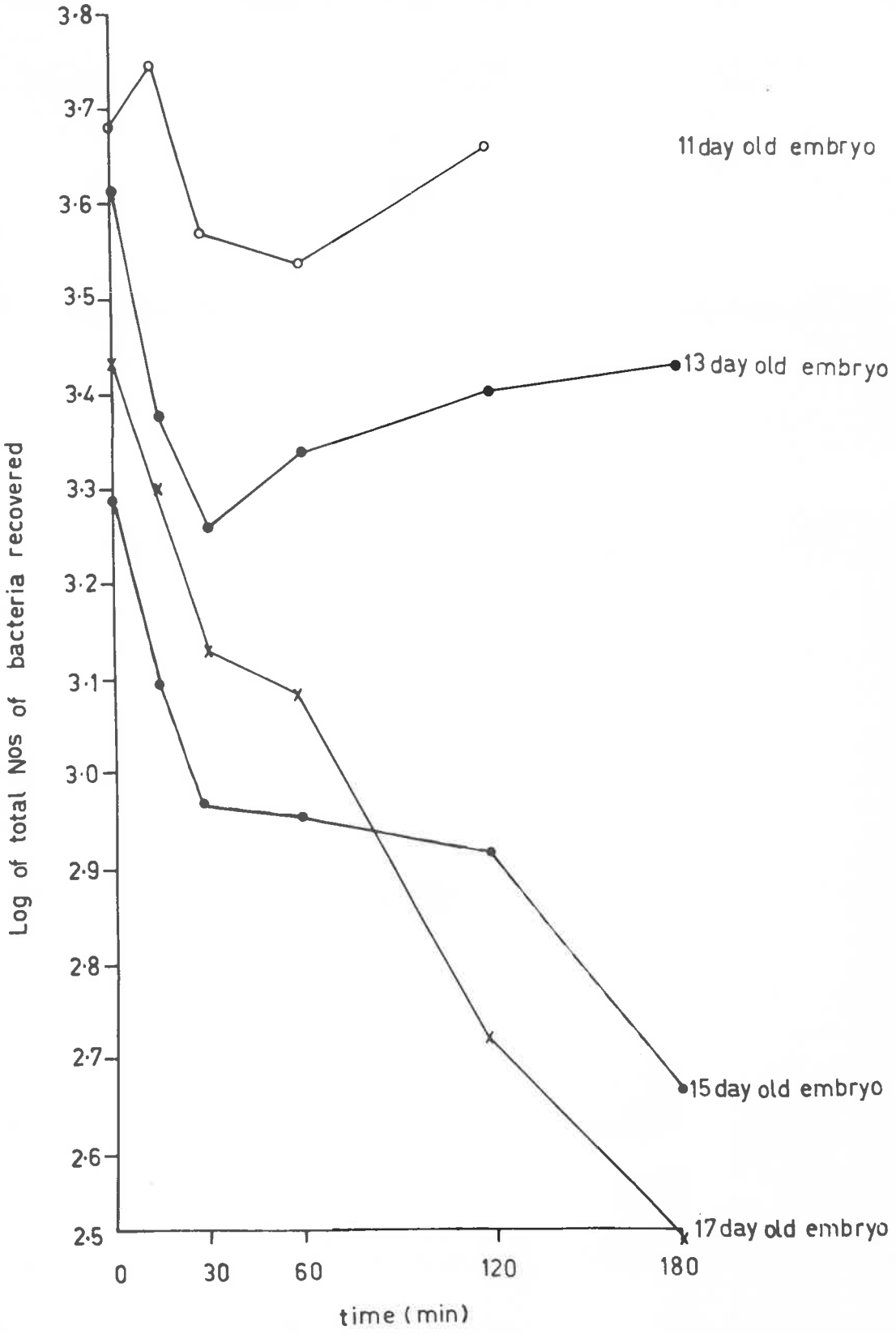
Age of Embryo	Time of Sampling	Distribution of Bacteria			Total Recovery of Bacteria
		Liver and Spleen	Membrane	Blood	
		Number of Viable Bacteria			
11 day	0	75	232	4575	4882
	15	665	452	4400	5577
	30	740	1168	1745	3653
	60	1070	845	1485	3400
	120	1335	955	2200	4490
13 day	0	167	184	3780	4131
	15	842	546	972	2360
	30	722	312	769	1803
	60	737	265	1134	2146
	120	1072	608	810	2490
	180	790	570	1323	2683

TABLE XX.

THE FATE OF LIVING E. COLI LILLY FOLLOWING INTRAVENOUS INJECTION IN THE CHICK EMBRYO AT DIFFERENT STAGES IN ITS DEVELOPMENT.

Age of Embryo	Time of Sampling	Distribution of Bacteria			Total Recovery of Bacteria
		Liver and Spleen	Membrane	Blood	
		Number of Viable Bacteria			
15 day	0	285	164	1500	1449
	15	800	234	195	1229
	30	466	222	225	912
	60	590	186	120	896
	120	615	132	75	822
	180	340	47	75	462
17 day	0	130	47	2528	2695
	15	806	62	128	996
	30	1157	110	64	1331
	60	1085	93	32	1210
	120	449	42	32	523
	180	264	59	-	323

Fig.9 The fate of living E. coli Lilly following intravenous injection into chick embryo at different stages in their development.


3·8
3·7
3·6
3·5
3·4
3·3
3·2
3·1
3·0
2·9
2·8
2·7
2·6
2·5
Log of total Nos of bacteria recovered
0
30
60
120
180
time (min)
11 day old embryo
13 day old embryo
15 day old embryo
17 day old embryo

opsonisation of the bacteria *in vitro* with adult chicken serum.

Only two age groups were studied, the 12 day old and 17 day old embryo, since from the previous studies with *E. coli* Lilly the greatest differences in the rate of killing were revealed at these ages.

The results of this experiment are given in Table XXI and the total recovery of bacteria plotted against time illustrated in Fig.10.

It is apparent that the bactericidal capacity of the phagocytic cells of the reticulo-endothelial system of the chick embryo at any one age varies according to the type of organism tested. In the 17 day old embryo *E. coli* Lilly was killed over a three hour period, whereas the opsonised *S. gallinarum* 98 showed only an initial slight decrease in the first 35 minutes. This was followed by a steady increase in numbers.

The differences between the rate of killing of *E. coli* Lilly and *S. gallinarum* 98 will be discussed more fully later.

The Virulence of *E. coli* Lilly and *S. gallinarum* 98 for Chick Embryos at Different Ages.

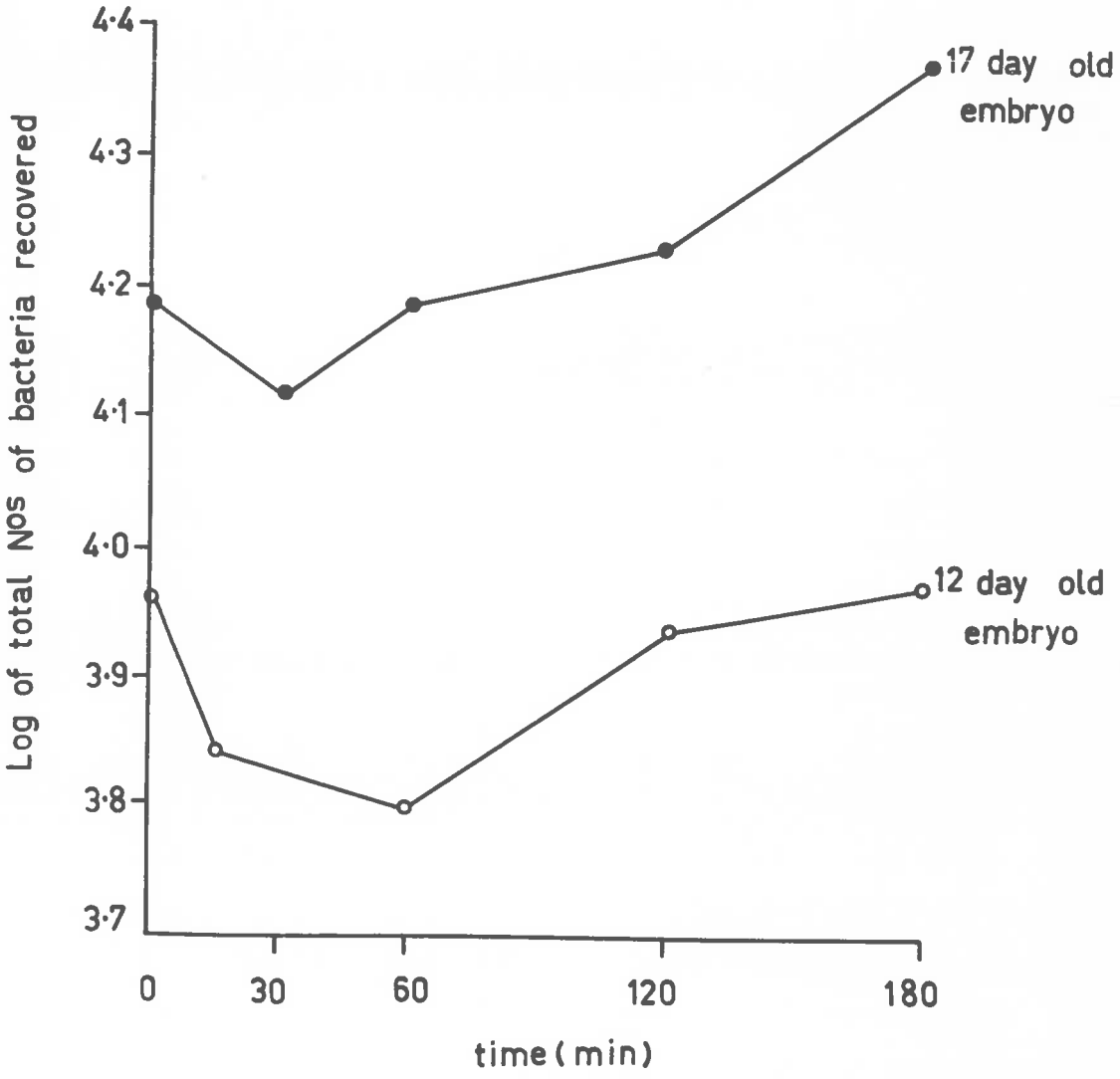
The previous experiments demonstrated that, depending on the age of the embryo, *E. coli* Lilly was inactivated by the phagocytic cells of the reticulo-endothelial system. *S. gallinarum* 98, however, was poorly phagocytosed whereas

TABLE XXI.

THE FATE OF LIVING S. GALLINARUM 9S OPSONISED WITH ADULT CHICKEN SERUM FOLLOWING INTRAVENOUS INJECTION IN CHICK EMBRYO AT 12TH AND 17TH DAY OF DEVELOPMENT.

Age of Embryo	Time of Sampling in Mins.	Distribution of Bacteria			Total Recovery of Bacteria
		Liver and Spleen	Membrane	Blood	
		Number of Viable Bacteria			
12 day	0	1180	1218	6620	9018
	15	1080	1662	4030	6772
	30	1530	2204	3710	7444
	60	2330	1875	2070	6275
	120	2880	1485	4180	8548
	180	2110	2509	4660	9279
17 day	0	1845	70	13400	15315
	15	9500	66	6450	16056
	30	10650	70	2230	12950
	60	2250	56	2840	15146
	120	12300	154	4500	16054
	180	13900	84	9600	23584

Fig.10 The fate of S. gallinarum 98 opsonised with adult hen serum following intravenous injection into the 12 and 17 day old chick embryo.


4·4
4·3
4·2
4·1
4·0
3·9
3·8
3·7
Log of total Nos of bacteria recovered
17 day old embryo
12 day old embryo
0
30
60
120
180
time (min)

opsonised S. gallinarum 98 was phagocytosed but not killed as efficiently as E. coli Lilly. These results suggested that the resistance of the embryo to infection by these two strains of bacteria would vary.

Various doses of S. gallinarum 98 opsonised with adult chicken serum and E. coli Lilly were injected into embryos of different ages. The experiments were terminated after four days for E. coli Lilly and after two days for S. gallinarum 98. The 50 per cent. lethal dose (L.D.50) was calculated by the method of Reed and Muench (1938).

The results in Table XXII show that there is considerable difference in the resistance of the chick embryo towards infection by these two organisms. The chick embryo was relatively more resistant to E. coli Lilly at a young age and its resistance increased rapidly until at the 18th day the L.D.50 was 6×10^4 bacteria. With S. gallinarum 98 and opsonised S. gallinarum 98 the embryos, however, were highly susceptible to challenge, and all were killed by less than twenty bacteria. The only difference in resistance that one could measure in chick embryos challenged with S. gallinarum 98 unopsonised and S. gallinarum 98 opsonised was that the latter group of embryos showed an increase in survival time of 24 hours. There was, however, no difference in over-all mortality between these two groups.

TABLE XXII.

THE VIRULENCE OF E. COLI LILLY AND S. GALLINARUM 9S FOR CHICK EMBRYOS AT DIFFERENT AGES.

Age of Embryo (Day)	Strain of Bacteria		
	E. coli Lilly	S. gallinarum 9S	Opsonised * S. gallinarum 9S
	50 percent. Lethal Dose		
11	9×10^2	< 20	
12			< 20
13	2.5×10^3	< 20	< 20
14	4.2×10^3		
15	1×10^4	< 20	
16			
18	6×10^4	< 20	< 20

< = less than 20 bacteria

* = opsonised with adult chicken serum

The Changes in Resistance of the Chick Embryo to Injection with S. gallinarum 9S on Hatching.

While S. gallinarum 9S is avirulent for the adult bird, the above experiment showed that this bacteria was highly virulent for the embryo [William-Smith, (1956)]. To understand the transition from susceptibility in the embryo to resistance in the adult bird, bacterial survival studies and host resistance studies were done using one day old and seven day old chickens.

Various doses of S. gallinarum 9S were injected intravenously into one day old and seven day old chickens post hatching. The chickens were observed for 21 days and the L.D.50was calculated (by Reed and Muench method).

It is clear that after hatching there is a dramatic change in the resistance of this species to infection with S. gallinarum 9S. Chick embryos that were susceptible to less than 20 bacteria have an L.D.50 of 2×10^4 at one day after hatching and an L.D.50 of 2×10^7 on the seventh day post hatching.

Bacterial survival studies were confined to examining the bactericidal potential of the phagocytic cells in two organs, the liver and spleen. A dose of approximately 2×10^4 S. gallinarum 9S in 0.1 ml. of saline was injected intravenously into the leg vein of one day and seven day old chickens. At determined time intervals samples of blood and homogenised liver and spleen were taken from two birds

and plated for viable count.

The results presented in Table XXIII show that the one day old chicken is able to kill the phagocytosed S.gallinarum 98 over a period of sixty minutes as compared with the continuous killing over three hours by the seven day old chicken. In this regard it is apparent that the chick embryo at 12 and 17 days of development deals as efficiently with E. coli Lilly as the one day and seven day old chicken deals with S. gallinarum 98.

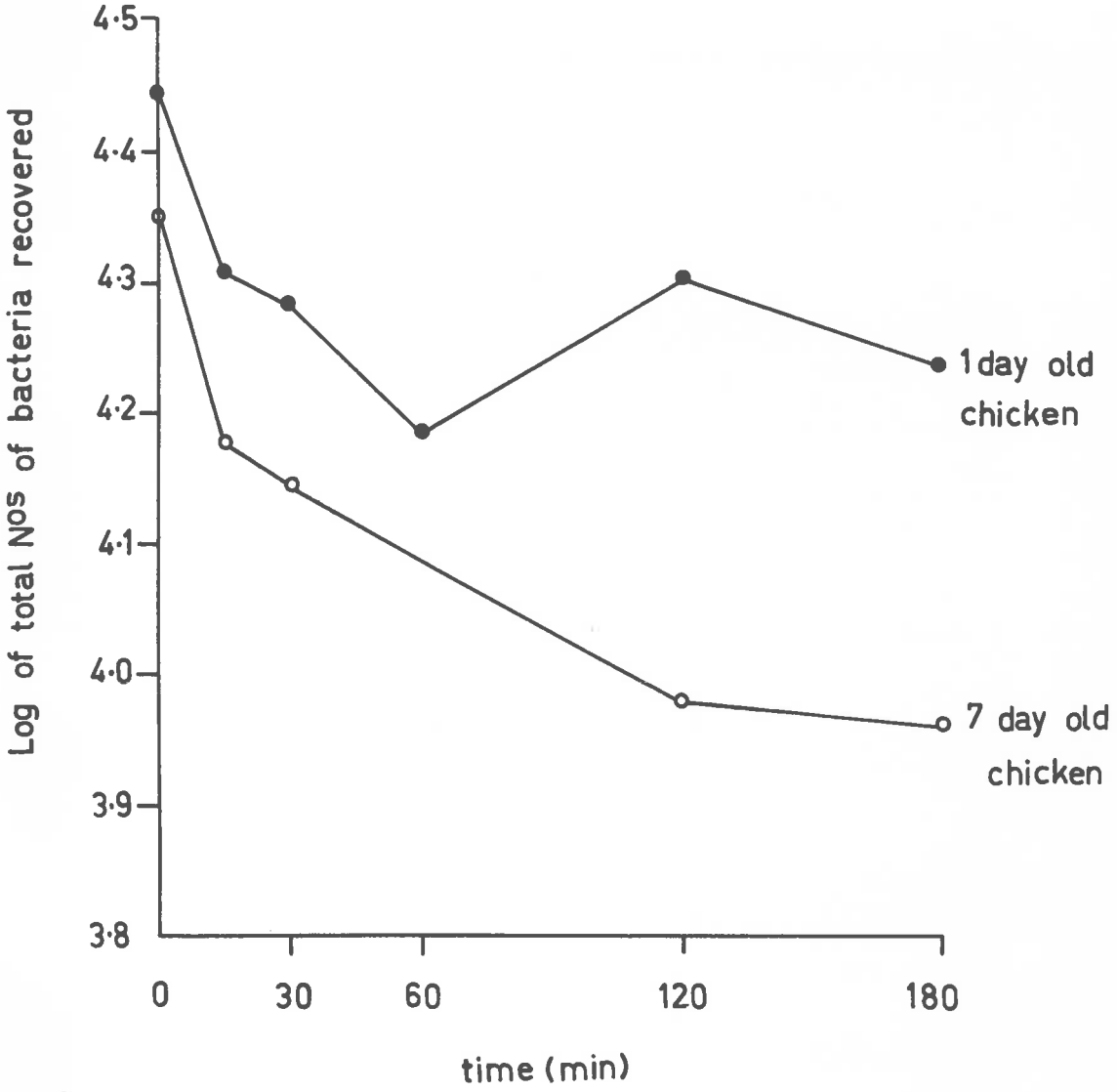
As in the study with other animals [Jenkin and Rowley, (1961); Biozzi and Stiffel, (1961)], the clearance of bacteria in the neonatal chicken is mainly confined to the liver and spleen. It can be seen from the Table XXIII that while the counts in the liver decrease due to killing, those in the spleen increase, indicating either multiplication of bacteria or accumulation of bacteria from the circulation. It is difficult, however, to separate killing from multiplication in absolute values, as a dynamic state exists between bacteria being removed from the circulation, bacteria being killed, bacteria multiplying and being released back into the blood circulation. It is only possible to demonstrate an overall increase or decrease which represents the resultant of these parameters (Fig.11). The results, however, give supporting evidence to the concept of a developing host defence mechanism.

TABLE XXIII.

THE CHANGES IN RESISTANCE OF THE CHICK EMBRYO TO INJECTION WITH S. GALLINARUM 96 ON HATCHING.

Age of Chicken Post Hatching	Time of Sampling	Distribution of Bacteria			Total Recovery of Bacteria
		Liver	Spleen	Blood	
		Number of Viable Bacteria			
1 Day	0	50	732	2720	27982
	15	8850	1492	1000	20342
	30	6600	1407	1100	19007
	60	12500	2490	455	15080
	120	18650	1010	20	20260
	180	15900	1000	15	16900
7 Day	0	850	925	20900	22675
	15	12900	875	1050	14825
	30	13000	325	700	14025
	60	16500	520	250	17270
	120	9400	0	0	9400
	180	9000	0	0	9000

Fig.11 The fate of S. gallinarum 9S following intravenous injection into chickens 1 day and 7 day post hatching.


4·5
4·4
4·3
4·2
4·1
4·0
3·9
3·8
Log of total Nos of bacteria recovered
0
30
60
120
180
time (min)
1 day old chicken
7 day old chicken

Conclusion.

In this chapter the intracellular bactericidal potency of cells that had phagocytosed E. coli Lilly and S. gallinarum 9S was examined. It was apparent that the capacity of the phagocytic cells to kill E. coli Lilly increased as the embryo matured. This ability was also reflected in the increase in resistance to this bacteria as the embryos grew older.

S. gallinarum 9S was not killed as efficiently as E. coli Lilly in embryos of all ages. The killing capacity of the phagocytic cells were, however, evident in hatched birds. This change was also followed by the increased resistance of the hatched birds to this organism.

CHAPTER VII.

THE NATURE AND ISOLATION OF THE OPSONINS DIRECTED AGAINST E. COLI LILLY AND S. GALLINARUM 9S.

It is quite clear that opsonins are required for the removal of bacteria from the blood of the embryo. Although opsonins for the rough and smooth strains of bacteria used in the present study occur in the adult chicken, the blood of the embryo contains opsonins only for the rough strains. The occurrence of these opsonins for the rough strain in the circulation of the embryo may be explained either by synthesis by the embryo or passive transfer from the mother. It has been clearly demonstrated that proteins are transferred from the mother to the embryo via the yolk sac fluid [Brierley and Hemmings, (1956); Patterson et al., (1962a)]. One possible explanation for the lack of transfer of opsonins for the smooth bacteria could be that the concentration of antibodies to the smooth strains in the serum of the adult bird was much lower than the titre of antibody to the rough strains. To test this hypothesis it was necessary to measure the titre of opsonins in the serum of the adult and embryo against these two strains.

Titre of Antibody in Adult Chicken Serum for E. coli Lilly and S. gallinarum 9S.

Because of its sensitivity, a haemagglutination method of assay for antibodies was used. Sheep red blood cells

were washed and sensitised with the lipopolysaccharide component of the 'O' somatic antigen extracted from both E. coli Lilly and S. gallinarum 98, as described (see Chapter II). Suitable dilutions of the sera were made and, after addition of the sensitised red cells, the mixture was incubated at 37°C for 60 minutes. The haemagglutination titre was read at the end of this time and after standing at 4°C overnight.

The results given in Table XXIV indicate that the adult serum possessed antibody against E. coli Lilly to a titre of 1/320 as compared to S. gallinarum 98 which was only 1/10. While it was possible to test for the presence of opsonins for E. coli Lilly by clearance studies in the 15 day old embryo (see Chapter V), it can be seen from Table XXIV that the haemagglutination assay was not sensitive enough to detect these antibodies.

The Passive Transfer of Opsonins for the Smooth Strain S. gallinarum 98 from Immunised Hens to the Chick Embryo.

It was found that the hen has a relatively low titre of opsonins for the smooth strain of S. gallinarum 98. It has been suggested that as a result of this low titre, only a small amount of antibody was transferred from the adult hen to the chick embryo, and that this would account for the extremely slow rate of clearance of this organism from the circulation.

Hens were immunised with a heat killed vaccine of

TABLE XXIV.

THE TITRE OF ANTIBODY IN THE SERA FROM ADULT HENS AND CHICK EMBRYOS AGAINST THE 'O' SOMATIC ANTIGEN OF E. COLI (LILLY) AND S. GALLINARUM 9S.

Source of Antigen	Source of Serum	Haemagglutination Titre*								
		1/10	1/20	1/40	1/80	1/160	1/320	1/640	C1	C2
E. coli Lilly	Adult Chicken Serum	+	+	+	+	+	+	±	-	-
	15 Day Old Chick Embryo Serum	±							-	-
S. gallinarum 9S	Adult Chicken Serum	+	±						-	-
	15 Day Old Chick Embryo Serum	-							-	-

* The haemagglutination titre is expressed by the reciprocal of the dilution of the serum and indicates macroscopic agglutination of the sensitised red cells.

C1 = Saline + Sensitised red cells.

C2 = Serum + Normal red cells.

S. gallinarum 9S over a period of three weeks to increase the titre of antibody. One month later the experimental fertile eggs were collected and incubation commenced. Embryos at various ages were then injected intravenously with isotopically labelled S. gallinarum 9S and the rate of clearance of this organism followed. The agglutination titres of serum of the adult hens were determined against both living and killed S. gallinarum 9S.

The results in Table XXV show the agglutination titre of the pooled sera from the immunised hens involved in the experiment. The agglutination titre for both living and killed bacteria was 1/1280.

The rates of clearance of S. gallinarum 9S in these embryos at various stages in their development are given in Table XXVI. It is apparent from these experiments that S. gallinarum 9S is cleared from the circulation of the embryos that were derived from immunised mothers. The rate of clearance also increases as the embryo grows older. This would perhaps suggest that the passive transfer of opsonins occurs where a sufficiently high titre of antibody is present in the adult hen. However, before coming to any definite conclusion, another important factor must be taken into account. Antibody may occur as two distinct molecular entities defined on their behaviour in the analytical centrifuge as 19S and 7S globulins. This may also account for the apparent selective transfer of antibodies to E. coli

TABLE XXV.

THE TITRE OF AGGLUTININS IN THE SERUM OF HENS IMMUNISED WITH S. GALLINARUM 98

Treatment of S. gallinarum	Source of Serum	Agglutination Titre *								
		1/10	1/20	1/40	1/80	1/320	1/1280	1/2560	1/5120	Cl
Living	Normal Hens	-								-
	Immunised Hens	+	+	+	+	+	+	±	-	-
Killed	Normal Hens	-								-
	Immunised Hens	+	+	+	+	+	+	±	-	-

* The agglutination titre is expressed by the reciprocal of the dilution of serum and indicates macroscopic agglutination of the bacteria.

Cl = Saline + Bacteria.

TABLE XXVI.

CLEARANCE OF ISOTOPICALLY LABELLED S. GALLINARUM 9S IN CHICK EMBRYOS FROM IMMUNISED HENS.

Age of Embryo	Source of Egg	Phagocytic Index K
14 day old embryo	Untreated adult	0.002
	Immunised adult	0.011
17 day old embryo	Untreated adult	0.003
	Immunised adult	0.054

Lilly in contrast to those for S. gallinarum 9S, selection being based on molecular size.

The Chemical Nature of the Opsonins to E. coli Lilly and S. gallinarum 9S.

The chemical nature of acquired specific antibody has been extensively studied since Tiselius (1937) first defined them as gamma globulins. However, until recently, nothing was known about the nature of the naturally occurring (natural antibody) opsonins present in normal serum. Rowley and Jenkin, (1962) and Turner and Rowley, (1963) were able to characterise the opsonin for S. typhimurium C5 in normal pig serum by physico-chemical and immuno-chemical methods, as a 19S macroglobulin. The present work has shown that opsonins are necessary for the clearance of the two strains of bacteria from the circulation of the chick embryo. The opsonins for E. coli Lilly are present in the serum of the adult hen and also in embryo serum. On the other hand, opsonins for S. gallinarum 9S are present in adult chicken serum, but are not present in embryo serum. When the adult bird is immunised against S. gallinarum 9S then opsonins are transferred to the embryo's circulation via the yolk sac.

In view of the possible selective transfer of antibodies based on molecular size, it seemed important to investigate more fully the chemical nature of the opsonins present in the adult hen and the chick embryo against both

these two strains of bacteria, i.e. E. coli Lilly and S. gallinarum 98.

Changes in the Concentration of Various Serum Proteins During the Development of the Chick Embryo.

Since the selective transfer antibodies may be based on molecular size, it seemed important as a preliminary step to investigate the changes in the concentration of various serum proteins during the development of the chick embryo. Particular attention was paid to those proteins whose electrophoretic mobility was similar to the two classes of antibodies described for adult animals, namely 7S and 19S globulins.

Several investigators have analysed both qualitatively and semi-quantitatively the serum proteins of the developing embryo [Moore, Shen and Alexander, (1945); Marshall and Deutsch, (1950); Heim and Schechtman, (1954)]. However, in most instances, the analysis was of a qualitative rather than quantitative nature. In this study the various serum proteins have been analysed by paper strip electrophoresis and the various components quantitated in relation to the total protein content of the serum using absorption spectrophotometry, as described in Chapter II.

The results of these experiments are given in Table XXVII. It is apparent that during development from the 12th to the 19th day, there is about a twofold increase in the total protein concentration. However, in this same

TABLE XXVII.

ANALYSIS BY PAPER STRIP ELECTROPHORESIS OF THE CHANGES IN THE CONCENTRATION OF VARIOUS SERUM PROTEINS DURING THE DEVELOPMENT OF THE CHICK EMBRYO.

Age of Embryos	Type of Serum Protein				Total Protein
	gamma	beta	alpha	albumin	
	Concentration of protein mg./ml.				
12 day	0.34	2.7	2.7	1.2	6.7
15 day	0.88	3.7	3.3	2.0	9.8
17 day	1.34	3.4	2.9	2.8	10.5
19 day	1.85	5.2	3.8	5.7	15.4
Adult	9.70	7.5	9.5	18.0	44.0

time period, there is a fivefold increase in the total amount of gamma globulin in the region of the 7S antibody. The beta globulin levels do not show a marked increase. It is in this region that the 19S macroglobulins may be found.

Fractionation of Serum from Adult Chickens and Chick Embryos by Ion Exchange Cellulose Column.

The column was prepared and the fractionation of adult and embryo serum carried out as described in Chapter II. Adult hen serum that had been dialysed overnight against the phosphate buffer used to stabilise the column was applied to the column and eluted using a phosphate buffer gradient of varying pH and molarity. The total amount of protein applied to the column varied from 175 mg. in the case of adult chicken serum to 107 mg. when embryo serum from 19 day old embryos was used. A series of constant volume eluates from the column (5 ml.) were assayed for their protein content by absorption at 280 mμ.

The elution curve for the protein was drawn as shown in Fig.12 and Fig.14. It is apparent from the table on Fig.12 and Fig.14 that practically 100 per cent. of the protein was recovered. On the basis of the elution curve the contents were pooled into the various fractions and concentrated by dialysis against "carbowax".

Assay for Opsonins in the Various Serum Fractions.

It has been shown in the previous chapter (Chapter V)

that, following intravenous injection of a relatively large dose of E. coli Lilly, it was possible to deplete the embryo of circulating opsonins for that strain. Hence, in the blockaded embryo, a subsequent small dose of E. coli Lilly was not cleared from the blood unless the bacteria were opsonised in vitro. For the assay of the biologically active fractions for E. coli Lilly, 14 day old embryos were blockaded with 0.1 ml. of 4×10^8 bacteria. After 60 minutes 10^9/ml. isotopically labelled E. coli Lilly that had been treated for 15 minutes at 4°C with 1 ml. of fraction were washed and resuspended in 0.5 ml. of casamino medium. A volume containing 0.1 ml. of 2×10^8 bacteria was injected into each embryo. The presence of opsonins was indicated by the ability of the cells of the reticulo-endothelial system to clear the labelled bacteria from the blood, at a faster rate than that observed in the control blockaded embryos injected with unopsonised E. coli Lilly.

In the case of H. gallinarum 98 it was not necessary to blockade the embryo as this organism was not cleared efficiently unless opsonised in vitro. Hence the presence of opsonins could be measured directly. At least three embryos were used to assay each fraction.

The biological activity of the various fractions has been expressed as follows. In the case of the blockaded embryos, the controls gave a K value of 0.005. This figure was subtracted from the experimental results and any increase

of 0.005 over that of the control was considered significant and given the arbitrary value of one biological unit. For example, if one of the fractions gave a K value of 0.025, this was equivalent to four biological units. Similarly, for S. gallinarum 9S the controls gave a K value of 0.003. Any result of 0.003 over that of control was expressed as one biological unit.

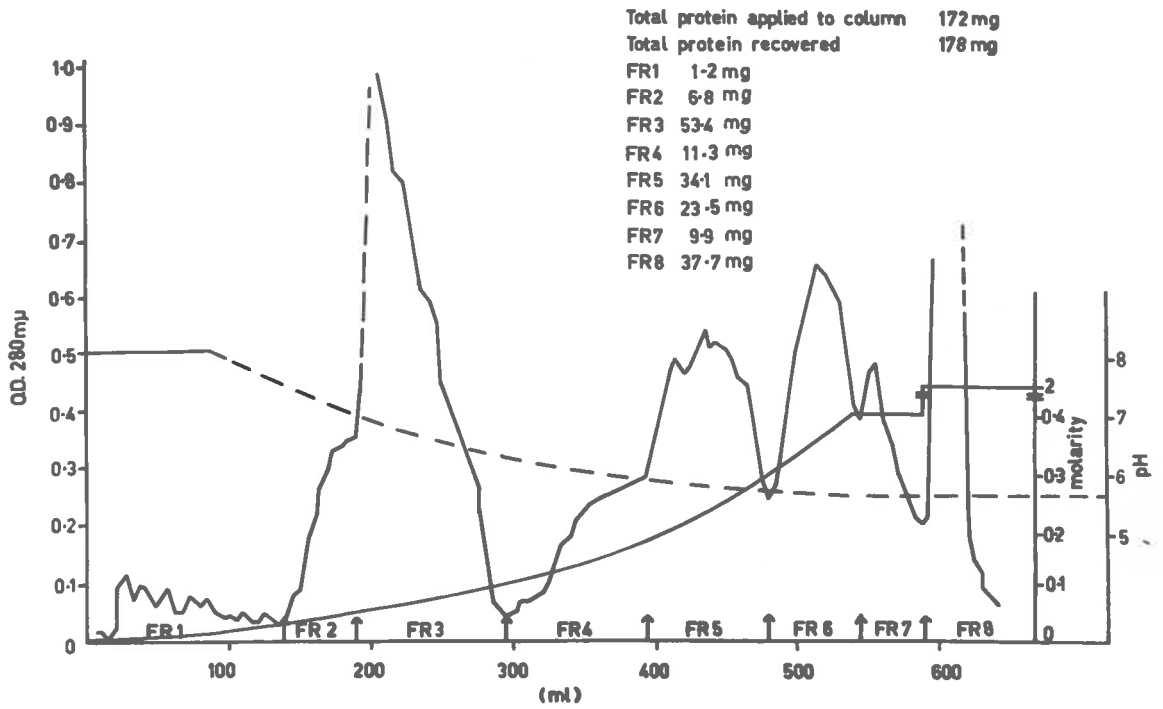
The results shown in Fig.13 and Fig.15 are the number of biological units contained in the total volume of each fraction.

The biological activity in adult chicken serum for E. coli Lilly and S. gallinarum 9S was eluted over two molarity ranges. For E. coli Lilly, activity was eluted between 0.01M to 0.1M and pH 8 to pH 6, as well as at 0.4M to 2M and at pH 5.6. Activity for S. gallinarum 9S was found in the eluates between 0.3M to 0.47M buffers and at pH 6 to 5.6. In the embryo serum activity for E. coli Lilly was eluted at 0.01M and pH 8.0.

From previous studies [Paney and Horbett, (1959); Turner and Rowley, (1963)] it has been shown that the 7S gamma globulins are eluted over the molarity and pH ranges - 0.01M to 0.1M and pH 8 to pH 6 respectively, while the 19S macroglobulins are eluted at high molarities and low pH (greater than 1.5M and pH 5). This would suggest that the activity for E. coli Lilly resided in both a 7S and 19S antibody, while only the 19S component of adult chicken

Fig.12 The elution pattern of adult chicken serum from a diethylaminoethyl cellulose column under increasing molarity and decreasing pH.
Table shows the total amount of protein recovered from each fraction (FR).

Fig.13 The total recovery of biological activity for E. coli Lilly and S. gallinarum 95 from fractions of adult chicken serum. The molarity range over which these fractions were eluted is also graphed.


Total protein applied to column 172 mg
Total protein recovered 178 mg
FR1 1·2 mg
FR2 6·8 mg
FR3 53·4 mg
FR4 11·3 mg
FR5 34·1 mg
FR6 23·5 mg
FR7 9·9 mg
FR8 37·7 mg
O.D. 280mμ
molarity
pH
FR1
FR 2
FR3
FR4
FR5
FR 6
FR7
FR8
(ml)

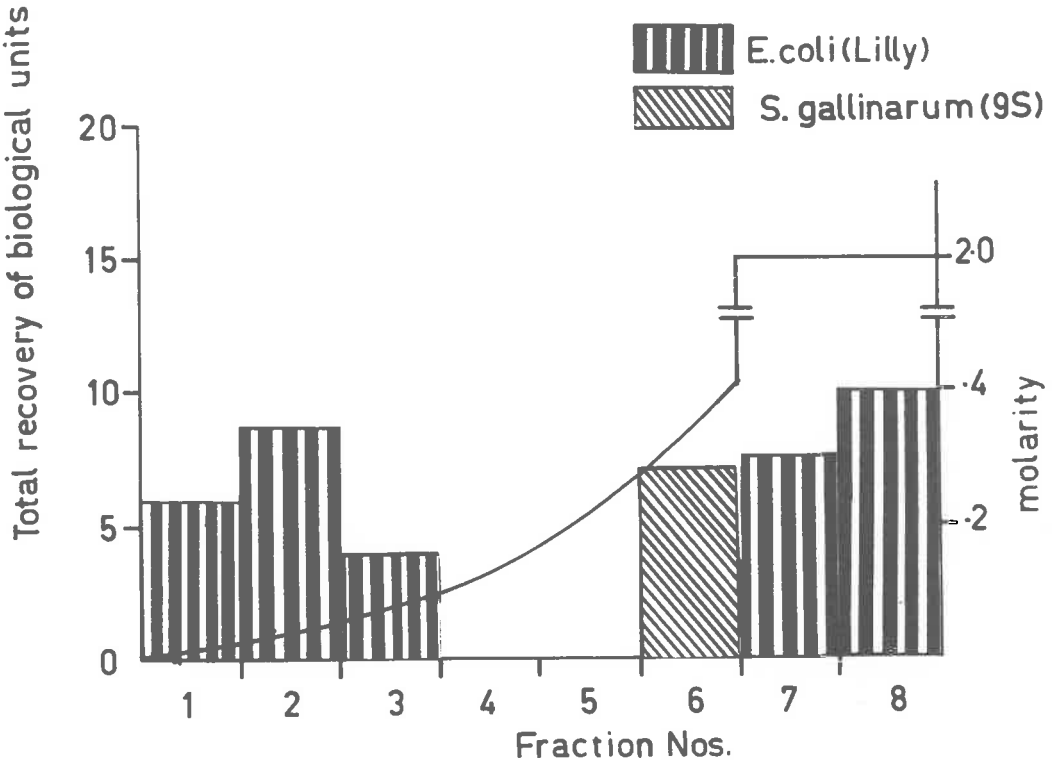
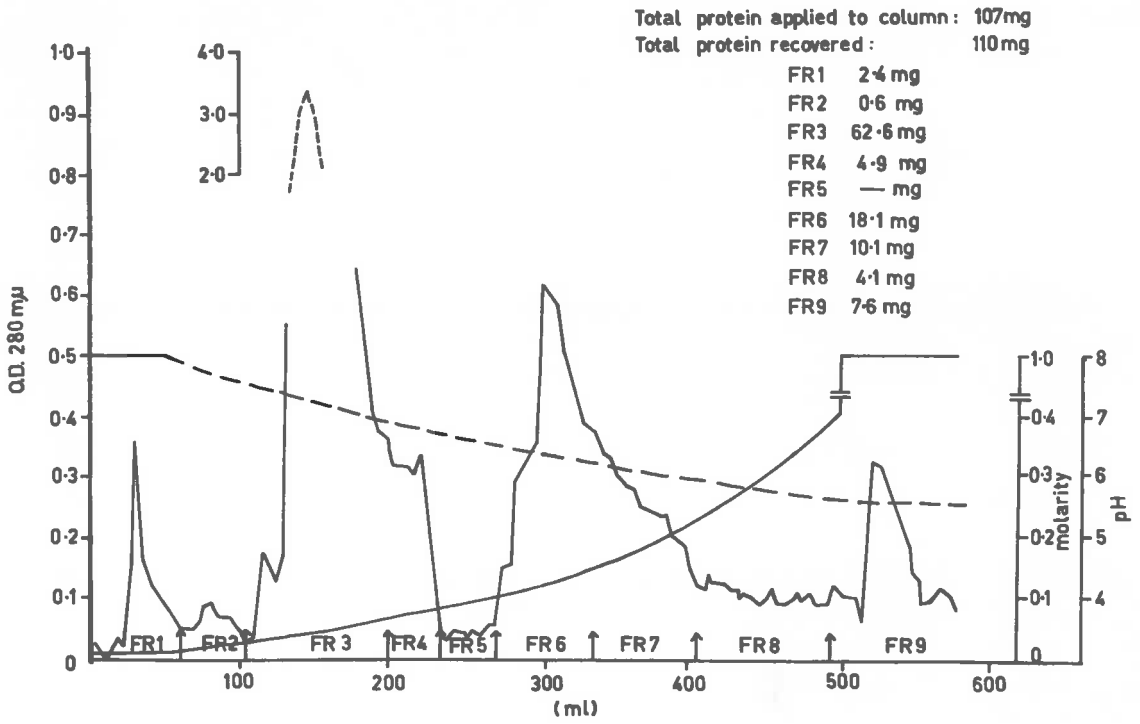
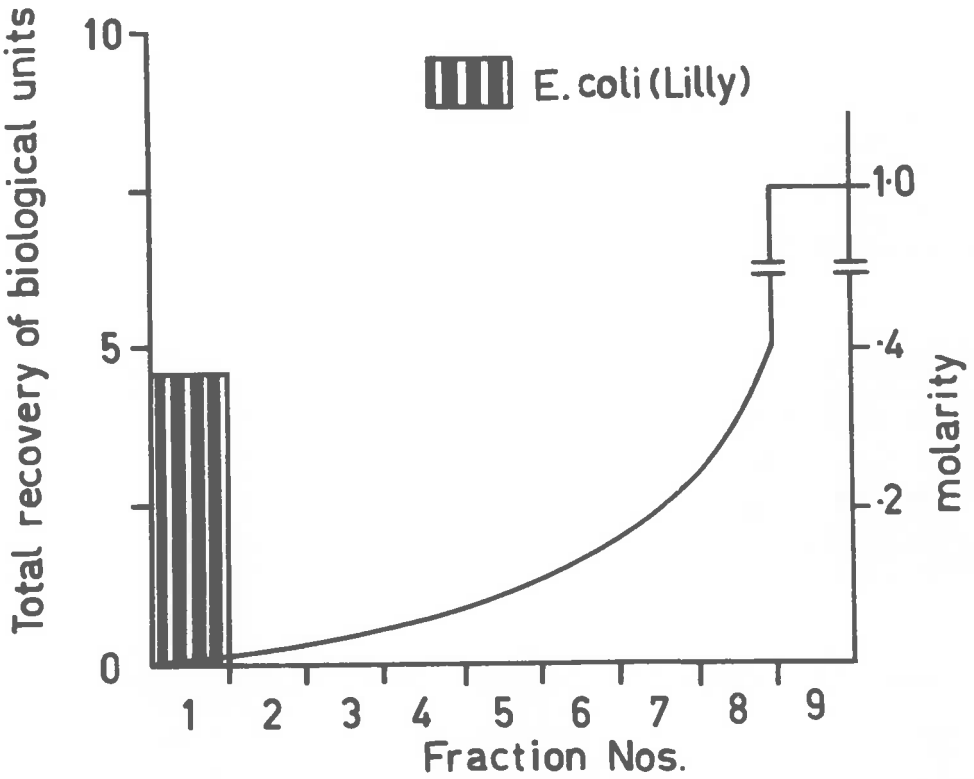

E.coli (Lilly)
S. gallinarum (9S)
Total recovery of biological units
molarity
Fraction Nos.

Fig.14 The elution pattern of 19 day old embryo serum from a diethylaminoethyl cellulose column under increasing molarity and decreasing pH.
Table shows the total amount of protein recovered from each fraction (FR).

Fig.15 The total recovery of biological activity for E. coli Lilly and S. gallinarum 98 from fractions of embryo serum.
The molarity range over which these fractions were eluted is also graphed.


Total protein applied to column: 107mg
Total protein recovered: 110mg
FR1 2·4 mg
FR2 0·6 mg
FR3 62·6 mg
FR4 4·9 mg
FR5 — mg
FR6 18·1 mg
FR7 10·1 mg
FR8 4·1 mg
FR9 7·6 mg
O.D. 280 mμ
molarity
pH
FR1
FR2
FR 3
FR4
FR5
FR6
FR7
FR8
FR9
(ml)


E. coli (Lilly)
Total recovery of biological units
molarity
Fraction Nos.

serum was opsonic for S. gallinarum 98.

Fractionation of Serum by Sucrose Gradient Ultra-Centrifugation.

It was suggestive from the results in the previous experiment that macroglobulins in the adult serum were opsonic for E. coli Lilly and S. gallinarum 98. The gamma globulin, which was present in adult serum and also in embryo serum, was only active for E. coli Lilly. However, when the one day old chicken was examined, these birds could clear S. gallinarum 98 efficiently, the K value being 0.045. This suggested that at one day there were opsonins in the serum of the neonatal bird to enable the phagocytic cells of the reticulo-endothelial system to clear S. gallinarum 98. It is at this stage that the L.D.50 for this organism increased to 2×10^4, while one day prior to hatching the embryo was susceptible to less than 20 bacteria (see Chapter VI).

It is clear that during hatching either opsonins were being rapidly synthesised by the chicken or these opsonins were being passively transferred via the gut due to absorption of the remaining yolk just prior to hatching.

Accordingly, the density gradient method described by Edelman, Kunkel and Franklin (1958) was used to trace the presence of opsonins for both E. coli Lilly and S. gallinarum 98 from normal adult chicken serum, the yolk fluid, embryo serum and one day old chicken serum. Various con-

centrations of sucrose were used to obtain a gradient that would give maximum separation of the high molecular weight 19S globulins from the low molecular weight 7S globulins. The gradients were prepared and a test sample of one ml. of adult chicken serum centrifuged and analysed for the sedimentation rates of proteins, as described (Chapter II). The elution pattern of the various proteins for the different sucrose gradients are shown in Fig.16. It is clear that the gradient consisting of 40, 35, 25 and 10 per cent. sucrose gave the best separation. In all subsequent analyses, this gradient was used.

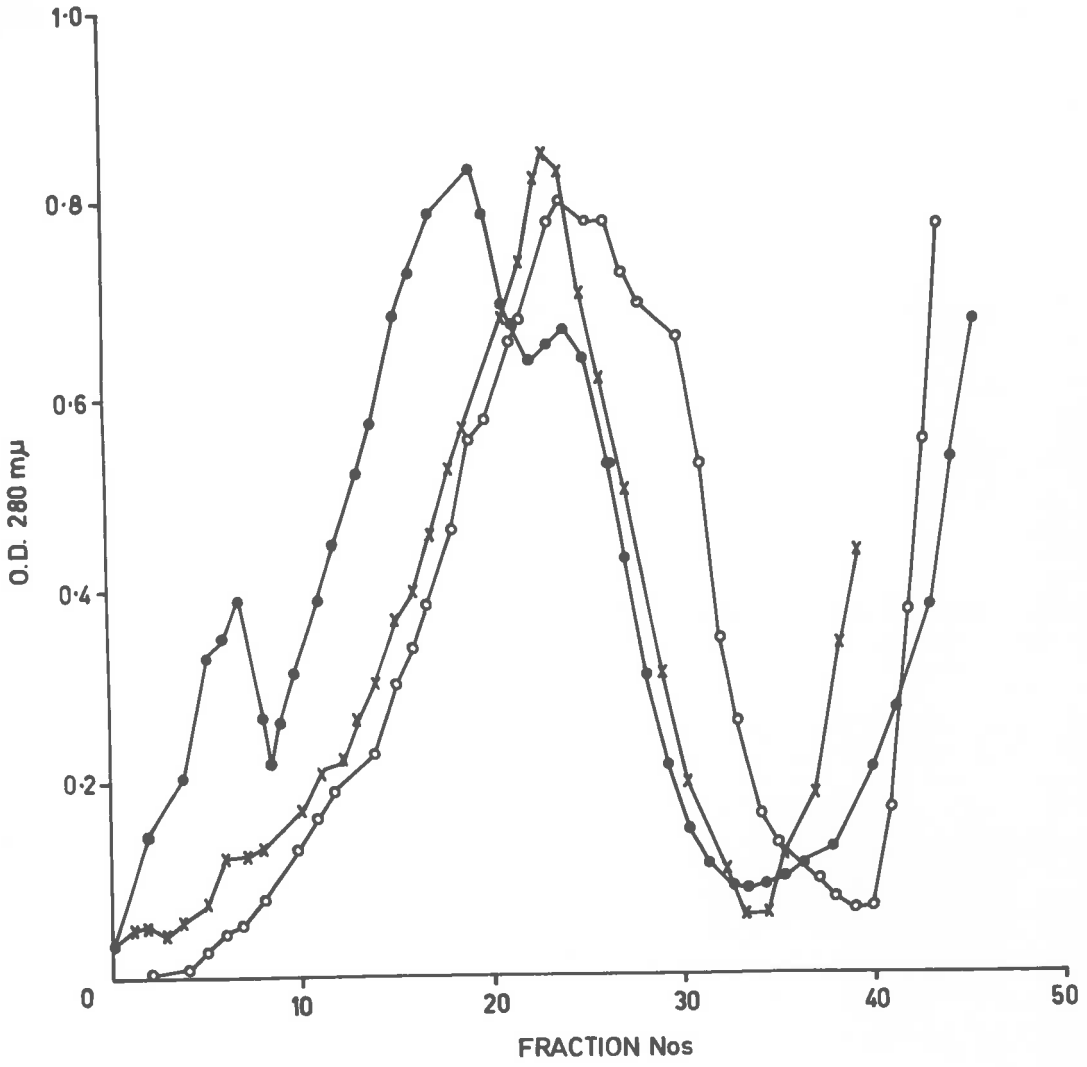
Serum from adult hens, one day old chickens and 19 day old embryos was centrifuged for 18 hours in a sucrose gradient as described. Native yolk fluid is not opsonic, either for E. coli Lilly or S. gallinarum 98. This may be due to either the low concentration of opsonins or opsonisation may be inhibited due to the high concentration of lipid. A prior fractionation step was indicated before subjecting the yolk fluid to density gradient centrifugation. Fresh yolk fluid was diluted 1:2 with 10 per cent. NaCl, overlayed with 0.15M NaCl, and centrifuged for five hours at 40,000 r.p.m. in a Spinco ultracentrifuge [Turner and Cook, (1958)]. The low density lipid-rich layer was removed and the residual yolk fluid was dialysed in 0.15M NaCl for 20 hours at 4°C. The dialysed extract was found to be opsonic for both E. coli Lilly and S. gallinarum 98 and was

Fig.16 Sedimentation pattern of proteins after centrifugation in various sucrose gradients.

o___o Sucrose gradient consisting of 40, 35, 25 and 10 per cent. sucrose.

x___x Sucrose gradient consisting of 40, 40, 30 and 20 per cent. sucrose.

o___o Sucrose gradient consisting of 50, 40, 30 and 20 per cent. sucrose.


1·0
0·8
0·6
0·4
0·2
0
O.D. 280 mμ
10
20
30
40
50
FRACTION Nos

therefore used as starting material for fractionation.

The sera and yolk fluid fractions were collected as outlined in Materials and Methods after sucrose gradient centrifugation and assayed for protein by absorption at 280 mμ. The optical density of each fraction was graphed as a function of tube number as shown in Fig.17-20. On the basis of the protein curve the tubes were pooled into three fractions. The faster sedimenting fraction was represented as FR1, the second as FR2 and the upper lipoprotein layer as FR3. Analytical ultracentrifuge patterns of these fractions from pig serum and human serum have shown that the peaks corresponding to FR1 and FR2 are macroglobulins and 7S globulins respectively. These macroglobulins are not only 19S molecules but may be a heterogenous mixture of globulins with sedimentation constants varying from 19S to 40S globulins [Kunkel, (1960); Turner and Rowley, (1963)].

The total amount of protein recovered from these samples is given in the respective figures (see Fig.17-20). The percentage of macroglobulins in the yolk extract and one day old chicken was 2 per cent., as compared to the adult chicken serum which had 11 per cent. The FR2 had about 80 to 90 per cent. of the total proteins.

Assay of Biological Activity of the Fractions from Sucrose Gradient Centrifugation.

The standard dose of 10^9 *E. coli* Lilly and *S. gallin-*

arum 9S was opsonised with 1 ml. of each fraction. volume of 0.2 ml. containing 2 x 10^8 treated bacteria was injected into each embryo and the rate of clearance followed. To assay the biologically active fraction for E. coli Lilly the embryos had to be depleted of opsonins as described previously.

The results shown in Fig.22, 24, 26 and 28, are the summation of results obtained from three embryos for each test. As the lipoprotein (FR3) showed no biological activity, these results have not been included. The third column represents the opsonic activity of the starting material. Biological activity of each fraction has been expressed as the increase in phagocytic index above that of controls, i.e. the clearance values of unopsonised bacteria.

It is apparent from the results in these figures that both 7S globulins and 19S globulins are opsonic for this bacteria. Though 19S globulins are not present in embryo serum, the presence of the 7S globulins enhances the clearance of E. coli Lilly in the chick embryo. On the other hand, the opsonin for S. gallinarum 9S is the 19S globulin. Even though this antibody is present in all fluids, including the yolk fluid, it is not present in embryo serum. Hence it seems possible that the failure of the 19S globulin to get across the yolk membrane because of its molecular size may be responsible for the inability

Fig.17 Sedimentation pattern of proteins of yolk extract after centrifugation in sucrose gradient.
Table shows total weight of protein recovered.

Fig.18 Sedimentation pattern of proteins of embryo serum after centrifugation in sucrose gradient.
Table shows total weight of protein recovered.

Fig.19 Sedimentation pattern of proteins of one day old chicken serum after centrifugation in sucrose gradient.
Table shows total weight of protein recovered.

Fig.20 Sedimentation pattern of proteins of adult hen serum after centrifugation in sucrose gradient.
Table shows total weight of protein recovered.

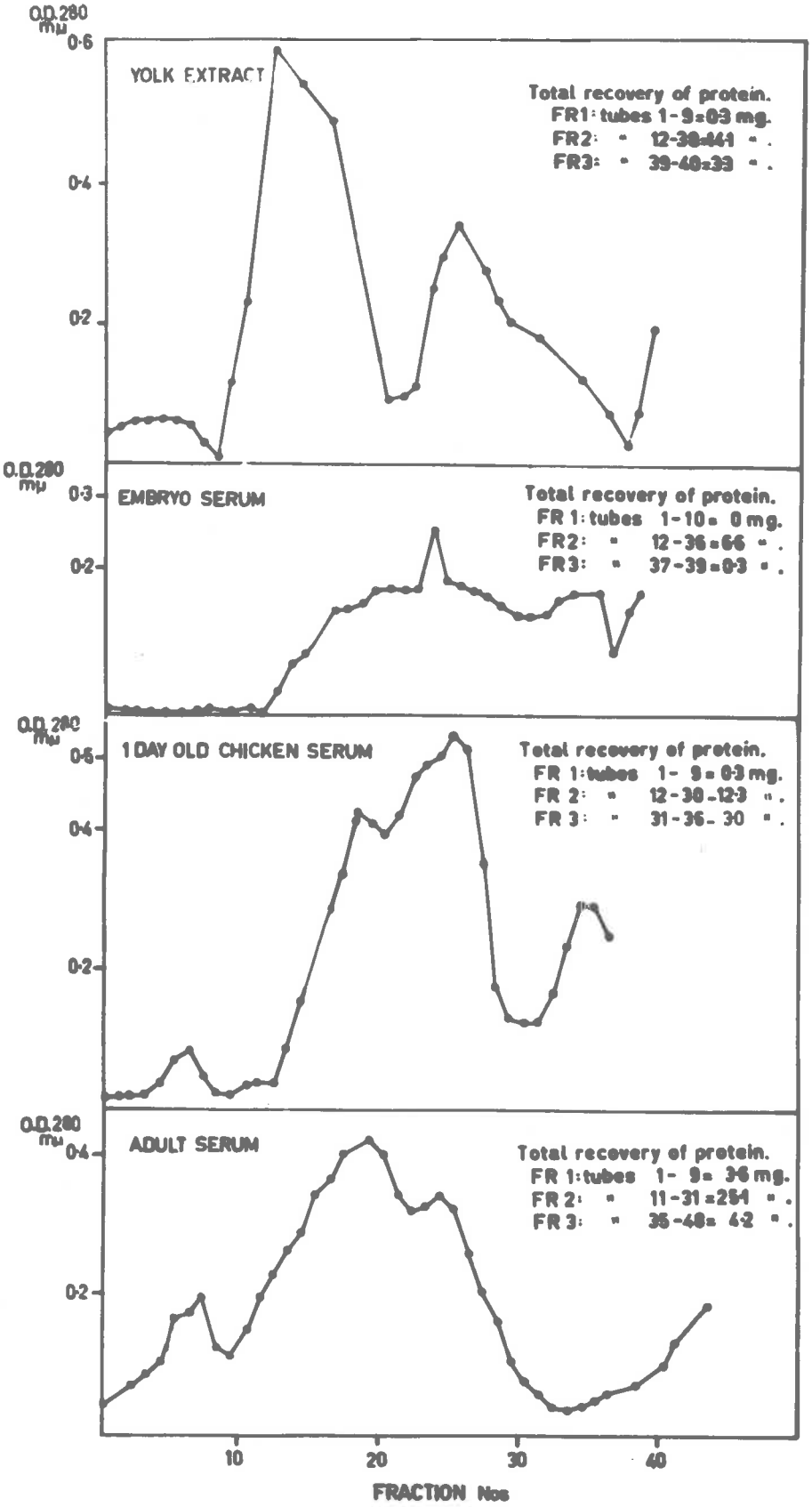
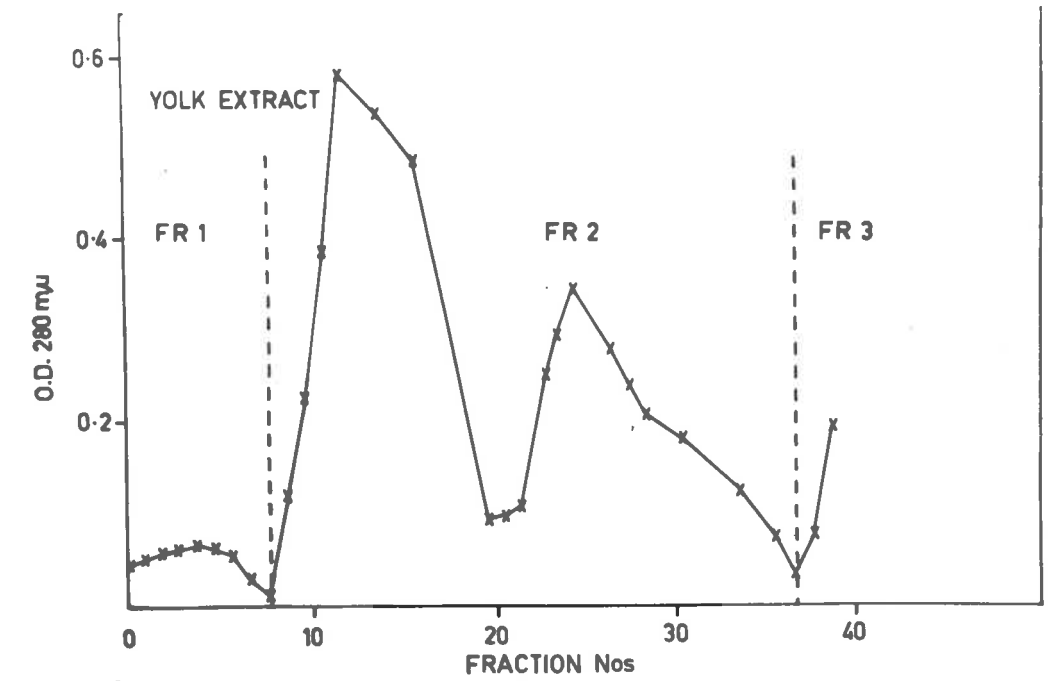

O.D.280 mμ
0·6
0·4
0·2
YOLK EXTRACT
Total recovery of protein.
FR1: tubes 1-9=0·3 mg.
FR2: " 12-38=44·1 " .
FR3: " 39-48=3·3 " .
O.D.280 mμ
0·3
0·2
EMBRYO SERUM
Total recovery of protein.
FR 1: tubes 1-10= 0 mg.
FR2: " 12-36=6·6 " .
FR3: " 37-39=0·3 " .
O.D.280 mμ
0·6
0·4
0·2
1 DAY OLD CHICKEN SERUM
Total recovery of protein.
FR 1: tubes 1- 9= 0·3 mg.
FR 2: " 12-30=12·3 " .
FR 3: " 31-36= 3·0 " .
O.D.280 mμ
0·4
0·2
ADULT SERUM
Total recovery of protein.
FR 1: tubes 1- 9= 3·6 mg.
FR 2: " 11-31=25·1 " .
FR 3: " 35-48= 4·2 " .
10
20
30
40
FRACTION Nos

Fig.21 Sedimentation pattern of proteins in yolk extract after centrifugation in sucrose gradient.

Fig.22 Total biological activity in 1 ml. of fraction.


0·6
0·4
0·2
O.D. 280mμ
YOLK EXTRACT
FR 1
FR 2
FR 3
0
10
20
30
40
FRACTION Nos

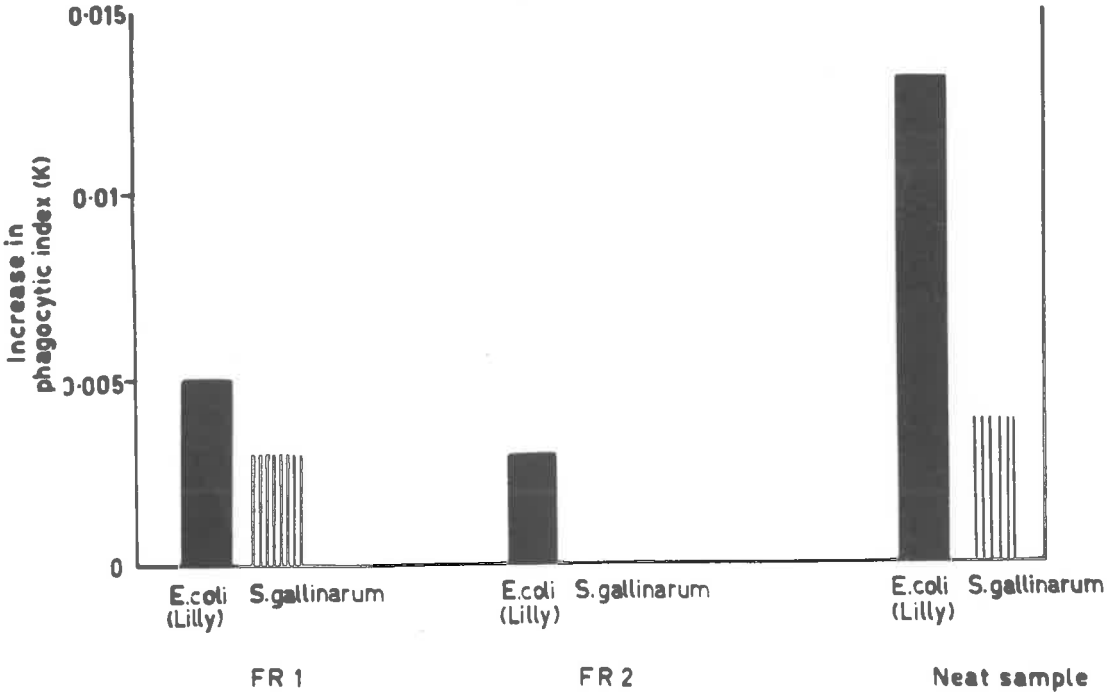

0·015
0·01
0·005
0
Increase in phagocytic index (K)
E.coli (Lilly)
S.gallinarum
E.coli (Lilly)
S.gallinarum
E.coli (Lilly)
S.gallinarum
FR 1
FR 2
Neat sample

Fig.23 Sedimentation pattern of proteins in embryo serum after centrifugation in sucrose gradient.

Fig.24 Total biological activity in 1 ml. of fraction.

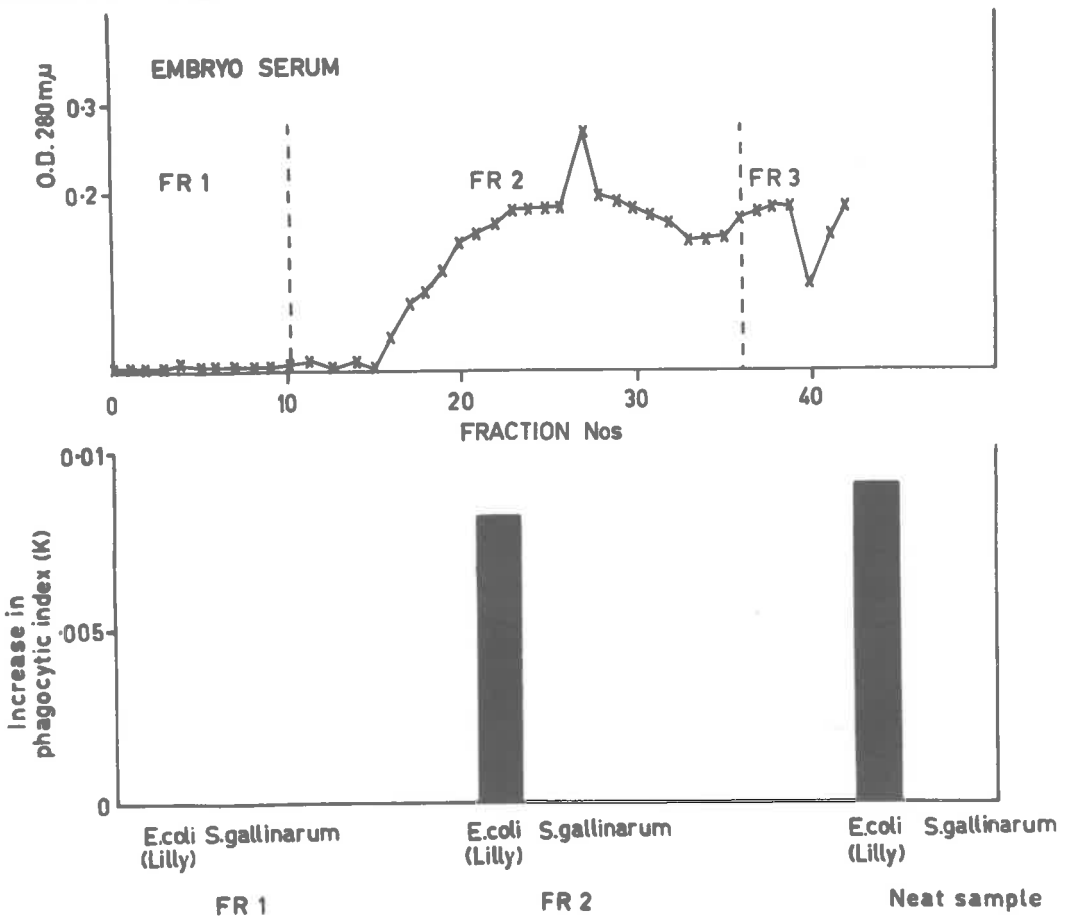

EMBRYO SERUM
O.D. 280 mμ
0·3
0·2
FR 1
FR 2
FR 3
0
10
20
30
40
FRACTION Nos
0·01
·005
0
Increase in phagocytic index (K)
E.coli (Lilly)
S.gallinarum
E.coli (Lilly)
S.gallinarum
E.coli (Lilly)
S.gallinarum
FR 1
FR 2
Neat sample

Fig.25 Sedimentation pattern of proteins in one day old chicken serum after centrifugation in sucrose gradient.

Fig.26 Total biological activity in 1 ml. of fraction.

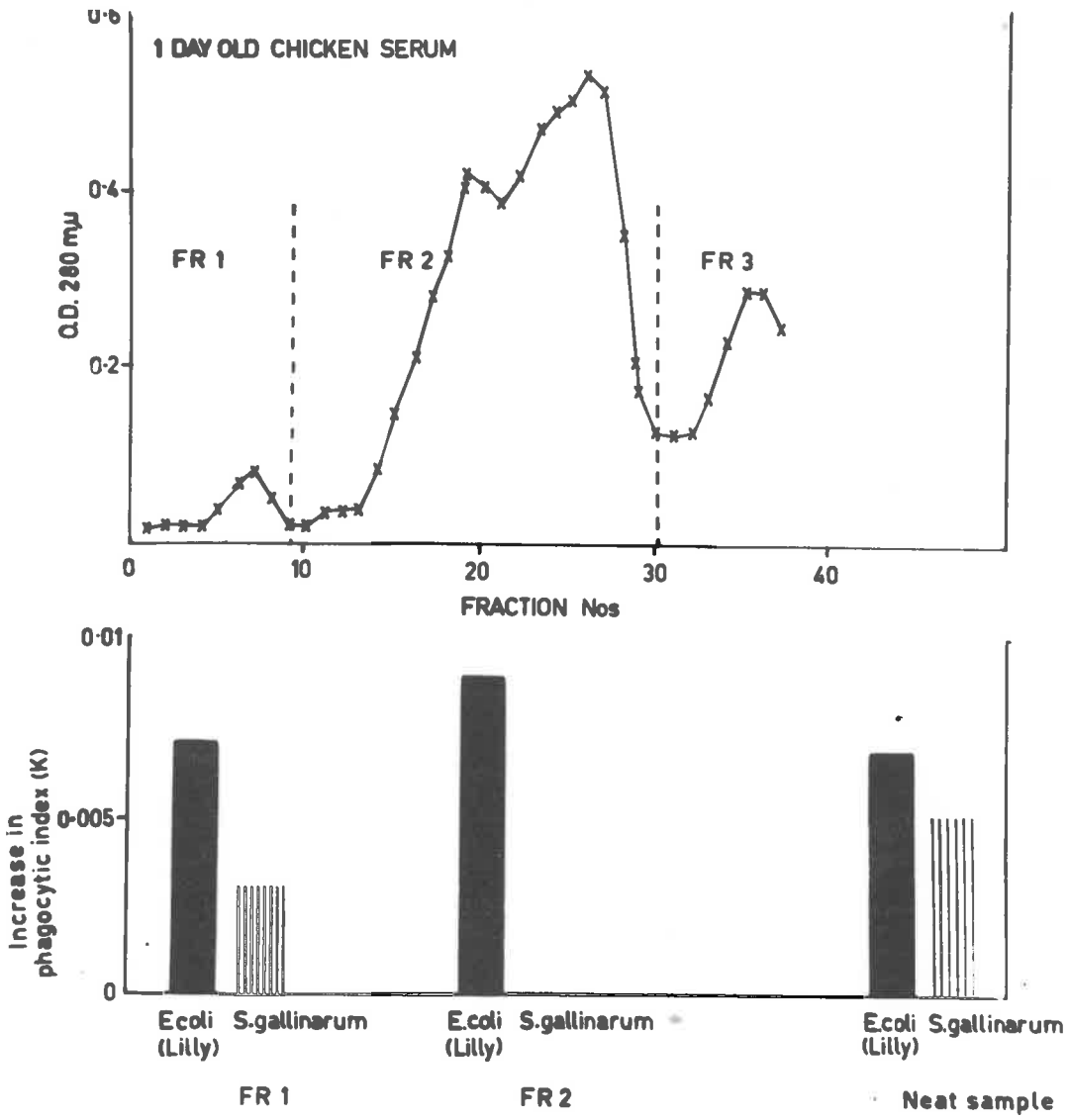
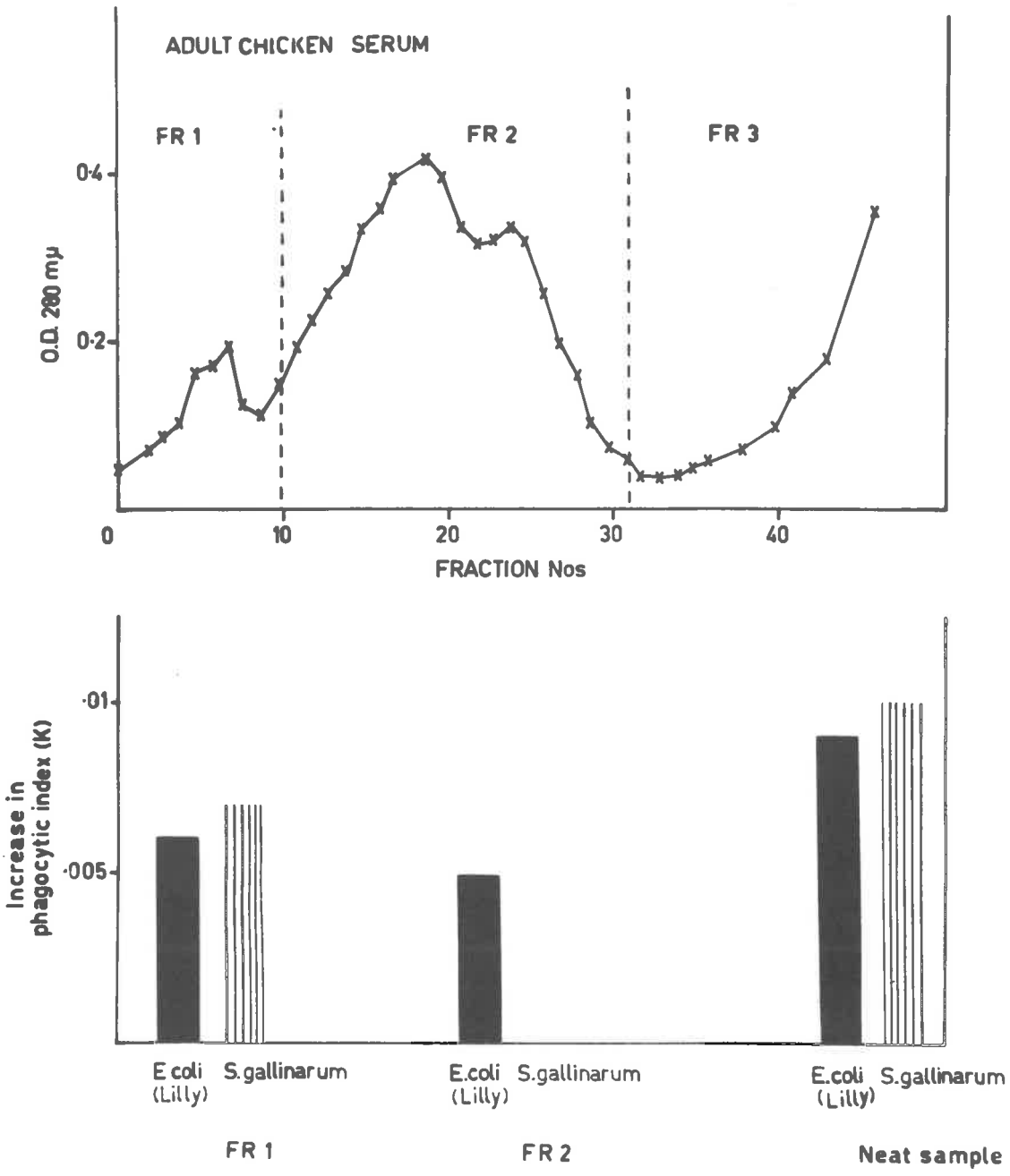

1 DAY OLD CHICKEN SERUM
O.D. 280 mμ
0·6
0·4
0·2
FR 1
FR 2
FR 3
0
10
20
30
40
FRACTION Nos
0·01
0·005
0
Increase in phagocytic index (K)
E.coli (Lilly)
S.gallinarum
E.coli (Lilly)
S.gallinarum
E.coli (Lilly)
S.gallinarum
FR 1
FR 2
Neat sample

Fig.27 Sedimentation pattern of proteins in adult hen serum after centrifugation in sucrose gradient.

Fig.28 Total biological activity in 1 ml. of fraction.


ADULT CHICKEN SERUM
FR 1
FR 2
FR 3
0·4
0·2
O.D. 280 mμ
0
10
20
30
40
FRACTION Nos
·01
·005
Increase in phagocytic index (K)
E coli (Lilly)
S.gallinarum
E.coli (Lilly)
S.gallinarum
E.coli (Lilly)
S.gallinarum
FR 1
FR 2
Neat sample

of the embryo to remove these bacteria from the blood.

Analysis of Immune Globulins in Serum from Adult Hens Immunised Against S. gallinarum 9S.

Several workers have shown that the injection of a variety of antigens into various animals produces firstly a rapidly sedimenting 19S macroglobulin which is then followed by the production of antibody molecules with a sedimentation constant of 7S [Benedict and Brown, (1962); Uhr, Finkelstein and Franklin, (1962)]. Apart from analytical centrifugation data, these two globulins may be identified by treatment with mercaptoethanol. The biological activity of macroglobulin antibodies is destroyed by such treatment while the smaller 7S globulins are, however, relatively resistant. In the previous experiments it was shown that only the 19S globulins were opsonic for S. gallinarum 9S. It was suggested that the slow clearance of this bacteria in the chick embryo was due to the failure of this globulin to cross the yolk sac membrane. However, when chickens were immunised with a heat killed vaccine of S. gallinarum 9S, the embryos that developed from eggs of these hens were able to clear the organism. It is possible that this enhanced clearance was due to the synthesis of 7S antibody by the immunised parent and transfer of these across the yolk membrane to the embryo as in the case of antibody specific for E. coli Lilly.

To test the validity of this argument, the opsonic

activity of both normal serum and immune serum was titrated in chick embryos (see Table XXVIII). As the result of this titration a dilution of serum was chosen for mercaptoethanol treatment that still gave just the maximum rate of clearance, i.e. $^1/_5$ for normal serum and $^1/_{20}$ for immune serum. A mixture containing equal volumes of serum and 0.2M mercaptoethanol was incubated at 37°C for one hour. Following incubation the mixture was dialysed overnight at 4°C against a phosphate buffer, ionic strength 0.2 at pH 7.0. These mercaptoethanol treated sera were then used to opsonise S. gallinarum 9S. Clearance was followed in 14 day old embryos and each sample was tested in three embryos and the results averaged.

It is apparent from Table XXIX that when normal serum is treated with mercaptoethanol, the biological activity for S. gallinarum 9S is destroyed, while immune serum still retains almost all its activity. This suggests that the hyper-immune serum contains mainly 7S globulins that are opsonic for S. gallinarum 9S, whereas in the normal serum, as shown previously by other methods, the opsonic activity is associated with macroglobulin antibody.

Conclusion.

The results from this chapter showed that specific 7S globulins and 19S globulins were opsonic for E. coli Lilly and S. gallinarum 9S. Under normal circumstances, E. coli Lilly was removed from the blood circulation of the

TABLE XXVIII.

TITRATION OF NORMAL AND IMMUNE SERUM IN 14 DAY OLD EMBRYOS.

Source of Serum	Dilution of Serum									
	Neat	1/2	1/5	1/10	1/20	1/50	1/100	1/500	1/1000	1/2000
	Phagocytic Index K									
Normal Hens	0.017	0.017	0.017	0.007	0.005	0.003	0.003			
Immune Hens			0.017	0.017	0.017	0.012		0.005	0.002	0.003

TABLE XXIX.

BIOLOGICAL ACTIVITY OF NORMAL AND IMMUNE SERA AFTER TREATMENT WITH 2 MERCAPTOETHANOL.

Sample	Treatment	Phagocytic Index K
Normal serum	None	0.017
	Mercaptoethanol	0.003
Immune serum	None	0.017
	Mercaptoethanol	0.017

chick embryo due to the presence of 7S globulins for this bacteria. The 7S globulins for S. gallinarum were not present and hence this organism was not cleared. However, 19S globulins were present in the yolk fluid for both organisms. It seemed that the macroglobulin antibody do not cross the embryonic membranes until at the time of hatching, when they are absorbed across the gut wall.

CHAPTER VIII.

DISCUSSION.

A study of the functional development of the reticulo-endothelial system is in part a study of the development of the host defence mechanism from embryonic to adult life. It is clear that in the adult animal this defence mechanism is dependent on the interplay of two systems; firstly, on the ability of an animal to actively produce antibody and secondly, in the presence of antibody (opsonin) the phagocytic cell should ingest and destroy the organism before it multiplies and kills the host. In 1961, Fishman implicated the phagocytic cell in yet another function. His work showed that when the macrophage ingests an antigen, an intracellular product is formed by the phagocyte which would apparently stimulate antibody producing cells (lymphocytes) to synthesise antibody.

From time to time the importance of the bactericidal activity of serum as a mode of defence *in vivo* against certain bacteria has been debated. Though antibody and complement constitute an efficient bactericidal mechanism *in vitro*, their efficiency *in vivo* is a matter of conjecture [Topley and Wilson, (1955)]. It has, however, been demonstrated that in the case of the chick embryo, should this mode of defence operate *in vivo*, it was not effective due to the small amount of complement in embryo serum (see ChapterIII).

The phagocytic cells of the reticulo-endothelial system of the chick embryo were able to remove efficiently the rough strains of bacteria, e.g. E. coli Lilly and E.coli K12, but only poorly the smooth strains, e.g. S. gallinarum 98, S. gallinarum 9240 and E. coli 2206. An inert particle like colloidal carbon was also phagocytosed. Phagocytosis by the cells of the chick embryo has been recorded by other workers. Earlier reports indicated that dye particles were phagocytosed by ectodermal, mesodermal and endodermal cells of the early blastoderm [Kiyono, (1918); Dabrowska, (1950)], but the phagocytic property of these cells disappeared two to three days after incubation. Perez del Castillo (1957) reported that carbon was not phagocytosed by the Kupffer cells of the liver in the 12 day old chick embryo. He found that efficient phagocytosis only took place on the 16th day of incubation. His results are in contradiction to the present work, where phagocytosis of carbon was recorded in the 12 day old embryo. Indeed, Nicol, Cox, Bilbey and Strachan, (1962) recorded phagocytosis of carbon by the 10 day old embryo. Kent (1961), using other inert particles like thorotrast and colloidal suspension of silver (Ag^{110}) was able to demonstrate phagocytosis in the four day old embryo. In general, the phagocytosis of particles by the cells of the chick embryo has been demonstrated histologically and is therefore of a qualitative rather than a quantitative nature. It is impossible from these results to obtain

any idea regarding the kinetics of phagocytosis of particles and their subsequent fate.

The results on the distribution of radioactive material showed that early in embryonic development the extra-embryonic membranes functioned as a phagocytic organ. Later the liver played a more prominent role. These results supported the findings of Kent (1961) who studied histologically the distribution of thorotrast and colloidal silver after intravenous injection.

In the present study it was found that, as the embryo matured, there was a concomitant increase in the rate of phagocytosis of rough strains of bacteria. It has been suggested by Benacerraf (1958) that an increase in the phagocytic index K may be due either to an increased number of phagocytic cells or to an increased efficiency of the same number of cells. To differentiate between these two aspects he introduced the corrected phagocytic index α which measured the phagocytic efficiency of the population of cells, making allowance for the increased number of phagocytic cells in relation to the increase in weight of the animal. If the increase in the rate of phagocytosis was merely due to the increase in the number of phagocytic cells related to the increase in liver size, then α should give a relatively constant value throughout development. However, if the α value increased, then this would indicate an increasing phagocytic efficiency of this system. When the

phagocytic index K was corrected in the chick embryo for the increased weight of the liver and spleen the α value was found to be fairly constant (see Chapter IV). This suggested that the increase in K as the embryo matured was due to the increased number of cells.

On closer analysis it is apparent that in the chick embryo the obtained corrected index α is not significant since, in the early stages of development, a considerable amount of the injected matter is phagocytosed by the extra-embryonic membranes. If one allows for this, it is apparent that the importance of the liver as a phagocytic organ increases markedly with the age of the embryo.

It is also apparent from this work that the corrected phagocytic index α is only of value in circumstances where serum factors are not limiting the rate of phagocytosis, a point ignored by Benacerraf (1958). This fact is apparent when one considers the rate of clearance of the smooth strains of bacteria. In this instance the phagocytic index K remains constant throughout the development and, if one were to apply the correction α to these results, the efficiency of the liver as a phagocytic organ would appear to decrease. Hence, if α is to be significant, one must study the rate of removal of a particle in circumstances where antibody does not limit the kinetics of this reaction.

It has been shown in the adult animal that a primary intravenous injection of a large dose of particles would re-

duce the clearance of a subsequent smaller dose of the same particle [Benacerraf, (1958); Jenkin and Rowley, (1961); Murray, (1963)]. Benacerraf, (1958) suggested that the reduced clearance of the second dose was due to the saturation of the phagocytic cells by the primary injection of particles. Jenkin and Rowley, (1961), Murray, (1963) have challenged this explanation and shown that when the second dose of particles was opsonised in vitro, then this dose was removed at the normal rate. They suggested that the first dose depleted the animal of opsonins, resulting in the slow clearance of the challenge dose due to the lack of opsonins rather than saturation of phagocytes.

Since recent investigations [Miler, (1963)] would suggest that the second explanation was more plausible, a similar method was used to detect the role of opsonins in phagocytosis by the cells of the reticulo-endothelial system in the chick embryo (see Chapter V). It was shown that with the range of particles tested, opsonins were necessary for the phagocytosis of both rough and smooth strains of bacteria in the chick embryo.

In the developing chick embryo the rough strains of bacteria were removed efficiently from the blood while the smooth strains were phagocytosed very slowly. The rapid removal of the rough bacteria was clearly due to the presence of opsonins for these bacteria in the circulation of the embryo, whilst the absence of opsonins for the smooth

strains explained the poor clearance of these strains of bacteria. It was also shown that the opsonins for the smooth bacteria, S. gallinarum 9S, appeared in the serum one day post hatching.

Examination of the serum from adult hens showed that there were opsonins for both the smooth and rough strains of bacteria tested, though the titre of antibody for the rough strains was higher than the titre of antibody against one of the smooth strains, S. gallinarum 9S.

There were several possibilities that might explain the presence of opsonins in embryo serum for rough strains of bacteria but not for the smooth strains. In view of the differences in opsonic titre of adult hen serum for these two phases of bacteria, one simple explanation could be that the passage of antibody from the hen to the developing ova in situ was dependent on a concentration gradient. However, subsequent work showed this to be an unlikely explanation.

Two other likelihoods remained. Firstly, it was proposed that the chick embryo could synthesise opsonins for E. coli Lilly during embryonic development, but the ability to synthesise opsonin for S. gallinarum 9S only appeared post hatching. Secondly, since antibody may exist in at least two distinct molecular sizes, it was reasoned that the opsonins for E. coli Lilly could be transferred from the yolk sac during foetal life, but the opsonins for S. gallinarum 9S could only be transferred after hatching, selection being

based on molecular size. It was shown that the second explanation was correct as the opsonins for E. coli Lilly were both small molecular weight globulins as well as large molecular weight globulins. By their sedimentation pattern in a density gradient these globulins resembled 7S and 19S molecules [Turner and Rowley, (1963)]. The opsonin for S.gallinarum 9S was a 19S globulin (see Chapter VII). It was shown that though the 19S globulin was present in the yolk fluid, this was not transferred across the yolk membrane, hence the failure of S. gallinarum 9S to be cleared by the embryo unless opsonised prior to injection.

The present study shows the selective transfer of proteins via the yolk sac to the chick embryo circulation. It seems that the transfer of antibody from hen to the embryo and newly hatched chick takes place in three stages. The first is the concentration of antibody, both of the 19S and 7S type by the follicular epithelium of the ovary into the yolk fluid during maturation of the ova. This concentration of protein is similar to the concentration of gamma globulin in the colostrum by the acinar epithelium of the udder of the cow [Dixon et al., (1961)]. The next stage is the transfer of the smaller globulin (7S type) across the yolk sac membrane. This situation resembles the yolk sac transfer in the rabbits and also the transfer of 7S globulins via the placenta in man and the primates [Brambell, (1958)]. The third step is the transfer of the 19S globulins via the

gut post hatching analogous to the transfer system in the ungulates [Dixon et al., (1961)]. In this case the yolk sac of the embryo is absorbed by the gut during the first five to seven days post hatching [Kent, (1961)]. During this time free absorption of the larger proteins take place. This is, of course, reflected by the ability of the one day old chicken being able to clear S. gallinarum 9S.

One of the earlier suggestions for the failure of the embryo to clear S. gallinarum 9S was the low titre of opsonins for this organism in the adult hen. It was argued that an increase in the titre by immunisation of the adult would increase the amount of opsonin incorporated in the yolk fluid of the embryo and hence transfer would ensue. The subsequent test of this hypothesis showed that embryos that developed from immunised hens did in fact clear S. gallinarum 9S. However, previous studies on the response of animals to an antigenic stimulus had shown that there is a primary synthesis of 19S globulins which is soon followed by the production of 7S globulins [Uhr, Finkelstein and Franklin, (1962); Benedict and Brown, (1962)]. Since these hens were immunised with several doses of S. gallinarum 9S and the eggs collected four weeks since the start of the vaccination course, it was reasonable to assume that the serum of the adult hen contained 7S antibodies for this strain of bacteria. These 7S globulins must have been transferred to the yolk fluid during egg formation and then been transferred to the embryo during development, as they were able to cross the membrane barrier.

Thus the phagocytic cells of the reticulo-endothelial system of the chick embryo were now able to clear S. gallinarum 9S from the blood. The validity of this argument was substantiated by the fact that when immune serum from vaccinated hens was treated with mercaptoethanol (0.1M of mercaptoethanol at pH 7.0 destroys the biological activity of the macroglobulin antibody), the opsonic activity for S. gallinarum 9S was retained. This indicated the relative absence of macroglobulin antibody in this sera. However, when normal hen serum was similarly treated, all activity against this smooth strain of bacteria was lost. This would suggest that in this instance as before, the selective transfer of antibody is not based on a concentration gradient but on molecular size.

In the adult animal, while opsonins are essential for phagocytosis, these opsonins seem also to be involved in the killing of bacteria inside the phagocyte. Jenkin (1963a) showed that S. typhimurium was able to multiply intracellularly in the absence of opsonins, though in the presence of these opsonins the bacteria were killed. It is clear that the phagocytic cells of the reticulo-endothelial system in the chick embryo are able to engulf bacteria from the blood. The rate of removal increases as the embryo matures and phagocytosis may be followed by killing of the ingested organisms.

The evidence of such intracellular killing came from

experiments in the older embryos where there was release of P^{32}-labelled particulate matter into the circulation 15 minutes after the clearance of viable bacteria (see Chapter IV). This is similar to the release of P^{32} from mouse peritoneal macrophages after ingestion of labelled bacteria [Jenkin, (1963b)]. When viable counts were made on samples of liver, spleen and membrane (the principal organs concerned with phagocytosis) there was definite evidence of very good killing, though in the early embryos (10 to 14 days of incubation) killing was not marked.

An apparent paradox in the present study is the slow rate of killing of the smooth strain S. gallinarum 9S following opsonisation in vitro (see Chapter VI). The failure of the phagocytic cells in the embryo to kill even very small doses of bacteria under apparently good opsonic conditions might be explained as follows. It has been shown that immunisation of mice with a heat killed vaccine of S. typhimurium does not offer protection to challenge with the live bacteria [Hobson, (1957)]. It has been suggested that in this case not all antibodies are effective in promoting destruction of the bacteria following phagocytosis. It seems that antibody to a heat labile antigen may be necessary to neutralise antigen sites on the surface of the bacteria that otherwise may interfere with the intracellular enzymatic degradation of the engulfed bacteria [Jenkin, (1964)]. This explanation may be applicable for the fail-

ure of S. gallinarum 9S to be killed by the phagocytic cells, i.e. the right type of antibody was not present in the serum used for opsonisation. Another reason may be the quantity of antibody needed to initiate the bactericidal mechanism. It could be argued that a minimum number of sites of antigen-antibody complexes on the bacterial surface are needed before the bacteria can be effectively degraded.

It is apparent that in any population of phagocytes there are always a number of incompetent cells that cannot kill ingested organisms [Jenkin and Benacerraf, (1960); Mackaness, (1960)]. These cells may be very immature phagocytes or senile phagocytic cells. This would imply that a number of bacteria will always multiply intracellularly. In the case where opsonins are not limiting, these bacteria would be re-phagocytosed after release from the initially infected phagocyte. If such a situation occurred in the adult animal it seems reasonable to assume that in the developing embryo a greater number of immature phagocytic cells would be present. Indeed younger embryos would contain more incompetent cells than older embryos. This was supported by the evidence that when E. coli Lilly was injected into embryos the older embryos were more competent to kill this organism than the younger embryos (see Chapter VI). Hence, even under conditions where opsonins were not limiting, the proportion of competent cells to incompetent cells may decide the fate of the host.

In the case of S. gallinarum 9S where opsonins are not normally present in the chick embryo, both the number of incompetent cells and the limiting amount of antibody would increase the likelihood of the death of the host. Thus, when opsonised S. gallinarum 9S is injected into the embryo, it is possible that not only a percentage of bacteria would multiply intracellularly, but also the progeny of these organisms would be unopsonised and eventually fatal bacteremia would result in the host. At best under these circumstances, host resistance can only be measured by the rate of clearance of the organism.

It has also been suggested that a minimal amount of opsonins are needed to be present on the bacterial surface before even competent cells can kill the bacteria. This is supported by the evidence that in the hatched chicken where antibody is present due to absorption of the yolk, the bacteria are effectively killed. However, when antibody is not present, as in the case of the embryos, the bacteria multiply. Hence an increase in the number of competent cells, together with increased supply of opsonins, could explain the resistance of the seven day old chicken to 2×10^7 S. gallinarum 9S while embryos were susceptible to less than 20 organisms.

It is apparent from the present study that the phagocytic efficiency of the reticulo-endothelial system develops as the embryo grows older. It is also obvious that the ability of certain cells of the embryo to phagocytose part-

icles is present at some of the earliest stages in development. It is possible that during development there is a selection for specialisation until, in adult life, only certain types of cells are involved in active phagocytosis. However, there is no doubt that these scavenging cells play an important role in the ingestion of effete self-components during ontogenesis [Romanoff, (1960)]. Since the present study suggests that recognition of foreigness by the embryo is dependent on serum factors, the question arises as to whether a similar mechanism also exists for the identification of functional self from non-functional self. Studies on the adult rat suggest that effete and damaged erythrocytes are removed by the liver only in the presence of serum [Jenkin and Karthigasu, (1962)]. If humoral factors are involved in the ontogenesis of the embryo, it would imply that specific recognition factors are present from the very early stage in development and possibly independent of passive transfer from the mother. This study emphasises the importance of antibody (opsonin) in aiding the recognition of injected foreign particles by the phagocytic cells of the reticulo-endothelial system of the chick embryo.

Since the phagocytic cell may be also involved in the pathway to antibody synthesis, it is important to investigate the intracellular events that follow phagocytosis. In this connection it is interesting to note that the population of macrophages in the early embryos are less com-

potent to kill ingested bacteria than the phagocytes of the older embryos. It is possible that, as the embryo matures, there is also a change in the intracellular functional capacity of the macrophage to deal with more antigens. This may be the reason why in some embryos like the foetal sheep only certain antigens can induce antibody synthesis [Silverstein et al., (1963)].

There is a great need for increased knowledge about the events that follow phagocytosis and its relationship to subsequent antibody synthesis, since it may well be that the fate of antigens within these cells determines the immunological competence of the developing embryo.

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