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THE FUNCTIONAL DEVELOPMENT OF THE RETICULO-ENDOTHELIAL SYSTEM IN RATS

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A thesis submitted for the degree of
Doctor of Philosophy

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ACKNOWLEDGEMENTS

ABSTRACT

A study was made of the development of the reticulo-endothelial system in foetal and natal rats.

A surgical method was devised whereby clearance studies using viable and non-viable particles were employed to ascertain the phagocytic ability of various populations of cells in foetal rats. It was shown that this property of the reticulo-endothelial system increased progressively during foetal life, remained static during neonatal life and then increased rapidly. The distribution of these phagocytic cells was demonstrated by histological methods using light and electron microscopy and also by the organ distribution of isotopically labelled bacteria.

The bactericidal ability of the cells of the reticulo-endothelial system in foetal and natal rats was investigated in regard to two strains of bacteria. It was shown to be absent in foetuses and became obvious towards the end of the suckling period.

The presence of 'natural' opsonins to *Salmonella typhimurium* C5 in serum from rats of different ages was investigated by quantitative and qualitative methods. It was shown, by mouse and chick assays, that opsonins were present in all of these sera and that the opsonic activity remained at the same level until shortly before weaning, from which time the activity increased rapidly.

Characterisation of these opsonins by biochemical techniques demonstrated a heterogeneity of these antibodies and showed that in adults the major proportion consisted of macroglobulins. In foetal rat serum the opsonic activity was mainly in the non-macroglobulin fraction. It was demonstrated, however, that foetal serum contained a similar proportion of macroglobulins to that found in adult rat serum. The preponderance of 7S-type antibody was attributed to a passive transfer of maternal antibody.

It was considered that this study provides a technique and background information necessary for future studies concerned with the quantitation of the production of serum proteins, especially antibodies, by foetal rats.

This thesis contains no material previously submitted by me for a degree in any University and to the best of my knowledge and belief it contains no material previously published or written by another person except when due reference is made in the text.

PETER C. READE

February, 1964.

CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

The way in which a host defends itself against infectious disease has considerable importance in the survival of individuals and of communities and has therefore attracted the attention of historians, philosophers and scientists alike.

Before the dawn of history man was unwittingly using micro-organisms to prepare forms of food and drink and he had also learnt to combat them by preserving food. The earliest known reference to infections was made in an Egyptian papyrus of about 2000 B.C., but it was not until the fourth century B.C. that Thucydides, the first writer of scientific history, reported that those who had suffered from smallpox were subsequently protected from the disease. Various prophylactic measures, particularly against smallpox, were practised in China in the 15th century and later in Asia Minor in the 16th century. The cause of infectious diseases and the means by which they spread was accurately predicted by Fracastoro in 1546. In the next century Leeuwenhoek (1676), renowned for his invention of the microscope, saw and described bacteria.

The first experimental evidence in the Western world for the value of prophylactic induction of an infectious disease came from the work of Jenner published in 1798.

Despite energetic investigation and much speculation it was nearly 150 years before Davaine (1850) reported the presence of micro-organisms in the blood of sheep dying from anthrax and also that high dilutions of this blood would produce the same disease in other animals, thereby positively implicating for the first time micro-organisms in the aetiology of infectious diseases.

It was Koch (1876) who because of his improved bacteriological techniques was able to isolate the anthrax bacillus in pure culture from the blood of infected animals and thereby substantiated the claims made by Davaine. Pasteur in developing the concept of the specificity of infectious diseases extended this knowledge and with classical experiments investigated the prevention of disease by introducing methods of immunization. From his work Pasteur in 1880 hypothesised that the state of immunity to microbial disease resulted from the elimination from the host of some essential nutriment at the time of the initial experience with the micro-organism. That the opposite to this was in fact the case was shown by Roux and Yersin in 1888. Their work soon to be supported and amplified by Behring and Kitasato (1890) demonstrated the advent of a specific substance in an animals blood following immunization with a toxin. The latter workers also demonstrated that this immunity could be conferred on another animal by injecting serum from an immune animal.

In 1893 Buchner demonstrated the presence in normal serum of a heat-labile bactericidal substance now known as complement. Soon after this Bordet (1895) showed that the serum from animals immunised with cholera vibrios had two antibacterial factors - one the heat-labile factor of Buchner and the other a specific heat-stable factor referred to as 'antibody'. Ehrlich (1896) soon introduced methods for standardising toxin and antitoxin and thereby greatly contributed to their rational use in the treatment of certain infectious diseases.

Another *in vitro* property of antibody was demonstrated by Pfeiffer in 1894 when he showed that the serum of animals immunised against the cholera vibrio was bacteriolytic for that organism. He also showed that transfer of this serum to another normal animal conferred immunity. Durham (1896) described the aggregation of bacteria by the specific immune serum and called this phenomenon agglutination, a reaction which Landsteiner (1900) soon employed to identify human blood groups. At about the same time (1897) Kraus described the formation of a precipitate when culture filtrate was mixed with specific immune serum. This reaction between soluble antigen and antibody is a test which has wide laboratory applications and is used in clinical and forensic medicine.

These accumulating facts made it reasonable to assume

that immunity to infectious diseases could be explained by the changes which took place in the serum of an infected host and gave rise thereby to the theory of Humoral Defence.

It is interesting that at the same time as this story of the part played in immunity by serum factors was unfolding importance was also being given to the properties of special cells in the body which were able to accumulate particulate matter introduced into the body by intravenous injection. From the middle of the 19th century numerous investigators have reported their findings on the fate of intravenously injected particles - Wyssokowitsch in 1886 being credited with having been the first to inject micro-organisms and to study their removal from the circulating blood. These early workers were aware of the part played by the liver, spleen, lungs and bone marrow - especially the first of these organs - in removing the bulk of injected material.

An enormous volume of literature concerning the cellular uptake of particulate matter amassed during this period with the emphasis being on the delineation of anatomical systems. This approach led to these predatory cells being given names according to their morphology or location as for example the 'sternzellen' of Kupffer (1878). Ranvier (1890) seems to have been the first to ascribe some purposeful physiological function to these cells and to distinguish them from other cells such as fibroblasts which sometimes

accumulate particles to a limited extent. He described these cells as a separate group and called them clasmatocytes. His observations of an in vitro system of these cells in which he saw their long processes breaking off were probably misinterpreted when he suggested that this was a step in the nutrition of fibroblasts.

It was Metchnikoff's (1887) interpretation of his almost legendary experiments which added to the existing knowledge of phagocytosis and which suggested to him the part played by phagocytic cells in host defence. Like Ranvier he conceived a system of cells disseminated throughout the body. To these cells, however, Metchnikoff ascribed a protective function. He suggested that leucocytes or microphages could be mobilised quickly in acute inflammatory processes while the large mononuclears or macrophages were more concerned with chronic inflammation and the uptake of large corpuscular material such as the Plasmodium of malaria. It was this interpretation which prompted him to introduce a theory of immunity - the theory of Cellular Defence - which depended upon the ability of certain cells to remove particulate matter from the blood and tissue fluids.

A verbal battle between the protagonists of these two apparently widely divergent views on the mechanism of immunity endured for many years. Because of the energy with which the antagonists sought to substantiate their arguments many important discoveries were made which

eventually led to the reconciliation of their differences.

Denys and Leclef in 1895 demonstrated enhanced phagocytosis of streptococci in immunised animals, and later, in 1903, Wright and Douglas expanded this knowledge and introduced the concept of serum factors which enhanced phagocytosis. They showed that the *in vitro* phagocytosis of *Staphylococcus pyogenes* by human leucocytes was much greater in the presence than in the absence of human serum. These workers concluded that as the serum had no bactericidal activity or direct action on the leucocytes it must contain substances which modified bacteria and rendered them more susceptible to phagocytosis. These substances were called 'opsonins' and it was implied that they played a primary role in the eventual destruction of micro-organisms. Many investigations have since demonstrated the importance of opsonins in the phagocytosis of bacteria and of inert particles such as carbon and starch (Nelson and Lebrun, 1956), and bentonite (Stollerman, 1961). Many of the fine details of the interaction between opsonin, particle and phagocytic cell have not yet been elucidated, but the general concept gives a good perspective of the part played by serum factors and phagocytic cells in host defence against micro-organisms.

Following the description by Metchnikoff of a widespread system of phagocytic cells for the purpose of both local and general defence there were many investigations of the cells of this system. These investigations were made more definitive

by the use of a group of dyes discovered by Ehrlich. These had the property of forming ultra-microscopic sols that were removed from the circulating blood by certain cells. As a result these cells became "vitally" stained. These observations were initiated in 1904 by Ribbert who used lithium carmine for vital staining. Further investigations were made in 1909 by Goldman who systematically examined many tissues after injecting a variety of these dyes into animals. The latter worker suggested that the various cells which accumulated these dyes from the circulation were of the same type. These cells which were apparently the macrophages of Metchnikoff were found amongst the lining cells of various vascular beds, distributed throughout the connective tissues of the body and forming a reticulum in organs such as the spleen, liver, bone marrow and lymph nodes. Maximow in 1906 called the macrophages in connective tissue "resting-wandering" cells because of their change from a fixed state to a migratory behaviour observed in local inflammation. In 1913 Aschoff and Kiyono after investigations with lithium carmine called these cells "histiocytes". It was at this same time that these latter investigators first suggested the idea of a 'reticulo-endothelial system' which was later further developed into a more definite concept by Aschoff and Landau in 1924.

Taliaferro and Mulligan, when reviewing this subject

in 1937 with particular reference to malarial infections, drew attention to the somewhat limited concept of Aschoff and his colleagues in that their view of the reticulum and endothelial elements overlooked the importance of the monocyte of the blood as part of the macrophage system. They reported that Maximow (1906) had demonstrated to their satisfaction that in local inflammatory disturbances lymphoid cells of the blood migrated into the lesion and rapidly transformed into macrophages. These cells they claimed behaved in a similar fashion to those cells derived from the reticulo-endothelial system as described by Aschoff et al. It was their suggestion therefore that this defence system of cells should be given the more comprehensive connotation of "lymphoid-macrophage" system to include both macrophages and their precursors.

In 1955 Rebuck and Crowley surveyed the literature concerning the transformation of lymphocytes to macrophages and also performed many experiments in which glass coverslips were applied to surgically excoriated skin. Migrating cells attached themselves to the coverslips which were removed at regular intervals, fixed and stained and examined microscopically. By this method they claim to have shown the transformation from lymphocyte to macrophage. Florey (1962) doubts this evidence: firstly, because Harris (1953) and Gowans (1961) have shown that lymphocytes unlike monocytes and polymorphonuclear leucocytes do not stick to glass:

secondly that electron micrographs have not positively demonstrated the emergency of lymphocytes from venules although monocytes and polymorphs have been seen frequently to emerge; and thirdly because these investigations are most frequently made with fixed tissues the transformation of one cell type to another has never been seen. Nor has it been seen in tissue culture (Medawar,1940) or in rabbit ear-chambers (Ebert, Sanders,and Florey,1940). In contrast to these observations Holub in 1958, described experiments in which he placed diffusion chambers containing lymphocytes from the cisterna chyli into rabbits. Within a few days macrophages appeared and by the sixth week these were the predominant cells within the chamber.

While Marchesi (1961) has now described the passage of lymphocytes from blood vessels to an extra-vascular location, the work of Holub remains as a single piece of evidence to support the transformation of lymphocyte to macrophage and thereby its connection with the phagocytic aspect of cellular defence mechanisms. Until more evidence is available it seems unreasonable to implicate the lymphocyte as a macrophage precursor.

Payling Wright (1953), described the reticulo-endothelial system as a collection of widely distributed mesenchymal cells characterised by the readiness with which they phagocytose small particles and coarse colloids. In 1956 Marshall attempted to define the reticulo-endothelial system on a

histochemical basis by using the method described by Rio-Hortega (1932). He had shown that the cells of this system had a pronounced affinity for silver and some other metals. While the chemical basis for this metal staining (metalophilia) was not known it was seen that the cells demonstrated by this method, although more numerous, were similarly distributed to the cells of the reticulo-endothelial system demonstrated by functional methods of particle uptake. Marshall suggested that the greater number of cells shown by the metal staining methods might be accounted for by the metals greater penetration or it could be that this method being chemical has greater sensitivity. Marshall also showed that lymphocytes and primitive reticular cells were unresponsive to silver and were not therefore of this system. Both blood monocytes, which were shown by Marshall to be feebly argyrophilic and by Ebert and Florey (1939) to transform into macrophages in rabbit ear-chambers, and histiocytes (Baillif 1941) seem to represent less active but immediate precursors of macrophages. At the present time, therefore, while the phagocytic function of this system has been well defined, the histogenesis of the component cells is in some doubt and more refined techniques need to be developed before this problem can be resolved.

From the time that it was first shown that particles were removed from the circulating blood attempts have been made to quantitate this phenomenon.

Some of the earliest references to the rate of removal

of bacteria were made by Bull (1915) and Manwaring and Coe (1916). The former investigator found that after intravenously injecting a rabbit with a suspension of Salmonella typhi the number of viable organisms that could be recovered from the circulating blood was frequently reduced to nil in fifteen to twenty minutes. The latter workers however, injected under similar circumstances, a dose of Streptococcus pneumoniae and found that twenty-five per cent of the organisms were still circulating up to one hour later.

Experiments such as those described above established that in the same animal species bacteria of different types were removed by the reticulo-endothelial system at different rates. In addition to these investigations Drinker and Shaw (1921) developed a quantitative assay of organ uptake using manganese dioxide and commented upon the rapidity with which this material was removed from the blood- mainly by the liver in the dog, rabbit, guinea pig, rat, chicken turtle and monkey, and the lung in the cat from which organ it was subsequently transferred to the liver. Foot (1919) and Wislocki (1924) quantitated the distribution of carbon in rabbits by histological methods and showed that most of this colloid was extracted from the circulation by the cells of the reticulo-endothalial system of the liver, spleen and kidney. Small amounts were found in other organs such as the bone marrow and lymph nodes. Cappell (1929, 1930) using colloidal

iron (saccharated iron oxide) studied histologically the organs of mice and rats after various doses and at various time intervals after injection of the colloid. He found that the colloidal iron was removed rapidly by the cells of the reticulo-endothelial system of the liver and spleen and was distributed in a similar fashion to that of carbon. Both Foot and Cappell remarked on finding carbon and iron respectively in some of the parenchymal cells of the liver as a part of the redistribution of the materials following the initial rapid clearance from the blood by the hepatic phagocytes.

In an effort to quantitate phagocytosis more accurately and to gain a more intimate knowledge of its mechanisms various workers from about the time of Leishman in 1902 have applied _in vitro_ techniques for this purpose. Leishman described a method with which he investigated the phagocytosis of bacteria by leucocytes by counting, with the aid of a microscope, the number of particles present in the leucocytes at various times following cell-parasite interaction. Later investigations, chronologically important amongst which appear to be those of Wright and Douglass (1903), Hamburger (1912), Fenn (1921), Fleming (1931), Mudd _et al._ (1934), Hanks (1940), Mackaness (1952), Rowley (1958) and Cohn and Morse (1959) have progressively refined and widened the application of _in vitro_ cell systems to the study of host-parasite interactions. The results of investigations of

this type have enabled a number of aspects of the activities of the cells implicated in host defence to be studied in precise, relatively simple, well controlled systems which avoid the many unknown parameters of _in vivo_ experiments but which are only of any significance in the understanding of host defence mechanism if they can be related to _in vivo_ situations.

It was from an _in vitro_ study of phagocytosis that Fenn (1921a) showed that a mathematical formula could be used to calculate the rate of phagocytosis. The equation that he derived was $K = \frac{A}{\log(A-X)} \cdot \frac{1}{t}$ where K = the rate of phagocytosis, A = the total number of particles and X = the number of particles ingested at time, t. He demonstrated with particles of carbon and quartz that the rate of phagocytosis (K) was dependent primarily upon the chance of collision between particle and cell and was independent within limits of the number of particles present.

Fenn (1923) demonstrated selective phagocytosis when he showed that under certain conditions carbon particles were phagocytosed _in vitro_ four times more rapidly than quartz particles. He commented that neither of these particles were phagocytosed to any extent in the absence of serum. Mudd _et al._ (1934) considered that this selection could be due, at least in part, to the differences in absorption of phagocytosis-promoting substances from the serum. In this connection it is interesting to note that Benacerraf _et al._

(1955) have shown that in *in vivo* experiments colloids are cleared at different rates in the same dose range. Although they omit reference to serum factors it seems possible, in this instance also, that this is a reflection of the difference in adsorptive properties of the colloids tested.

Maxfield and Mortensen in 1941 used radioactive colloidal thorium dioxide (Thorotrast) in elegant experiments to test their hypothesis that the removal of small particles was a matter of chance resulting from their random movement in the circulating blood and therefore their random chance of contacting a phagocyte. They assumed that because of the relatively small dose of particles injected phagocytosis was almost instantaneous once the particle had contacted the phagocytic cell and that the capacity of the cell to continue phagocytosing particles was not measurably affected until the ingested particles constituted a mass of the order of a few per cent of the cell colume. This could mean the ingestion of literally millions of thorium dioxide particles 10 Angstroms in diameter per phagocyte. In verifying these assumptions Maxfield and Mortensen showed that the particles were removed from the circulation in a triphasic exponential fashion with a constant fraction of the total number of particles being removed for any given short period of time.

The suggestion that radioactive markers could be incorporated in micro-organisms so that these could be used in clearance studies for more adequate quantitation of

phagocytic activity was made by Berry and Spies in 1949. It was not until 1959 however that Benacerraf and his colleagues, who had been engaged in extensive study of the *in vivo* function of the reticulo-endothelial system since 1953, applied this technique to further investigate the kinetics of removal of bacteria by this system. Bacteria grown in a phosphorous-free medium to which radioactive phosphorus (P^{32}) was added in the form of orthophosphate incorporate this label, and their removal from the circulating blood may be demonstrated therefore by assaying serial blood samples for radioactivity. This method is essentially the same as described by Halpern *et al.* (1953) who used carbon particles in their extensive experiments. Blood samples were haemolysed in dilute sodium carbonate and the resultant suspension assayed for carbon content by spectrophotometry. The results of *in vivo* phagocytic experiments from this large group of workers have been expressed as the phagocytic index (K) and have been derived after plotting the $\log_{10}$ of the concentration of particles against time by an equation similar to that proposed by Fenn - $K = \frac{\log \text{conc. a} - \log \text{conc.b}}{tb - ta}$ where t = time at a and b. From this group of workers has come information concerning the clearance of various particles - both vital and non-vital - in mice, rats, guinea pigs and rabbits. They have shown that in relation to any one particle the functional activity of the reticulo-endothelial system varied in these species, but that the organ distribution of injected colloids was similar. They demonstrated that the

rate of clearance was dose dependent and that above a minimal level, K varied inversely with the dose (D) injected so that $K \times D$ = a constant. These workers derived the formula $= \frac{W}{W.l.s.} \cdot \sqrt[3]{K}$ where W, l, and s represent the weight of the entire animal, of the liver and spleen respectively illustrated that a more meaningful phagocytic index was obtained by taking into account the size of the liver and spleen.

Members of this group also studied the effects on the phagocytic capacity of the reticulo-endothelial system of portal blood flow (Benacerraf _et al_, 1955), of age (Benacerraf _et al_, 1957), of blocking doses of colloid (Benacerraf _et al_, 1955; Biozzi _et al_, 1957), and of a mixture of particles (Benacerraf _et al_, 1955 and Biozzi _et al_, (1956). The effect that particle size has on the rate of clearance has been investigated by Dobson (1957) and it has been shown that cells of the reticulo-endothelial system can phagocytose particles which vary greatly in size - from 12 Angstroms (Smulders, 1951) of some vital dyes to 11 microns for pigeon red cells (Halpern _et al_., 1957). In general terms it has been found that the larger the particle the more rapid was the rate of clearance.

In all of these experiments, as commented upon by Benacerraf (1958), Chevremont (1948) and numerous workers before them, the major part of the phagocytosis of particles is attributable to the hepatic phagocytes of the blood sinusoids of the liver. These cells are strategically situated to monitor the blood stream. In _in vivo_

studies the liver has been shown (Beracerraf _et al_., 1957) to remove approximately 90 per cent of an injected dose of colloid within 30 minutes. Wardlaw and Howard (1959) compared the clearance of 13 species of bacteria suspended in serum and perfused through isolated rat livers. They found that the percentage removal varied between 36 and 87, illustrating a selective uptake. Howard (1961) suggests that the apparent resistance of bacteria to phagocytosis, commented upon by Biozzi _et al_, (1960), might be explained by the finding that surface charge affects the susceptibility of bacteria to phagocytosis (Mudd _et al_., 1934). Choucroun (1936) showed that the charge on individual bacteria in a culture varied considerably and followed a Gaussian distribution. It is considered that this variation in charge might account for the variation in time over which the exponential phase of blood clearance of particles occurs.

Since Denys and Leclef (1895) demonstrated enhanced phagocytosis in immunised animals, and Wright and Douglas (1903) introduced the concept of opsonins, numerous experiments have demonstrated the significance of these antibodies, whether in normal serum or in immune serum. The importance of opsonins in host defence has been emphasised by Jenkin and Rowley (1961) who have examined the part that opsonins play in phagocytosis of vital and non-vital particles and in so-called "R.E.S. blockade". It is generally held that opsonins act by coating particles and thereby make them

more amenable to phagocytosis, and it seems therefore that their action is one of changing the surface properties of the particle, perhaps the surface charge.

There are a variety of events which can follow the phagocytosis of a particle, and these are of considerable significance to a host in its defence against micro-organisms. If the particle is indigestible it can remain in the cells of the reticulo-endothelial system for a very long time (Florey, 1962); it can be deposited in an extra-cellular situation (Casley-Smith, 1962); or be transferred to parenchymal cells as already mentioned. If, however, the phagocytic cell can digest the particle it can be completely disrupted and disposed of by macrophages and polymorphonuclear leucocytes (Florey, 1962). The fate of ingested bacteria, is not so simply decided and depends upon the type of bacteria (Suter, 1956), and the presence (Robertson and Van Sant, 1939) and probably the titre (Jenkin and Rowley, 1961) of opsonins. Suter described three groups of bacteria with regard to their fate within phagocytic cells. Firstly obligate extracellular parasites such as *Staphylococcus aureus* which are generally unable to multiply or survive after phagocytosis (Kapral and Shayegani, 1959); secondly, facultative intracellular parasites such as *Mycobacterium tuberculosis* and *Salmonella typhi* which are capable of multiplication within R.E. cells and in this way produce disease; and thirdly obligate intracellular parasites

such as _Mycobacterium leprae_ which can only multiply within the cytoplasm of cells.

Most of this enormous field of study in which the humoral and cellular aspects of the reticulo-endothelial system have been energetically investigated over the past century has been directed towards the defence which natal animals exhibit towards possible pathogens. It has been tacitly assumed that this defence mechanism starts at or about birth and that before this the embryo is immunologically incompetent (Burnet and Fenner, 1949). Billingham _et al_. (1956) with elegant experiments provided what was apparently direct confirmation of this prediction of immunological unresponsiveness to contact by antigenic material in foetal or early neonatal life and was termed by them "acquired immunological tolerance". They injected newborn mice of an inbred strain with spleen cells from another inbred strain of mice and found that the injected mice in later life could not mount an immunological reaction to grafts from the donor strain. The findings of these workers apparently provided an answer to Burnet and Fenner's question as to why animals are unresponsive to their own constituent antigens. This phenomen of immune tolerance has many similarities to immunoligical paralysis first described by Felton (1949) in regard to pneumococcal polysaccharides in mice. Immune paralysis is specific and can be induced in adult animals with doses which vary with the antigen used. Both of these phenomena may have a

finite duration, are to some extent dose dependent and appear to present the converse of the immune response.

It has also been assumed, however, that a developing embryo relies upon immune responses of its mother for its survival both *in utero* and *post partum*, and that this protection is provided by way of passive maternal transfer (Brambell *et al*.,1951)

The study of maternal transfer of immunity was established by Ehrlich in 1892 when he showed that immunity against tetanus could be transferred from immunised mice to young mice both before and after their birth. This demonstration established from the beginning that in mammals at least immune substances could be transferred from a mother to her young by at least two routes; via the uterus before birth and by mammary secretions after birth. From the animals that have been investigated a somewhat confusing picture emerges: dogs, rats and mice have both uterine and mammary transfer, rabbits, guinea pigs and man appear to have an almost exclusively uterine transfer while the domesticated ungulates investigated appear to transfer immunity to their young by way of the colostrum in the first few hours of suckling (Brambell, *et al*.,1951).

On the surface, the mechanism of maternal transfer in mammals appears easy to explain - the placenta on the one hand perhaps acts as a filter through which maternal antibodies percolate along with other substances necessary for

the animals nutrition and growth, while on the other hand antibody and other proteins are secreted into the colostrum and milk which the embryo drinks and absorbs via its gut wall. The latter mechanism appears in essence to be true, but there is evidence which suggests that the former is an erroneous simplification.

The mammalian allanto-chorionic placenta seems to have evolved to bring maternal and foetal circulations to close approximation for the purpose of exchange without mixing. Grosser in 1909 proposed a scheme for the classification of placentae according to the number and type of tissues intervening between foetal and maternal bloods. He observed that in certain animals the foetal chorion was directly apposed to the uterine epithelium, while in others there were varying degrees of erosion of maternal tissues. Mossman in 1926 added to this classification by describing examples in which foetal tissues were also removed. There existed therefore a range of placental types from that for example of the horse and pig in which the six tissues persist - the capillary endothelium, mesenchyme and trophoblast of the foetus and the epithelium, connective tissue and capillary endothelium of the uterine mucosa - to the simplest type of placenta of the rat, mouse, rabbit and guinea pig in which only foetal endothelium separates the two circulations.

This rather mechanistic approach coupled with the fact that antibody passed to the foetus led to attempts to

correlate the transfer of passive immunity and the structure of the placenta. It was assumed that the fewer the layers separating the two circulations the greater was the probability of diffusion from one to the other. This gains some credence when results show that there is an absence of foetal transfer in the horse and pig which have a six layered barrier, that there is considerable foetal transfer in rabbits which have a single layered barrier and that dogs take up an intermediate position in which some antibody is transferred via a four layered barrier. The human, however, with a three (or two, nearer parturition) layered barrier and exclusively foetal transfer, and rats and mice with a single layered barrier but with both foetal and neonatal transfer, are not completely reconcilable with Mossman's concepts. If it is considered that transfer occurs by simple diffusion these variations are not surprising, however, since the rate of diffusion across a number of semi-permeable membranes is limited by the least permeable layer and not by their total numbers.

Krukenberg in 1888 injected a barium sulphate suspension and non-pathogenic micro-organisms into pregnant rabbits and recovered neither of these from the conceptuses. Similarly Wislocki (1921) administerdd carbon particles to a pregnant dog, and to pregnant cats, rabbits and guinea pigs and obtained the same results. He commented on the absence of carbon particles from the endothelial cells which line the maternal vessels in the placenta of the dog and cat while

their counterparts in the liver, spleen and bone marrow were loaded with phagocytosed carbon particles. Goldman (1909) however used trypan blue in similar experiments on mice and rats and found that this ultra-microscopic sol had in fact penetrated to some of the foetal tissues to be phagocytosed by trophoblastic giant cells, chorionic epithelium and the epithelium of the vitelline membrane, but had apparently been prevented from entering the foetuses. Wislocki further reports that trypan blue must be on the border line of transmission, for traces can enter the foetal circulation in the rabbit and guinea pig.

Recent experimental evidence has provided information which calls for a revision of the earlier mechanical somewhat teleological concepts. Brambell et al. (1951) showed that by interfering with the vitelline circulations of rabbit conceptuses transfer of passive immunity was prevented. It was suggested therefore that the route of transfer was via the uterine lumen and through the yolk sac (which is morphogenetically part of the gut wall) to the foetal circulation. Brambell and Halliday (1956) and Barnes (1957) with similar studies have shown that rats and guinea pigs have a similar mechanism, and in addition, that rats transfer antibody via the gut both before birth - apparently by drinking embryonic fluids - and postnatally from mammary secretions. To add to this evidence of yolk sac transfer, Dempsey (1960) has shown that the cells of the yolk sac are actively pinocytic and that

labelled antibody introduced into the uterine lumen can be shown to pass via these cells into the foetal tissues. Further evidence against the early ideas of simple diffusion has collected since it was shown by Hartley in 1951 that the transmission of antibodies from the maternal to the foetal circulation is a selective process. He showed that homologous diphtheria antitoxin prepared in guinea pigs and administered passively in late pregnancy passed readily into the foetus; horse diphtheria antitoxin on the other hand did not, and neither did pepsin digested homologous antiserum although of much smaller molecular weight. According to Wiener and Berlin (1947) molecular size perhaps plays some part, for they have shown that complete Rh agglutinins which are estimated to have a molecular weight of about 500,000 do not pass the human placenta at all, whereas incomplete Rh antibodies, with a probably molecular weight of about 170,000 appear to pass this placental barrier with ease. Brambell <u>et al</u>. have however shown that 7S equine diphtheria antitoxin reaches concentrations of not more than 2 per cent of those achieved by either 7S or 19S homologous antibody. Valquist (1958) suggests that the permeability is in some way connected with certain gamma globulin fractions. Complete Rh antibodies, isoagglutinins and typhoid O antibodies are known to be prevented from entering the foetal circulation and these have been shown by Enders (1944) to be more closely related to beta- than to gamma-globulins.

It can be seen that passive maternal transfer of immuhity has a place of singular importance in the concept of an animals early defence against the external environment. and that it appeared to be a reasonable natural consequence of the proposition of embryonic immunological immaturity. In contradistinction to this proposition, however, evidence has accumulated in recent years of the way in which immunological competence develops in the chick embryo (Karthigasu and Jenkin, 1963) and in foetal sheep (Silverstein, Uhr, and Kraner, 1963; Silverstein, Thorbecke, Kraner and Lukes, 1963). The former workers demonstrated by _in vivo_ phagocytic experiments that the phagocytic index (K) of the rough strains of bacteria used increased with increasing embryonic age and that the rates of clearance appear to be dependent upon the presence or absence of serum opsonins. The origin of these opsonins was not established, but it was argued that some of these immune substances could be produced by the embryo, and if this was so the embryo was to some extent at least immunologically independent of maternal transfer. Silverstein and his co-workers stimulated _in utero_ a series of foetal lambs with a variety of antigens and at determined times collected their cord blood. The blood samples which were assayed for antibody against the experimental antigens clearly demonstrated the presence of specific antibody. Immuno-electrophoretic techniques showed that both 7S gamma-globulins and 19S beta-2N-macroglobulins were produced.

These are the types of antibody produced in adults animals (Uhr and Finkelstein, 1963). The experiments further showed that both of these types of antibody were produced in spleen cells and to a lesser extent in the cells of the lymph nodes draining the initial site of injection of antigen.

While evidence such as this has widened and complicated the field of investigation and negated the assumption that foetal animals are immunologically incompetent, it has not detracted from the significance of passive maternal transfer.

In addition to demonstrating a continuously developing foetal immunological competence during the period of gestation, these foregoing experiments appear to support the contention that the reticulo-endothelail system is not only an organ of defence but has the primordial property of disposing of, or preparing for re-utilisation, the hosts particulate waste products. These latter physiological functions are frequently overlooked, or at the best given second place to the pathological aspects. Mammalian embryos in particular therefore find themselves confronted with what appears to be, from the present state of our knowledge, an insoluble problem. On the one hand, during gestation there is an intimate relationship of two genotypically dissimilar animals, and on the other the need for the embryo to dispose of or re-utilise its effete products by a mechanism which, because of its immunological implications, should cause its destruction. The modus operandi of this non-aggression pact between mother and foetus is not clear. Hemmings (1961) points out that

the dangers of pregnancy have been traditionally overemphasised, but that the hazards of being gestated are very real.

Despite our lack of knowledge the physiological aspects of the reticulo-endothelial system have received only sporadic attention. It has been shown that the widespread nature of the system makes it difficult to define, and that most of the investigations of its functions have been interpreted in regard to the part that they play in host defence mechanisms. In any biological community whether of animals or cells disposal of waste is a constant requirement. It is a fundamentally necessary utility and thereby considerably more important than defence. If these two activities can be expressed, however, by the same system this seems to be the ultimate in biological economy. There is a considerable amount of literature, much of it at a fairly superficial level, which deals with the physiological functions of the reticulo-endothelial system. Doan (1957) suggests that the physiological functions are twofold and have a cellular aspect highlighted by phagocytosis and a humoral aspect such as the production of normal serum globulins.

The role of physiological phagocytosis has been reviewed by Miescher (1957) in regard to haematoclasia. It is his view that the reticulo-endothelial system has a passive role in this process and represents no more than a blood corpuscle cemetery. Vannotti (1957), however, in the light of extensive investigations, considers it proven that the

reticulo-endothelial system plays an active part in the intermediary metabolism of iron.

The production of serum globulins by the cells of the reticulo-endothelial system is less well documented but gains limited support from Sabin's (1939) investigations and by inference from the concept of antibody production by the reticulo-endothelial system. In this latter respect again it can be seen that defence mechanisms being the most readily discernable because of their magnitude have been used as a basis for arguing a physiological problem. Whether cells of the reticulo-endothelial system are directly responsible for antibody production is currently doubted by Howard (1961) and yet it seems possible that they can produce small quantities of these substances (McKenna and Stevens, 1960). Burnet and Fenner (1949) predicted that cells of this system were necessary for the train of events which precede antibody production and this phenomenan was demonstrated in an <u>in vitro</u> system by Fishman (1959).

Dixon and Weigle (1957) concluded from experiments in which competent lymphocytes were transferred to neonatal rabbits that the neonatal animals were not able to provide an environment in which these competent cells could express their immunological activities. They suggested that the paucity of immunological activity at this age might result from a deficiency in environment rather than a deficiency in the cells responsible for antibody production.

Silverstein *et al*. (1963) have drawn attention to the unique opportunity which the mammalian foetus provides for the study of immunological phenomena in an immunological 'virgin', uncomplicated by a host of unrelated activities in which most so-called 'normal' animals are inevitably involved. It was against this background then that a study of the development of the reticulo-endothelial system in foetal animals (using the rat as a mammalian model) was undertaken.

This introduction and historical survey illustrates the importance to animals, in particular mammals, of the reticulo-endothelial system and indicates areas in which a lack of information seriously limits an understanding of this system. One such area has been chosen for exploration in this present study.

CHAPTER II

MATERIALS AND METHODS.

The subject matter of this chapter is divided into three sections:

A. Biological Aspects

B. Histological Aspects

C. Biochemical Aspects

A. BIOLOGICAL ASPECTS

1. Bacterial Strains

(a) *Salmonella typhimurium* C5: this is a smooth strain which is virulent for mice, LD50 = 100 organisms (Furness and Rowley 1956), but it was found to be avirulent for rats.

(b) *Salmonella typhimurium* M206: this smooth strain was described by Jensen (1929) and is avirulent for mice, the LD50 being approximately 10^6 organisms. It was found to be avirulent for rats.

(c) *Salmonella gallinarum* 9S: a smooth avirulent mutant of *Salmonella gallinarum* 9240 obtained from Dr. H. Williams-Smith, Lilystone Hall, Essex, England. It was found to be avirulent for rats.

(d) *Salmonella enterididis* Se 795: a smooth strain isolated from fur seals. It was obtained from Dr. E. Ribi, National Institute of Allergy and

Infectious Diseases, Rocky Mountain Laboratory, Hamilton, Montana, U.S.A., as a highly virulent organism for mice and was found to be avirulent for rats. The organism was described by Milner *et al.* (1957).

(e) *Escherichia coli* 2206: this is a smooth strain which is avirulent for mice and rats and was referred to by Rowley (1954).

(f) *Escherichia coli* (Lilly): this is a rough strain which is avirulent for mice and rats and was supplied by Dr. A.C. Wardlaw, Connaught Medical Research Laboratories, Toronto, Canada.

(g) *Escherichia coli* K12: this is also a rough strain which is awirulent for mice and rats and previously referred to by Rowley (1954).

2. Maintenance of bacterial strains

Freeze dried preparations of cultures of these strains were received in sealed ampoules. When required the ampoules were opened with aseptic precautions and their contents emulsified in one ml. of nutrient broth. This suspensions was then sub-cultured in 10 ml. of nutrient broth and incubated at 37°C for 18 hours. To verify the purity of each culture a loopful of liquid from the incubated broth was streaked onto a nutrient agar plate, incubated at 37°C and examined

for homogeneity of colony form. If the culture appeared pure several colonies were picked off and inoculated into 10 ml. of nutrient broth. This was incubated for 18 hours after which one ml. aliquots of the culture were dispensed into sterile ampoules, sealed and snap-frozen by immersion in solid carbon dioxide/ethyl alcohol mixture. These ampoules were then stored at -20°C.

When a particular bacterial strain was required, an appropriate ampoule was thawed and the contents placed in 10 ml. of nutrient broth and incubated for 18 hours. A sample of the culture was then streaked onto nutrient agar slopes contained in one ounce screw-capped bottles and incubated at 37°C for 18 hours. These agar slopes were then stores at 4°C. Each slope was used no more than ten times.

3. Radioactive isotope labelling of bacteria

Bacteria were grown in a supplemented casamino acid medium described by Benacerraf et al. (1959). This medium was autoclaved, adjusted to pH 7.0 and dispensed in 50 ml. amounts. When required this amount was added to a 500 ml. Elenmeyer flask which was placed in an enamel basin and packed about tightly with cotton wool. To this flask was added 0.5 milli-curie of radioactive phosphorus (P^{32}) as orthophosphate and 0.1 ml. of casamino acid medium

containing approximately 10^7 bacteria. This medium was then agitated at one stroke per second in a hot room at 37°C for a period of 18 hours.

After this time the bacteria were harvested by centrifugation at 5000 r.p.m. for 10 minutes and washed three times with 50 ml. of physiological saline in firmly stoppered polythene centrifuge tubes. The supernatant from the third washing was shown to contain negligible quantities of radioactivity.

After this last washing the bacteria were resuspended in casamino acid medium at a concentration of 10^9 organisms/ml. The concentration was estimated by means of optical density (O.D.) using a Shimadzu photo-electric spectrophotometer, model Q.R.50, at a wave length of 675 milli-microns. It was shown that an O.D. of 0.16 at this wave length would give the required concentration of organisms. These suspensions were kept at 4°C and were not used for more than five days. They were rewashed each time before use.

4. Carbon

A shellac free preparation of India ink (Ink No. C11/1431a) prepared by Gunther Wagner, Hanover, Germany, was treated according to the method described by Biozzi *et al.* (1953) giving a particle size of less than 500 Angstroms at a concentration of 32 mg./ml.

in 2 per cent gelatine at pH 7.4. The various doses of carbon were prepared by warming the suspension of 37°C until it liquefied and making appropriate dilutions in physiological saline. As the more concentrated solutions solidified at room temperature these and the syringes used for injection were maintained at 37°C during the experiments.

5. Polystyrene latex

This material was obtained from the Dow Corning Corporation, Midland, Michigan, U.S.A. The particles were spherical, of regular size electronmicrographically and measured 800 Angstroms in diameter. A stock suspension of 106.5 mg./ml. was kept at 4°C and diluted in physiological saline as required.

6. Thorium dioxide

The material used was 'Thorotrast' - a preparation of thorium dioxide manufactured by Testagar and Co., Inc., Detroit, Michigan, U.S.A., and was batch no. 1314, expiry date January 1968. The suspension contained 24 per cent to 26 per cent of stabilised colloidal thorium dioxide by volume, 25 per cent aqueous dextrin, and 0.15 per cent methyl parasept as a preservative.

7. Opsonisation of bacteria

Equal volumes of the bacteria (10^9 orgs./ml.) and the serum sample to be investigated were mixed and kept at

$4^{o}C$ for 20 minutes. During this time there was no apparent microscopical agglutination nor was there a reduction in the viability of the organisms. The mixture was then centrifuges at 5000 r.p.m. for 10 minutes and the deposited bacteria were washed three times with 10 ml. of physiological saline. Following this the organisms were suspended in a volume of casamino acid medium equal to the volume of bacterial suspension originally used for the procedure of opsonisation and were then ready for immediate use.

8. Strains of animals

The animals were kept in metal cages in air-conditioned rooms maintained at $22^{o}C$. Food and water were available *ad libitum*. The two strains of animals used extensively in this study were:-

(a) Hooded Wistar rats at various stages of growth and pregnancy, and

(b) Swiss white mice of either sex and an average body weight of 18-20 gm.

Other animals used were laboratory flop-eared rabbits and white Leghorn eggs from a local chicken farm.

9. Determination of stage of pregnancy of rats

Initially one adult buck was placed in a cage overnight with six does each over four months of age and known to be non-pregnant. This procedure however frequently produced no pregnancies. Thiessen and Rogers (1961)

have suggested that such failures can be due to the female rats having entered an extended phase of dieostrus because of prolonged segregation from bucks and that it can take several days for this to pass after the doe is introduced to the buck. To overcome this problem a small wire cage to contain the buck for three days was constructed within a larger cage in which the does were housed. At the appropriate time the buck was placed overnight with the does and an expected number of pregnancies resulted. Conception could then be dated to within approximately 12 hours. The gestation period for this strain of rats was found to be approximately 22½ days. An average number of conceptuses was 10 to 12 with an even distribution in each horn of the bicornuate uterus.

10. Preparation of pregnant rats for experimental procedures

The conduct of this study depended upon the development of a technique which would allow foetal rats to be kept alive and in as near normal condition as possible for several hours while the various operative procedures were performed. There were thus both anaesthetic and surgical problems to be overcome.

It was first of all necessary to find a method by which a pregnant rat could be maintained anaesthetic for many hours without significant physiological disturbances. To this end a number of anaesthetic

methods were investigated. The barbiturates, phenobarbitone and pentobarbitone, dial and urethane mixture, urethane and chloralose mixture and curare-type relaxants combined with an automatically respirated gaseous anaesthetic such as nitrous oxide and oxygen delivered by way of a tracheal cannula, were all extensively tested and found to be unsatisfactory.

A most satisfactory method for maintenance of anaesthesia was however eventually devised and was used unchanged throughout the course of the study. The anaesthetic procedure was as follows:

After lightly anaesthetising a pregnant rat in a closed container filled with ether vapour, an area of skin approximately one centimetre in diameter was removed with curved scissors from the middle of the anterior part of the neck. This uncovered the strap muscles of the neck and the trachea could then be exposed by blunt dissection. A small transverse incision two to three mm. long was made between cartilagenous rings in the vicinity of the fourth or fifth tracheal ring and a bevelled nylon cannula, approximating to the internal size of the trachea, was inserted for approximately one cm. A black silk ligature was placed around the trachea to fix the cannula in place, care being taken to avoid closely related nerves and vessels. To this cannula was fitted

a T-piece tube to one arm of which was affixed a flexible tube for the delivery of anaesthetic gases while the other arm was open to allow the voluntary respiration to be at atmospheric pressure. The 'dead space' in the T-piece was estimated to be similar to that in the respiratory system of the experimental rats. The mixture of anaesthetic gases was supplied in metred quantities from metal cylinders of compressed gases at approximately the same flow rate as the tidal air flow of the rats. This was calculated to be approximately 200 ml./minute. The anaesthetic machine was supplied by Commonwealth Industrial Gases, Torrensville, South Australia, and was slightly modified with small volume flow meters and a Goldman Halothane vapouriser (Fig.1). It was with this machine that the maintenance anaesthetic was supplied at a flow rate of 100 ml./minute oxygen and 100 ml./minute nitrous oxide. Half to three quarters of this mixture was passed through the Goldman vapouriser containing anaesthetic ether. The resultant mixture was supplied to the animal which could be maintained by this means at the upper limit of the plane of surgical anaesthesia for more than 24 hours if required. In an effort to establish that these and the later surgical procedures produced as little change in the animals' physiology as possible carotid blood pressure recordings were obtained using standard physiological

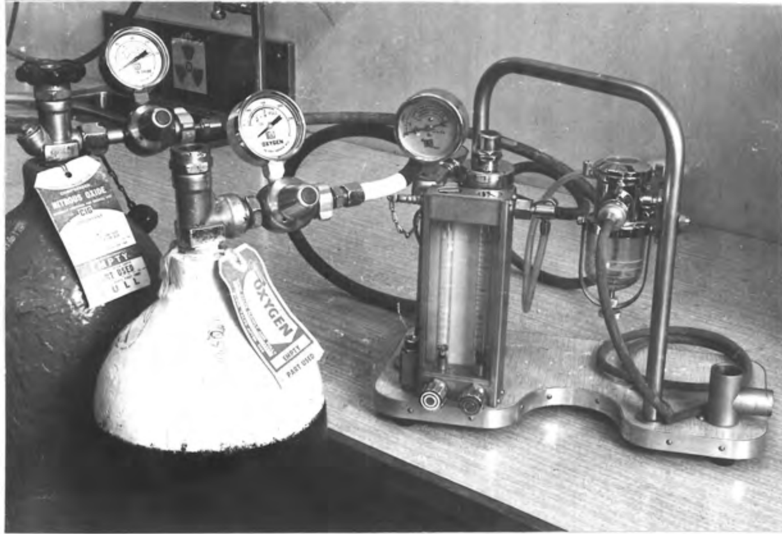


Fig.1. C.I.G. anaesthetic machine with flow-meters and ether vapouriser.

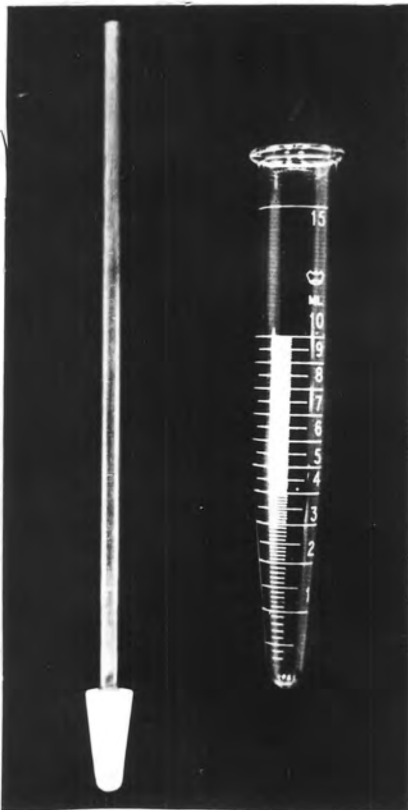


Fig.2. Tissue grinder used for small organs.

techniques. Insignificantly small variations were observed. This confirmed the more general observations of the animals continuing satisfactory state while under the described conditions of anaesthesia.

Following tracheal cannulation and connection to the anaesthetic machine the animals were fixed to a rigid 'Perspex' support in a supine head raised position. The tube to the anaesthetic machine was clamped to this support and the immobilised animals were then transferred to a bath of Ringer Lock solution at 37°C. The bath, made from Perspex sheet cemented with a chloroform solution of Perspex, measured 43 cm. x 43 cm. x 10 cm. and was partly filled with 12 litres of the above solution. The bath was warmed by means of heated water pumped via a thermostatically controlled water bath through loops of glass tubing lying on the floor of the bath. A centigrade mercury thermometer was fixed in the bath as an added precaution in temperature control.

The rat was submerged in this bath to the lower border of its thoracic cage for the blood pressure recordings had shown that considerable changes in blood pressure could be induced by slight pressure on the chest wall.

The surgical procedures were then carried out:-

An antero-posterior incision approximately three cm. long was made through the skin, abdominal musculature and peritoneum to expose the lower abdominal viscera. The gravid bicornuate uterus was gently exteriorised and supported on a submerged Perspex table which was perforated to allow convection to maintain a temperature of 37°C within this support (Fig.3). The uterine wall was then divided by a longitudinal incision antimesiometrally from the apex of one uterine horn to the apex of the other and the conceptuses thereby presented. The foetal membranes (yolk sac splanchnopleure and amnion) were divided distal to the placenta to avoid sectioning the larger vitelline vessels. By this means the foetuses were exposed connected by way of their cord vessels to their placentae, the connections of which to the uterine wall were undisturbed. They were supported, in the order of their attachment to the uterus, on the table and submerged to a depth of one to two centimetres in the warmed physiological saline (Fig. 4). It was following this surgical preparation that all experimental operative procedures on rat foetuses continued.

These procedures were mainly the intravenous injection of various substances and the aspiration of blood samples.

For the intravenous injection a 30 gauge needle was used. The bevel of the needle was reduced to about

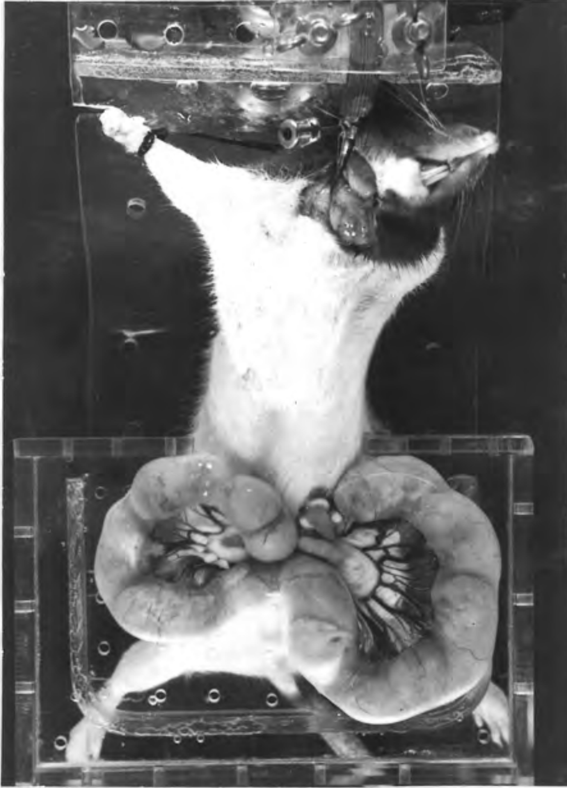


Fig. 3. A pregnant rat with gravid uterus exteriorised and placed on a support in physiological saline at 37°C

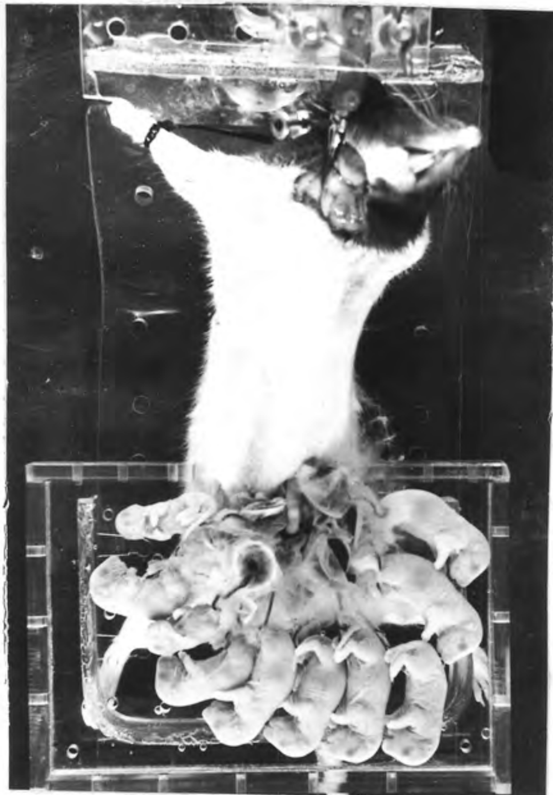


Fig. 4. Foetal rats removed from the uterus and from the foetal membranes.

45°, and the needle was cemented to 35 cm. of fine polythene tubing. This was attached to a foot operated one ml. tuberculin syringe, and was used to introduce the test material into the orbital branch of the anterior facial vein (Fig. 5). Aliquots of 0.05 ml. were used exclusively as the volume injected into the rat foetuses with dilutions being arranged according to this volume. The tuberculin syringe was loaded for each injection taking care to exclude air. The volume delivered by the syringe was considered to be adequately constant as indicated by radioactive counts made on successive deliveries from the syringe. There was no doubt whether or not the material had been injected intravenously, for if a solution was deposited outside the vein a bleb of considerable proportions was immediately evident. If this occurred (as it did in about one in ten injections into foetuses older than 14 days) the particular foetus was discarded. Satisfactory injections into the veins of foetal rats younger than 14 days was virtually impossible with these macroscopic methods.

When blood was to be removed from the foetuses for various assay procedures it was obtained by cardiac aspiration (Fig. 6). The blood was collected by using specially prepared glass pipettes (0.01 ml. or 0.02 ml,

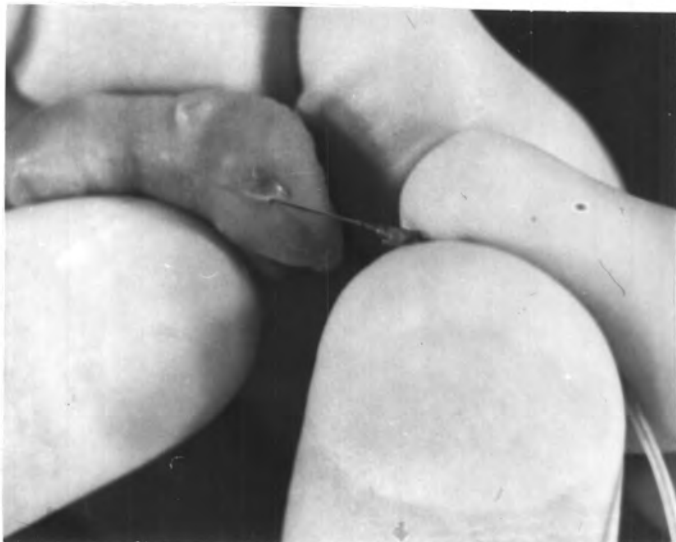


Fig. 5. Injection with a 30 gauge needle into the orbital branch of the anterior facial vein of a foetal rat.

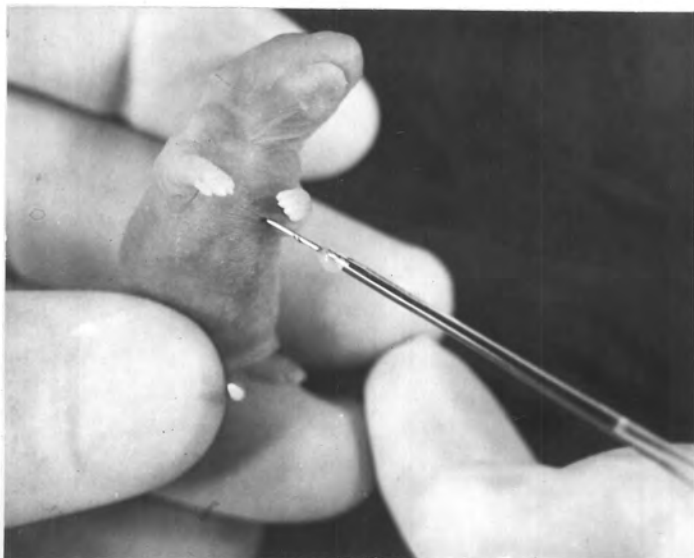


Fig. 6. Aspiration of blood by cardiac puncture with a 26 gauge needle cemented to a 0.01 ml. glass pipette

capacity) fitted with approximately one cm. long 26 gauge needles. The bevels of the needles were reduced to about 45°. The pipettes were mouth operated and could be sterilised by boiling or autoclaving. The bleeding procedure was rarely unproductive after the 16th day of gestation. Before this stage bleeding by cardiac aspiration or by any other means for that matter was commonly unsuccessful. The scope of this investigation was therefore limited to a study of rat foetuses in the period after the 14th and 16th day from conception. Successive aspirations were not employed in any of the experiments and each sample assayed therefore represents one embryo and thereby considerably decreases the inherent error of using single animals for successive aspirations.

11. Washing glassware

All glassware and caps used to contain blood, serum or embryonic fluids was carefully washed according to the following regimen:

(a) Wash containers and caps separately in boiling Calgon (Albright and Wilson (Aust.) Pty. Ltd.) and sodium metasilicate solution for 30 minutes; (b) Wash ten times in tap water; (c) Wash ten times in distilled water: (d) Wash three times in deionised water; (e) Replace caps and autoclave.

12. Collection of rat serum

Blood was collected by cardiac puncture from both

foetal and natal rats considered to be in good health. Foetal rats were bled by the pipettes described previously and rats from birth to two to three weeks of age were etherised, pinned out supine on a cork dissecting board and after opening the thorax were exsanguinated by bleeding from the heart with a Pasteur pipette. Rats above this age were bled from the heart with a 25 gauge needle fitted to a 10 ml. glass syringe. Blood obtained in these ways was collected in glassware washed as described above, was stood on the bench for 15 minutes to allow clotting and then kept at 4°C for 30 minutes to encourage clot retraction for maximal recovery of serum. After this time the serum was withdrawn with a pipette and spun in conical glass centrifuge tubes at 1500 r.p.m. for five minutes. The clear straw coloured serum was pipetted from the packed cells and stored in approximately three ml. volumes in labelled five ml. screw top bottles at -20°C. These serum samples were thawed not more than twice and were used as near to the time of collection as possible and in any case not longer than eight weeks after collection. All serum samples unless otherwise stated were pooled samples from at least five animals.

13. Collection of rat embryonic fluid

This is the extra-foetal fluid enclosed by the placental membranes and includes therefore the exocoelomic and amniotic fluids. For the purpose of the present study it was not considered necessary to study these fluids separately and they were therefore pooled. The intact conceptuses were detached from the wall of the uterus, dipped in sterile isotonic saline to remove traces of blood and wiped with absorbent paper They were then held over a bottle and the membranes incised with a scalpel blade to permit the fluid to be collected. An attempt was made to avoid the large vitelline vessels. The embryonic fluid from foetuses of the same litter was pooled and this clear colourless fluid was stored at -20°C. It was thawed not more than twice and was used as near to the time of collection aspossible and in any case not longer than eight weeks after collection.

14. Collection of pig serum

The pig serum used was prepared from the blood of healthy pigs slaughtered at the Adelaide Metropolitan Abbatoirs and was handled and stored in a manner identical to that described for rat serum.

15. Design of experiments

The design of the experiments concerned with this aspect of the study are described under the following headings:-

(a) Bacterial clearance studies

(b) Carbon clearance studies

(c) Assay of opsonic activity of serum and serum fractions:

(i) mouse assay

(ii) chick embryo assay

(d) Organ distribution of injected particles

(e) Bactericidal activity of rat serum

(f) Bacterial survival in foetal and neonatal rats.

(a) Bacterial clearance studies

Foetal rats were prepared as has already been described. A dose range of from 10^7 to 2.5×10^8 isotopically labelled Salmonella typhimurium C5 per 0.05 ml. was used in a dose dependence clearance study by the method to be described. From the results of this study it was decided to use 5×10^7 orgs./ml. for all clearance studies in foetuses of all ages.

After injecting this dose one sample of 0.01 ml. was aspirated by cardiac puncture from each foetus. At least five foetuses were used in each assay, blood samples being taken at 1,2,3,4 and 5 minutes after

injection for bacteria cleared rapidly and at 1,2,5, 7 and 10 minutes for bacteria cleared more slowly. Rats from birth to 7 days of age were injected with the same volume and concentration of organisms as the foetuses and a similar amount of blood was aspirated by cardiac puncture at 1,2,5, 7 and 10 minutes after injection. As rats above the age of one week were furred it was necessary to use another injection site. The tails of these animals were frequently heavily pigmented and as a consequence had obscured tail veins. It was therefore decided to lightly anaesthetise the the animals with ether, remove the skin on the inner aspect of one hind leg and expose the femoral vein. Intravenous injection into the vein was made with a 25 gauge needle fitted to a 1 ml. tuberculin syringe. The doses of organisms for animals above one week old were adjusted according to their weight and are set out in Table 1.

TABLE I

Dose of organisms for rats above 7 days old

Age (days)	Dose of Organisms
14	10^8
21	10^8
28	2×10^8
56	4×10^8
84	6×10^8

At least five blood samples were taken from each natal rat above two weeks of age by bleeding from the retro-orbital venous plexus into a 0.02 ml. glass pipette according to the method described by Biozzi et al. (1953). The blood samples were taken at time intervals similar to those used for studies on the foetal rats.

The blood samples that were collected both from the heart and from the retro-orbital venous plexus were immediately deposited onto circular discs of Whatman No. 3 filter paper previously cut to fit the available metal planchette holders. The amount of radioactivity present in each sample was then assayed using a thin end-window Geiger counter installed in a Nuclear Chicago automatic sample changer C110A

(Nuclear Chicago, Chicago), with an automatic timer C111 coupled to the 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 (General Electric Co., Schenectady, New York) which increased the sensitivity of the machine threefold.

If instead of the above method of measuring the clearance of isotopically labelled micro-organisms viable counts were used to study their clearance the blood samples were taken in a similar way to the above, diluted to an adequate level in sterile isotonic saline and a determined aliquot plated onto dried nutrient agar plates. The inoculated plates were incubated at 37°C until the colonies could be counted. The results obtained from these two methods of counting were then plotted as log concentration against time and this exponential expression was used to calculate the phagocytic index (K) where $K = \frac{\log C1 - \log C2}{T2 - T1}$ and where C1 and C2 are the concentration of bacteria (or other colloids) at times T1 and T2.

(b) Carbon clearance studies

The injection of carbon was the same as described in (a) above. The dose of carbon injected into natal

rats was of the order of 8 mg. of carbon/100 gm. of body weight which was shown by Biozzi et al. (1953) to be on the plateau of a dose dependence curve. A constant dose of 0.4 mg. of carbon in 0.05 ml. was administered to the foetuses of all ages and this was equivalent to 8 mg./100 gm. of body weight for the foetuses near term. Blood samples were also obtained as in (a) above (at times ½, 1, 2, 3, 4, 5 minutes for the natal animals and times 1,2,3,5, 7, 10 minutes for the foetal animals) and were immediately deposited into 0.1 per cent Na_2CO_3 for haemolysis to occur. The samples of foetal blood were lysed in 0.5 ml. of the Na_2CO_3 solution and the samples of natal blood were lysed in 3 ml. of the same solution. The optical densities of the 3ml. lysates were read in 1 cm. glass cells in a Shimadzu spectrophotometer at a wave length of 650 millimicrons using 0.02 ml. of normal blood lysed in 3 ml. of 0.1 per cent. Na_2CO_3 solution as a blank. The concentrations of carbon in these specimens were then determined by reference to a standard curve (Fig. 7) which had been prepared by determining in the same way the optical density of lysed blood samples containing from 16 micrograms to 32 micrograms of carbon per 0.02 ml.

The optical density (as a drum reading) of the

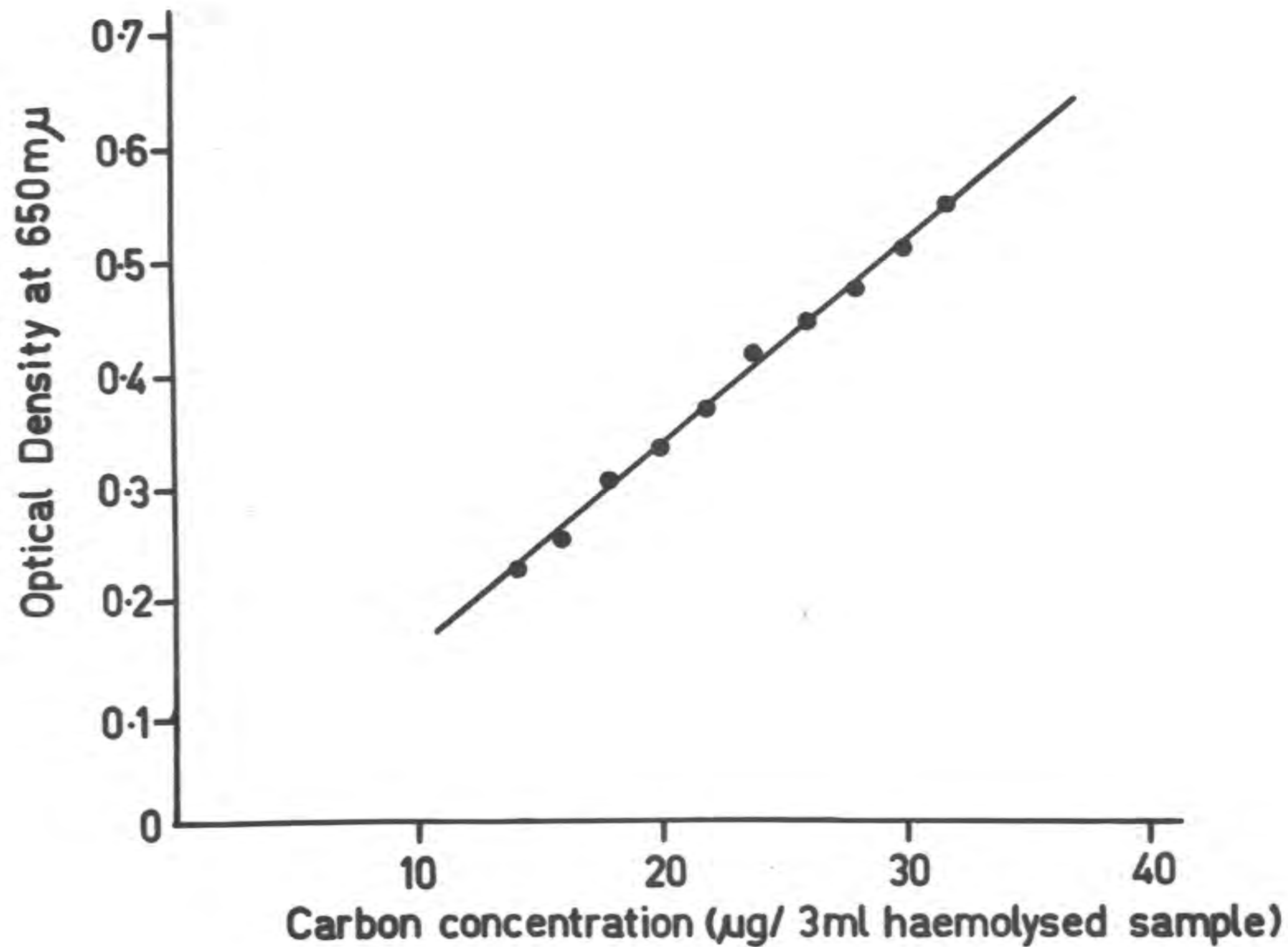


Fig.7 Standard curve for carbon concentration at O.D. 650 mμ on a Shimadzu spectrophotometer.

0.5 ml. samples, because of their smaller volume, were estimated in a 1 cm. glass micro-cell in a Hilger Spekker spectrophotometer (Adam Hilger Ltd., London) using a red filter (Ilford Spectrum Filter 608). The concentrations of the samples were calculated by reference to a standard curve prepared as previously and using 0.5 ml. of each of the previously described 3 ml. standard lysates (Fig. 8). The concentrations were plotted against the recorded drum reading.

The phagocytic index (K) was then calculated as described in (a) above.

(c) Assay of opsonic activity of serum and serum fractions

The various serum or serum fraction samples were used to opsonise isotopically labelled *Salmonella typhimurium* C5 as described above.

(i) Mouse Assay

The technique for mouse clearance studies was essentially that described by Biozzi *et al.* (1953). Following opsonisation the labelled bacteria were injected into a tail vein of 18 to 20 gm. mice at a concentration of 10^9 organisms per 100 gm. of body weight. At suitable time intervals 0.02 ml. blood samples were withdrawn from the retro-orbital venous plexus, placed onto discs of filter paper and assayed for radioactivity

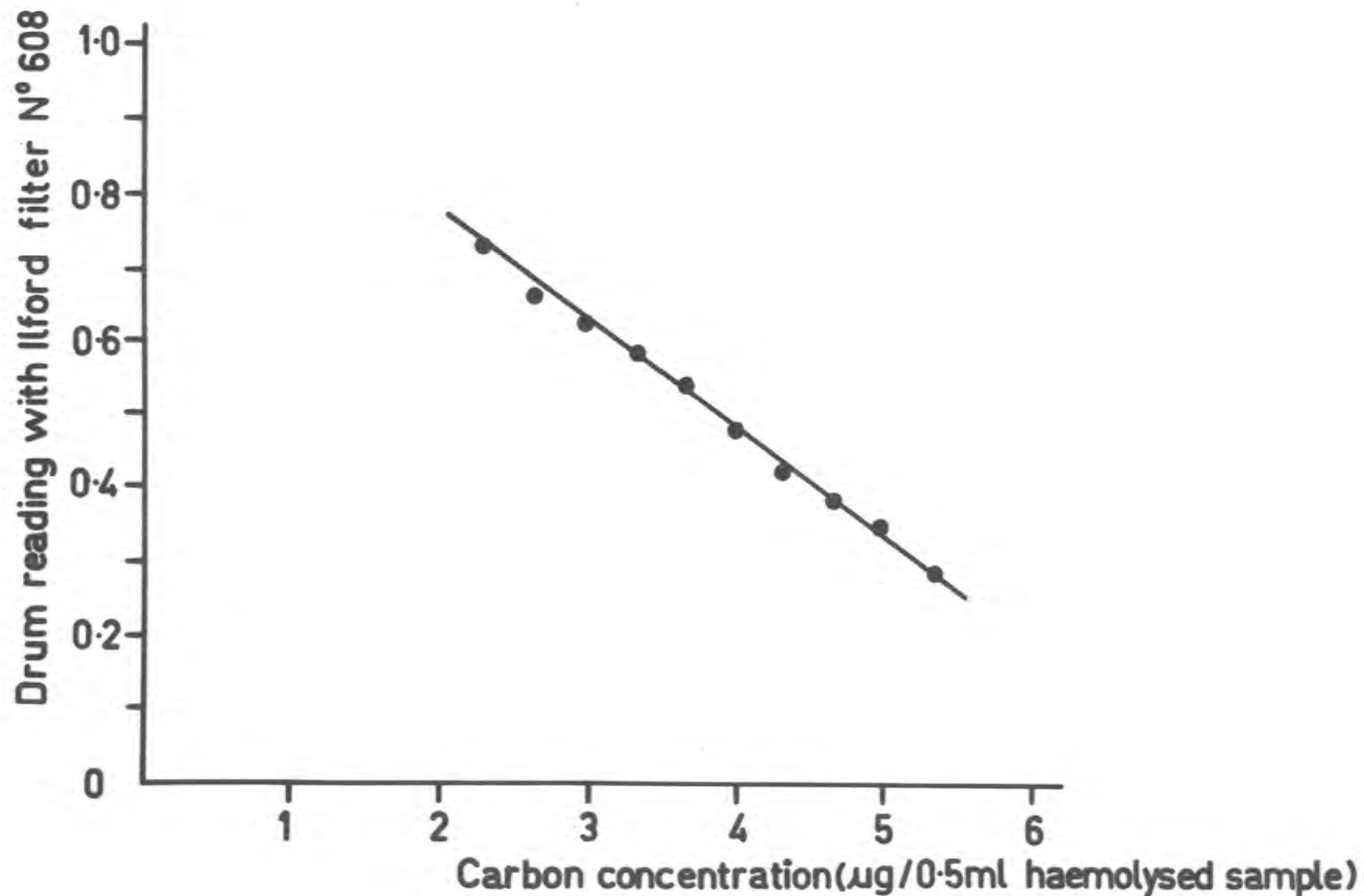


Fig.8 Standard curve for carbon concentration with ILFORD filter N° 608 in a Spekker spectrophotometer.

as described above. The phagocytic index (K) was calculated and gave an estimation of the opsonic activity of the serum towards the test organisms. At least three mice were used for each assay and the result expressed as an average of these.

The antibody activity of the serum and serum factions as determined by mouse clearance studies were expressed as biological units of activity. This unit was defined by Turner et al. (1963), "as that amount of antibody which would increase the rate of clearance (K) of the virulent strain of Salmonella typhimurium by a factor of 0.01 over that observed in the controls". The total biological units of activity of each sample could then be obtained by multiplying thevolume of the sample by the value obtained from the above calculation. The most meaningful results were obtained when the samples were of such a dilution that a decrease in phagocytic index (K) was proportional to increasing serum dilutions.

(ii) Chick embryo assay.

The technique used for clearance studies in chick embryos was that described by Karthigasu and Jenkin (1963). Labelled bacteria (2×10^8 orgs. suspended in 0.2 ml. casamino acid medium),

some samples of which were opsonised with foetal rat serum, or fractions thereof were injected into the previously exposed chorio-allantoic vein of 16 day old chick embryos. Blood samples were aspirated from this vein at times 2, 5, 15, 30, 45 and 60 minutes and 0.02 ml. deposited on filter paper planchettes and assayed for radioactivity as described. The phagocytic index (K) was calculated for each serum sample and was the result of clearances in at least three eggs. The chick embryo clearance studies were also expressed as biological units of activity by defining the unit in this instance as that amount of antibody which would increase the rate of clearance (K) of Salmonella typhimurium C5 by a factor of 0.001 over that observed in the controls.

(d) Organ distribution of isotopically labelled bacteria

This study was restricted to foetal animals. Their aged from conception were 14, 18- and 22- days.

Injection of 5×10^7 isotopically labelled Salmonelia typhimurium C5 at a determined radioactive count was made according to the method described above. At time intervals of 1, 5, 10, 20, 60 and 120 minutes, 0.01 ml. blood samples were aspirated for assay of radioactivity and the respective conceptuses removed and dissected. The blood volume of the foetal rats

was calculated by determining the dilution of the inoculum of the isotopically labelled organisms in the blood of the foetus by extrapolating the clearance curve to zero time. The various organs, placenta, liver, lung, gut, kidney, brain and spleen, and the remaining carcass of each foetus, were incubated separately for 18 hours at 37°C in an appropriate volume of 10 per cent NaOH. If after this time digestion was not complete gentle heat was applied to dissolve the remnants. The volume of each digest was measured and 0.01 ml. samples were assayed for radioactivity. A balance sheet accounting for the total radioactivity injected was then assembled to illustrate the percentage distribution of the radioactivity in the various organs at various time intervals.

(e) Bactericidal activity of rat serum

Serum was obtained from natal animals and serum and embryonic fluid from foetuses as described. *Escherichia coli* (Lilly) was used as the test organism. Into carefully washed small test tubes with aluminium caps was placed 0.1 ml. of the test serum, 0.4 ml. of basal medium (to give a serum dilution of 1/5) and approximately 10^3 organisms from an 18 hour broth culture of 10^9 orgs./ml. in 0.02 ml. of basal medium. Embryonic fluid was used undiluted and therefore

approximately 10^3 organisms in 0.02 ml. were added to 0.5 ml. of this fluid.

A blank of 0.5 ml. of basal medium, to which this same number of organisms were added, was used as a control. The samples were incubated in a 37°C water bath during the experiment and duplicate 0.04 ml. samples were taken from each tube at time 0, 10, 20, 40 and 60 minutes and plated onto dried nutrient agar plates. These were then incubated at 37°C for approximately 18 hours until the colonies could be readily counted. The count resulting from each sample was expressed as a percentage of the zero time count.

(f) Bacterial survival in foetal and neonatal rats

The rats were prepared as previously described and injected intravenously with approximately 10^4 *Salmonella typhimurium* C5 from an 18 hour broth culture of 10^9 orgs./ml. At times 0, 15, 30, 45, 90, 120 and 180 minutes 0.01 ml. of blood was aspirated, with sterility precautions, from the heart and plated on a dried nutrient agar plate. The conceptus from which the blood sample was aspirated was separated from its uterine connections and placed in a sterile glass Petri dish. The placenta, and liver were then dissected from the conceptus, with care being taken to prevent contamination, and placed in sterile tissue grinders. The placentae and foetal livers were homogenised in 1 ml. sterile isotonic saline and the

neonatal livers were homogenised in 4 ml. of this solution. Specially designed tissue grinders were made to cope with the small organ size, small volumes and the necessity for accurate redovery df bacteria. Each tissue grinder (Fig. 2) consisted of a graduated conical 'Pyrex' centrifuge tube with a 'Teflon' pestle turned to fit accurately the apical 2 cm. of the tube. Into the base of the Teflon was threaded a brass handle 5 mm. x 10 cm. The volume of the placenta and liver could then be measured in the graduated tube. After grinding the organs, samples of 0.1 ml. of the homogenates were plated onto dried nutrient agar plates. These were incubated for approximately 18 hours at 37°C and the colonies counted. The total number of viable organisms in the various organs could then be calculated at any given time by reference tò the total volume of the homogenate. The results were presented as the $\log_{10}$ of the concentration of organisms plotted against time.

B. HISTOLOGICAL ASPECTS

Histological examinations were made at two levels:

1. Light microscopy
2. Electron microscopy.

1. Light microscopy

The foetuses examined were aged 14-, 18-, 20-, and 22-days and the matal animals were 3 months old.

Specimens were taken of normal tissue and at 1,2,4,6, 8, 10, 15, 30, 60, 90, 120 and 180 minutes after injecting carbon and bacteria by the method and in the doses already described.

All of the material was taken from anaesthetised animals or from animals immediately after their death. Without delay it was placed in an appropriate volume of 10 per cent buffered formal saline for fixation. After at least 24 hours in this fixative the material was trimmed and subjected to routine wax embedding procedures and was then sectioned on a rotary microtome at a thickness of seven microns. The sections were then floated onto glass slides and stained in a routine way with haematoxylin and eosin, polychromatic methylene blue, grams stain or saturated aqueous picric acid. Cover slips were applied with 'De Pex' (George T. Gurr Ltd., London) which was then allowed to solidify in a 37°C incubator. The slides were examined and photographed with a 'Photomicroscope' (Carl Zeiss, Germany) microscope with a range of powers to 2000 times magnification.

2. <u>Electron microscopy</u>

The animals examined were 20 day old rat foetuses and 3 month old rats. Injection procedures were the same as those which have been described. Two suspensions were injected into the foetuses (Suspension A and B) and one suspension (Suspension B) was injected into

the 3 months old animals. The constitution of these suspensions was:-

Suspension A 0.2 ml. Ferritin (250 mg./ml.)

0.06 ml. Carbon (32 mg./ml)

0.06 ml. Polystyrene latex (10.65 mg.per cent)

0.19 ml. Sterile isotonic saline.

Inject 0.05 ml. of this suspension into each foetus.
Samples were taken at 1, 4 and 16 minutes.

Suspension B 2.0 ml. Carbon (32 mg./ml.)

4.0 ml. 1.2×10^{10} orgs./ml.

0.2 ml. Thorotrast (250 mg./ml.)

One ml. of this suspension was injected into the 3 month old rats and 0.05 ml. into the foetal rats.
Samples were taken at 1, 5, 25 and 15 minutes.
The organisms used in Suspension B were either Salmonella enteritidis Se 795 or Escherichia coli (Lilly)
Once removed from the animal the specimen was immediately transferred to a card on which a drop of the fixative (2 per cent OsO_4 in 0.15M phosphate buffer) had been placed. The specimen was placed in the fixative and finely divided by a scalpel blade. This material was then placed in a stoppered bottle containing more fixative and stored in this for at least 2 hours.
The specimens were dehydrated through a series of alcohols and embedded in either 'Araldite' (Ciba Ltd.,

Basle) or a mixture of methyl and butyl methacrylate. The embedded specimens were section on a Huxley microtome at a thickness of 500 Angstroms. They were stained with either uranyl acetate, one per cent phosphotungstic acid or two per cent lead hydroxide. The sections were examined in a Phillips E.M. 100B electronmicroscope.

C. BIOCHEMICAL ASPECTS

In this section the biochemical materials and methods used to examine the serum of natal rats and the serum and embryonic fluids of foetal rats are described. Many of these investigations were directed towards ascertaining both qualitatively and quantitatively the opsonic activity of these sera for Salmonella typhi murium C5. The methods used in this study involved the following procedures:-

absorption spectrophotometry; paper-strip electrophoresis; starch-gel electrophoresis; amylase assay; reduction by sulphydryl reagents; gel filtration chromatography; density gradient ultracentrifugation; ion exchange chromatography and immuno-electrophoresis.

1. Absorption spectrophotometry

The protein content (mg./ml.) of various serum and embryonic fluid samples and serum fractions was estimated by reference to standard protein curves prepared in the following manner. A sample of 10 ml. of pooled 3 month old rat serum was dialysed with

gentle agitation against 0.15M NaCl at 4°C for 24 hours. Duplicate 4 ml. samples of the retentate and the dialysate were dried overnight in a 70°C oven and were then vacuum dried at this temperature to constant weight. It was determined by this method that this serum contained 64.6 mg./ml. of protein by dry weight. Duplicate serial dilutions of the retentate of 1:200 dilution in isotonic saline were prepared and the optical densities measured by U.V. absorption at a wave length of 280 millimicrons in a Shimadzu spectrophotometer. The samples were read in one cm. silica cells using isotonic saline as a blank. A standard curve was drawn by plotting optical density against micrograms of protein/ml. and the linear relationship obtained is illustrated in Fig. 9.

2. Paper strip-electrophoresis

Serum analysis by this method was employed to separate both foetal and natal rat serum and embryonic fluids into their major components - gamma-, beta-, and alpha-globulins and albumin. This method allowed the percentage distribution of these various protein groups to be calculated. The technique used was similar to that described by Jencks, (1955). The serum and embryonic fluid samples were analysed on paper strips (Whatman 3 mm.) in a Spinco (Model R) paper eletrophoresis cell. This cell was operated for 16 hours at 150 volts and contained veronal buffer,

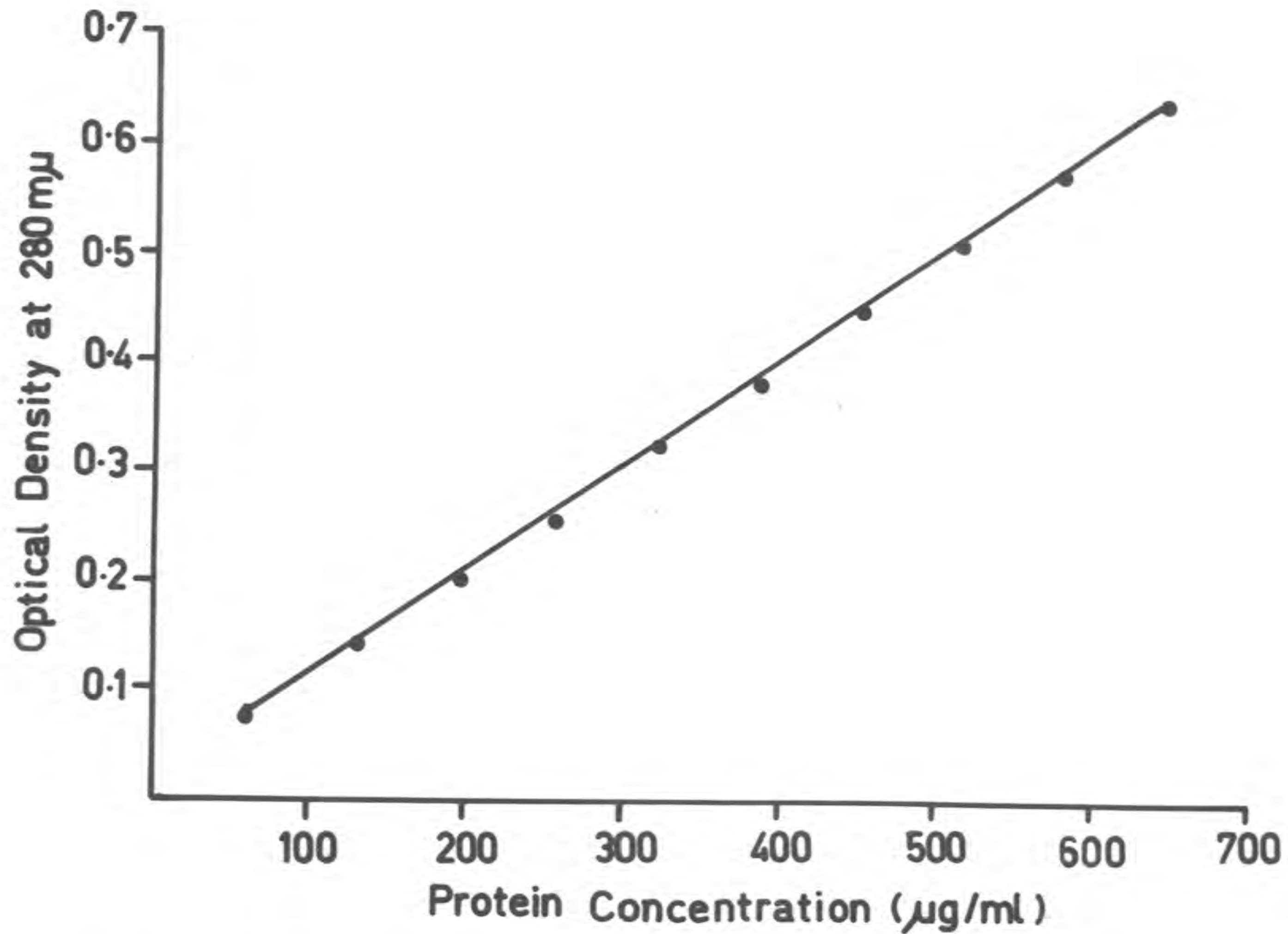


Fig.9 Standard curve for Protein Concentration at O.D. 280 mμ

ionic strength 0.075, pH 8.6. Aliquots containing approximately 600 micrograms of protein were applied to the paper. Following the period of electrophoresis the paper strips were removed from the cell, dried at 110°C for 30 minutes and then rinsed for 6 minutes in methanol, The strips were stained for six minutes in 0.1 per cent bromphenol blue in methanol, washed in 5 per cent acetic acid to remove the background stain and dried at 110°C for 15 minutes. The stained strips were then immediately placed in a closed vessel containing ammonium hydroxide to allow maximum colour development. They were removed from this container as required for quantitation in a Spinco Analytrol (Model RB) from which analysis the various groups of protein were expressed as a percentage of the total serum protein. The total protein concentration of each sample was known and the amounts of these fractions in mg./ml. was thereby calculated.

3. Starch-gel eletrophoresis

This method of serum fractionation was essentially that described by Smithies (1959) and was used in this study for two purposes. Firstly as a method of screening various rat sera and embryonic fluids to show age differences, and secondly as a preparative method for isolating various serum fractions for biological assay.

The method for both of these purposes was identical except that a larger volume of serum was used in the preparative procedure. The gel was prepared by heating a mixture of 60 gm. of hydrolysed starch and 500 ml. of 0.02M borate buffer at pH 8.6 until the starch solution reached the requisite viscosity. This mixture was then exhausted of air by subjecting it to reduced pressure for 45 seconds, poured into a Perspex former where it remained under a weighted lid for three hours at room temperature. The previously lubricated lid was removed after the serum samples (approximately 0.05 ml. per well for the screening procedure and 1.2 ml. per gel for the preparative method) were pipetted into the preformed wells. Liquid vaseline was poured over the surface of the gel to prevent dehydration and to retain the serum samples in the wells. The gel was then clamped vertically with the base standing in the anode electrode vessel and a paper wick connecting the cathode electrode vessel to the upper end of the gel. A potential of 4.5 volts/cm. was applied for 18 hours at room temperature. After this time the apparatus was dismantled and the vaseline removed. The gel was sliced for the screening procedure and stained in amido black 10 B for 60 seconds after which it was washed in methanol/water/acetic acid solvent to remove background stain. The preparative starch-gel was cut

into horizontal strips approximately one cm. wide, with reference to a stained longitudinal strip from the side of each gel, to include in each horizontal strip a major protein band. These strips of starch-gel were finely macerated with a scalpel blade and then extracted three times in a blender with 10 ml. of 20 per cent $(NH_4)_2SO_4$ at pH 6.5. The supernatant was filtered through a sintered glass funnel. Each fractionation was then dialysed with gentle agitation against 0.15M Na Cl at $4^{\circ}C$ for 24 hours. The retentates were freeze dried to approximately 10 ml. aliquots, redialysed against 0.15M Na Cl and then taken to dryness by freeze drying. Each fraction was dissolved in one ml. of buffer at pH 7.4 and assayed for protein content and for opsonic activity against Salmonella typhimurium C5.

4. Amylase Assay

The method used was based on that described by Van Loon et al. (1952). To a one ml. sample of a 1:10 saline dilution of rat serum or embryonic fluid was added 4 mg. of starch in a 0.1 per cent solution. Five ml. of phosphate buffer at pH 7.0 was added and the mixture incubated at $37^{\circ}C$ for 15 minutes. This was cooled by adding 50 ml. of distilled water. Four ml. of 0.01N iodine solution was added and the mixture made to 100 ml. with water. The samples were assayed for optical density in a Unicam spectro-

photometer at 650 millimicrons using a 1:1000 dilution in saline of the original sample as a blank. The results of this assay were expressed in arbitary units/ml. calculated from the expression $\frac{(O.D.Blank - O.D.Test)}{O.D.\ Blank}$ x 800 = Units/100ml.

5. Reduction by sulphydryl reagents

Samples of three month old rat serum were reduced by 2-mercaptoethanol. A 0.2M solution was prepared by adding 0.72 ml. of 2-mercaptoethanol to 50 ml. of phosphate buffer at pH 7.0. One ml. of this solution was added to one ml. of the test serum. This mixture was incubated at 37°C for 60 minutes and then dialysed against two litres of 0.15M NaCl at 4°C for 24 hours. The sample was then used to opsonise _Salmonella typhimurium_ C5 as previously described and assayed for biological activity.

6. Gel filtration chromatography

The method of serum fractionation by gel filtration used in this study is essentially that of Killander and Flodin (1962). The material used, Sephadex G-200 (A B Pharmacia, Uppsala, Sweden) is a crosslinked polydextran. The Sephadex was sieved to a 150-250 mesh fraction and this fraction was then soaked in 0.1M tris-hydroxy-methyl-amino-methane (TRIS) - H CI buffer pH 8.0 for 24 hours to allow swelling. The turbid

supernatant was removed by decantation and the material packed into a vertical column measuring 4 cm. x 50 cm. in the following manner. The column was closed at its base and then one third filled with buffer. A thin suspension of the gel was introduced and allowed to settle onto a glass wool pad situated at the base of the column. Excess buffer was drained from the outlet and the process of filling and draining was continued until the column was packed to within 5 cm. of its top, care being taken to prevent drying. A reservoir was then placed on the top of the column and the gel washed with three times its own volume of buffer. The gel was allowed to equilibrate at 4°C overnight and the remaining elution procedures were carried out at this temperature. A Whatman No. 1 disc of filter paper was placed on the surface of the gel and the excess buffer removed by pipette. The serum sample was then slowly applied and allowed to diffuse into the gel. The sides of the column above the gel were washed with buffer and a one litre reservoir placed on top of the column and filled with buffer. Elution proceeded by gravity at a flow rate of 20-25 ml./hour in 5 ml. aliquots metred by an electronically regulated drop counter and collected on a fraction collector. The optical densities of these fractions

were read at a wave length of 280 millimicrons in one cm. silica cells in a Shimadzu spectrophotometer. An elution curve plotting optical density of eluate against progressive volume of eluate was drawn from this data and the fractions were pooled according to the major peaks of this elution pattern. These pooled fractions were concentrated by pressure dialysis in L.K.B. ultrafilters(AB Pharmacia Upssala, Sweden). The concentrated fractions were then assayed for protein concentration and for opsonic activity towards Salmonella typhimurium C5.

7. Density gradient ultracentrifugation

The separation of proteins into high and low molecular weight components has been described by Kunkel (1960) whose method was employed in this study.

A sucrose gradient with concentrations of 40,35, 25 and 10 per cent sucrose in 1M Na Cl was prepared in ½" x 2" 'Lusteroid' tubes. One ml. aliquots of each of these sucrose concentrations were layered successively in each of these tubes commencing with the most concentrated solution. The solutions were then allowed to equilibrate for 24 hours at 4°C after which time each tube was layered with one ml. of serum sample with a protein concentration of approximately 30 mg./ml. The tubes were assembled in a SW/39 swing-out rotor and centrifuged for 18 hours at 35,000 r.p.m.

in a Spince (Model L) ultracentrifuge. After this time the sample was removed from each tube by bleeding in two drop aliquots from a hole pierced in its base. Each aliquot was collected in 5 ml. bottles to which had been added previously 3 ml. of 0.15M Na Cl.

The protein content of each of these samples was assayed by optical density as previously described. An elution curve was drawn by plotting optical density against the sample number. Fractions for assay were obtained by pooling the contents of the bottles with reference to the major peaks of the elution curve. These pooled samples were concentrated by pressure dialysis, assayed by spectrophotometry for protein concentration and assayed for their opsonic activity. to Salmonella typhimurium C5

8. Ion exchange chromatography

The method described by Turner and Rowley (1963) for the fractionation of serum proteins by di-ethyl-amino-ethyl (D.E.A.E.) cellulose ion exchange column chromatography was employed in this study. The D.E.A.E. cellulose was pretreated and washed by the method of Peterson and Sober (1960). The cellulose, suspended in 0.01M phosphate buffer at pH 8, was packed into a column, 1.2 cm. x 50 cm., under nitrogen at a pressure of 10 lbs./square inch.

A reservoir was then added to the column which was washed with 100 ml. of the 0.01M, pH 8.0 phosphate buffer.

During the remainder of the experiment the column was kept at a constant temperature of 4°C by water circulating through the outer jacket of the column. Five ml. of rat serum which had been dialysed against 500 ml. of the above phosphate buffer was slowly layered on top of the column. The serum was then eluted from the column by passage of 100 ml. of the 0.01M pH.8.0. phosphate buffer. Gradient elution was then provided from a six chambered mixing apparatus similar to that 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 0.01 M to 0.4 M. The final traces of protein were eluted with 50 ml. of 1M phosphate buffer at pH 5.6. The eluate from the column was collected in a fraction collector (Paton Industries, Beaumont, South Aust.) in 5 ml. aliquots at a flow rate of one ml./minute. These aliquots were maintained at 4°C throughout the course of the experiment. The fractions were assayed for protein content and an elution pattern drawn from which the contents of the tubes were pooled in accordance with the various peaks. The pooled samples were concentrated by pressure dialysis and assayed for protein content and opsonic activity to *Salmonella typhimurium* C5.

9. Production of antiserum

(a) Adult rabbits were immunised by six successive bi-weekly injections of 0.5 ml. of pooled adult rat serum into each buttock.. The animals were rested for 14 days following which 2 ml. of adult rat serum were given intravenously. The serum was collected 12 days later.

(b) Adult rats were injected four times intra-peritoneally with 10^6 viable Salmonella typhimurium C5 at two day intervals. They were rested for 14 days and again injected by the same route and with the same concentration of living organisms. The serum was collected 12 days later.

10. Immuno-electrophoresis

The method followed in these studies was essentially that described by Scheidegger (1955).

Bacto-agar (Difco Laboratories, Detroit, Michigan, U.S.A.) was washed with distilled water and then made to a one per cent gel with distilled water. This stock gel was maintained at 4°C until required when it was liquified by minimal exposure in a steamer. It was then diluted with an equal volume of veronal buffer, ionic strength 0.10 and pH 8.6. Three ml. of this hot solution was pipetted onto 3 inch x 1 inch glass slides, which had previously been coated with 0.15 per cent agar, and allowed to gel. Two mm.

wells were then cut 1½ inches from the end of the slide and ½ inch apart and the samples applied. The gel was then inverted and subjected to electrophoresis at 6 volts/cm. for 75 minutes at room temperature. A longitudinal trough was then cut in the gel midway between the wells and filled with antiserum. Forty-eight hours at 4°C in a humid atmosphere were allowed for the development of the precipitin bands. The excess antiserum was leached out at 4°C with numerous washes of veronal buffer, ionic strength 0.10 pH 7.4 followed by 0.15 M Na Cl. The buffer salts were removed by washing with distilled water at 4°C and the precipitin bands fixed by immersing the gels in one per cent acetic acid for 15 minutes. They were then stained for 15 minutes in amido black 10B and the excess dye removed by repeated washings in 2 per cent acetic acid. They were then dried over P_2O_5 and examined.

CHAPTER III

CLEARANCE STUDIES IN FOETAL AND NATAL RATS

a. Introduction

The mechanisms which protect an animal against the omnipresence of micro-organisms have a place of singular importance in determining the survival of the animal. The reticulo-endothelial system of mammals has been described as providing a major part of this protection by acting as a widespread system of cells which have the property of phagocytosis. It was suggested in an earlier chapter that this function of the reticulo-endothelial system could be a further ontogenic step by a system which has evolved to discriminate between the 'wanted' and 'not wanted' substances of an animals own body. It is considered reasonable to propose that the method by which the immune mechanism is expressed in the recognition of, to use Burnet's (1959) terms 'self' and 'not self' is in all probability an eminently economical application of the more primitive 'wanted' or 'not wanted' discrimination.

The function of the reticulo-endothelial system at the immunological level has been energetically investigated in natal animals by numerous workers. One of the main methods has been by using vital or non-vital particles in clearance studies. These studies are considered to test the phagocytic capacity of the

reticulo-endothelial system. Until the time of this study the development of the reticulo-endothelial system, and by implication immunological competence, of foetal animals had received little attention. It was assumed in fact that the mammalian foetus was immunologically incompetent and that any evidence to the contrary could be directly attributed to a passive transfer of immune substances from the maternal animal.

The results of investigations concurrent with this study have demonstrated the immunologic ability of foetal sheep (Silverstein _et al_., 1963) and the development of immunological competence in chick embryos (Karthigasu and Jenkin, 1963). It was considered that a study of foetal mammals would contribute to a greater understanding of the development of the immune response and its relation to other biological phenomena. The clearance from the blood of intravenously introduced particles has therefore been used in this study to investigate the phagocytic ability of the reticulo-endothelial system of foetal and natal rats and to gain some understanding of the way in which this ability develops from the foetal to the natal animal.

b. _Dose level in foetal rats_

Salmonella typhimurium C5 was the organism selected for the detailed clearance studies that were done in foetal and natal rats. This organism was chosen because of its well known characteristics in mouse clearance studies

(Jenkin, 1961) and because preliminary studies had shown that it was avirulent for rats. It was therefore anticipated that this organism would be cleared efficiently from the blood of adult rats and that if the younger animals varied in this capacity difference in clearance rates between animals of different ages would be readily obvious. A difference in clearance rates was in fact demonstrated at the dose levels stated in Chapter II. A dose of 5×10^7 orgs./0.05 ml. was chosen for foetal rats; it was based on the dose:weight ratio (10^9 orgs./ 100 gm. body weight) used for mouse clearance studies. In order to demonstrate that there was no dependence in this range a dose level varying from 10^7 orgs./0.05 ml. to 2.5×10^8 orgs./0.05 ml. were used in a dose dependence study in 20-day foetal rats. Table 2 shows the similar K values obtained at the various concentrations of _Salmonella typhimurium_ C5 that were used in this dose dependence study.

TABLE 2

The results of the dose dependent experiments using _Salmonella typhimurium_ C5 in 20-day foetal rats:

	Dose of _S.typhimurium_ C5/0.05 ml.				
	10^7	2.5×10^7	5×10^7	10^8	2.5×10^8
K	0.053	0.056	0.054	0.049	0.056

c. Clearance of Salmonella typhimurium C5 in foetal and natal rats.

Fig. 10 shows a typical clearance surve for 5×10^7 orgs./ 0.05 ml. for a series of 22-day foetal rats. The time of the experiment was extended to 120 minutes to illustrate the initial rapid exponential phase of clearance, during which approximately 80 per cent of the bacteria are removed, and the very much slower removal of the remainder. The rate of clearance of particles from the circulating blood was found to be similar by both viable and radioactive assay methods and can be calculated from the exponential section of this curve by the equation $K = \frac{C_1 - C_2}{T_2 - T_1}$ where C is the $\log_{10}$ concentration of either viable or radioactive counts at time T. The results from the series of clearance studies carried out using Salmonella typhimurium C5 in foetal and natal rats are presented in table form and as a composite graph in Fig. 11. These data illustrated that the phagocytic index (K value) varies according to the age of the rat. It can be seen that there was an initial rapid and progressive increase in K (from K = 0.026 to K. = 0.055) as the foetus matures from 16- days to 22- days and that the rate of clearance was maintained at a level similar to that at birth until about the time of weaning. The animals wean at approximately 21- days post partum and from this time until two months of age there is an approximate

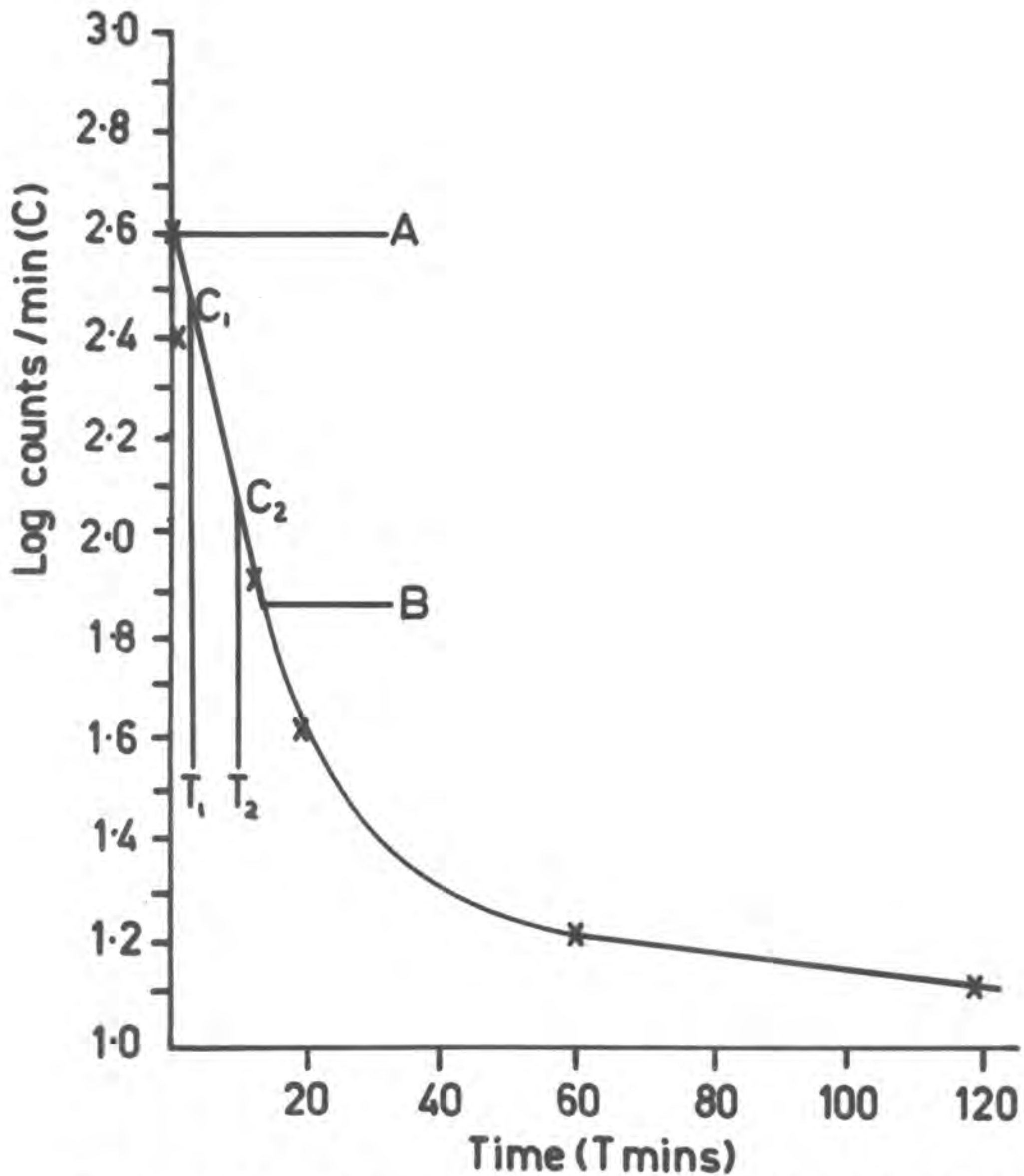
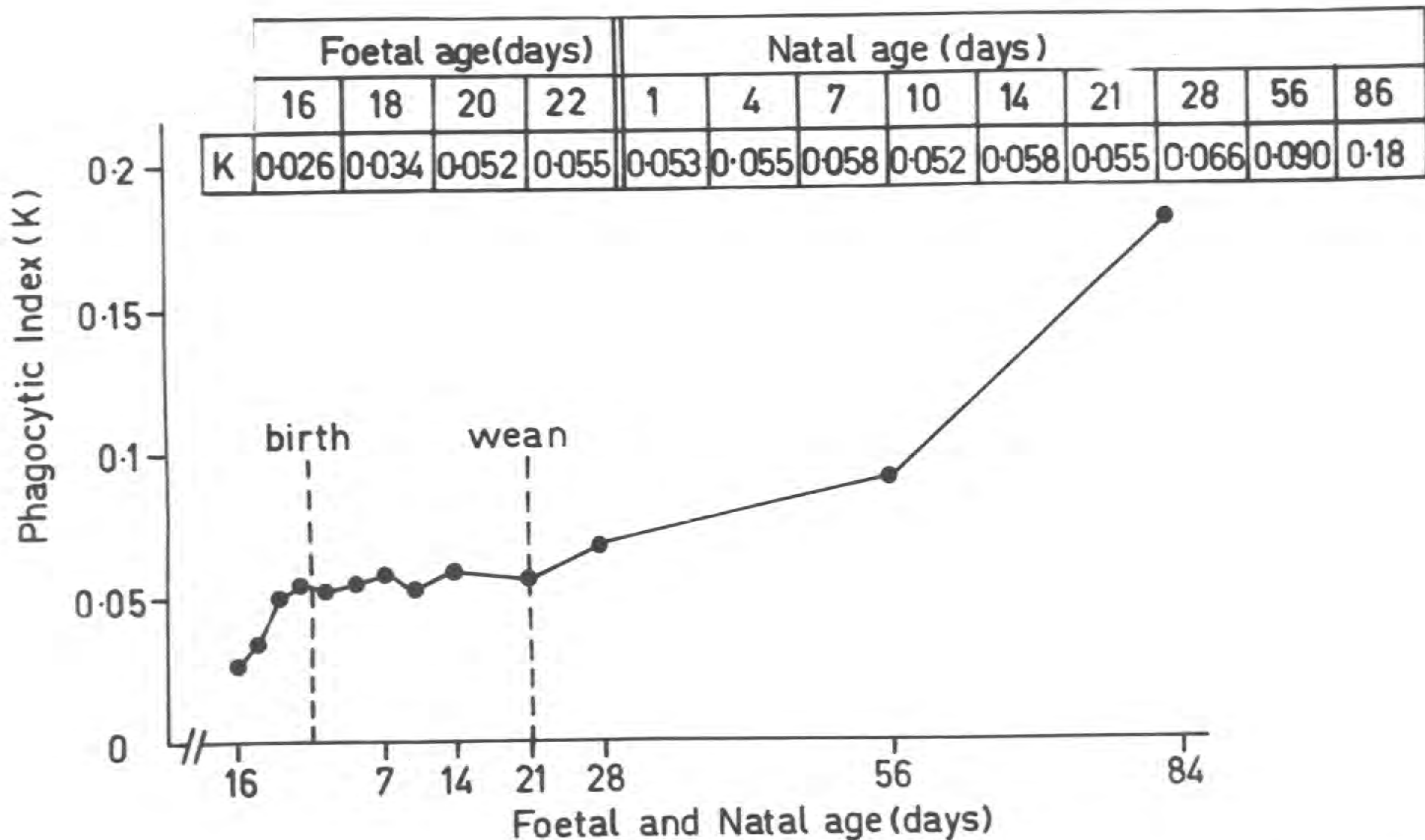


Fig.10 A typical clearance curve demstrating the linearity of the curve from A to B where the blood clearance is exponential with time and where the expression $K = \frac{C_1 - C_2}{T_2 - T_1}$ is applied



	Foetal age(days)				Natal age (days)								
	16	18	20	22	1	4	7	10	14	21	28	56	86
K	0·026	0·034	0·052	0·055	0·053	0·055	0·058	0·052	0·058	0·055	0·066	0·090	0·18

Fig. 11 A table and composite curve which illustrate a changing Phagocytic Index (K) in relation to the clearance of S. typhimurium as the animals mature.

doubling in clearance rate from K = 0.055 to K = 0.090. During the next month, until the animals are three months old, the clearance rate again doubles to reach K = 0.18.

d. Clearance of various other organisms in foetal rats

Other strains of Gram negative bacteria were used in clearance studies in foetal rats and the results, including those for *Salmonella typhimurium* C5 are shown in Table 3.

TABLE 3

A comparison of the K values obtained with a selection of Gram negative organisms used for clearance studies in foetal rats.

Organisms used in clearance studies	K values for Foetal Rats aged:		
	18 days	20 days	22 days
Salmonella typhimurium C5	0.034	0.052	0.055
Salmonella typhimurium M206	0.046	0.044	0.057
Salmonella gallinarum 9S	0.018	0.071	0.088
Salmonella enteritidis Se 795	0.076	0.13	0.15
Escherichia coli (Lilly)	0.072	0.12	0.15
Escherichia coli K12	0.15	0.25	0.28
Escherichia coli 2206	0.034	0.049	0.051

From the study of the clearance of this group of organisms in foetal rats aged 18- 20- and 22- days the increasing capacity that the maturing foetuses display for clearing bacteria from their circulating blood was again made

obvious. The extraordinarily rapid clearance of the rough *Eschenichia coli* strains was evidenced and the similarity in K value for smooth *Salmonellae* (with the exception of *Salmonella enteritidis* Se 795) and smooth *Escherichia coli* 2206 was demonstrated.

The previous work of Karthigasu and Jenkin (1963) has highlighted the importance of opsonins in the clearance of micro-organisms in chick embryos. These workers have shown that the addition of serum containing opsonins for an organism which by itself was not cleared would substantially increase its clearance after opsonisation. It was therefore decided to test whether the relatively slow clearance rate of some of the smooth organisms by the rat foetus was due to a deficiency of serum opsonins. *Salmonella typhimurium* C5 was opsonised for this purpose with adult rat serum which was known from mouse assay studies, (see Chapter VI) to contain considerable opsonic activity for this organism. These bacteria were then used for clearance studies in 22-day foetal rats. The results from this study showed that both the opsonised and unopsonised bacteria were removed at the same rate (K= 0.064) and this suggested therefore that opsonins were not necessarily the limiting factor in the slower clearance of the smooth Gram negative bacteria.

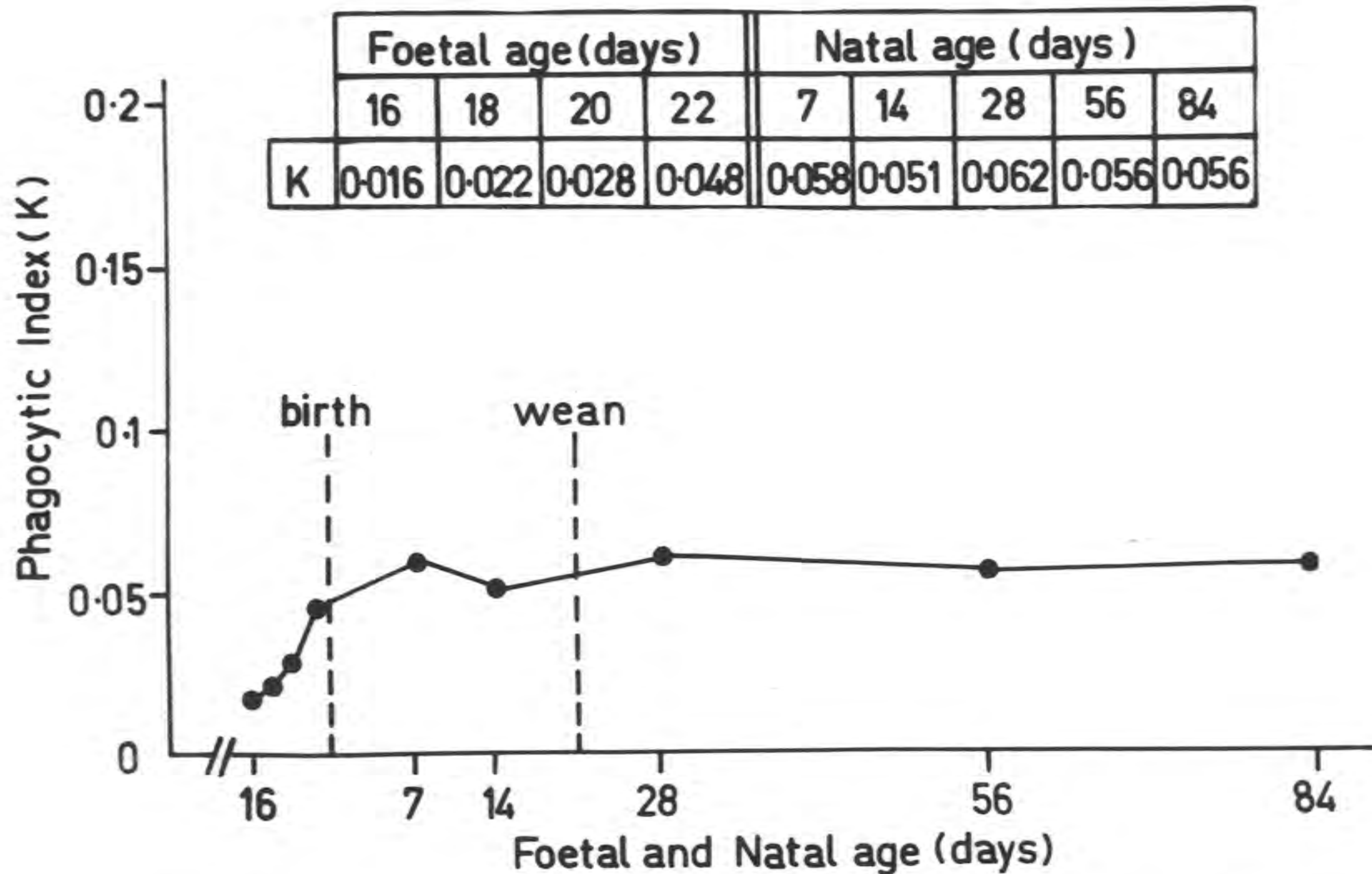
e. Clearance of carbon in foetal and natal rats

Following these studies of bacterial clearance,

shellac-free gelatin-suspended carbon was used in a similar way to examine the clearance of non-vital particles. It is acknowledged, however, as many studies have demonstrated, that carbon is not a biologically bland substance. Nevertheless its use provided a graphic method of demonstrating blood clearance especially in foetuses where the absence of fur and pigmentation allowed changes in skin and organ colour to be easily observed. The K values for the clearance of carbon in both foetal and natal animals using a dose of 0.4 mg. carbon for the foetal animals and a dose equivalent to 8 mg./100 gm. of body weight for the natal animals are shown in Fig. 12. These results demonstrate again the increasing functional capacity of the foetal phagocytic system and also demonstrate the relatively constant way in which natal rats clear a body weight determined dose of carbon.

f. <u>The relationship between changing clearance values and body and organ weight</u>

Reference was made earlier (Chapter I) to the derivation by Benacerraf <u>et al</u>. (1953) of a so called "corrected phagocytic index" (alpha value) which was introduced to take into account the individual variation in weight of the main organs in which phagocytic elements of the reticulo-endothelial system are found in animals of the same age and total body weight. The alpha value is obtained from the equation $\frac{W}{W.l.s.} \sqrt[3]{K}$ where W is the



	Foetal age (days)				Natal age (days)				
	16	18	20	22	7	14	28	56	84
K	0·016	0·022	0·028	0·048	0·058	0·051	0·062	0·056	0·056

Fig.12 A table and composite curve which illustrate the increasing Phagocytic Index (K) for a constant dose of 0·4 mg. of Carbon in foetal rats and a relatively constant K value for a weight determined dose in natal animals

total body weight, W.l.s. the weight of the liver and spleen and K is the phagocytic index.

Because it has been observed in this study that the liver and spleen account for approximately 90 per cent of the particulate matter phagocytosed, the ratio $\frac{W}{W.l.s.}$ is one which can be used to give an estimate of the relative amount of phagocytic system available in animals of different ages. From Table 4 it can be seen that foetal rats from 18-days to 22-days of age have approximately the same $\frac{W}{W.l.s.}$ ratio and that this ratio increases in natal animals to 7-days of age, then decreases to 28-days, to increase again during the next two months. It is interesting that Tables 4 and 5 which tabulate the results of clearance studies with Salmonella typhimurium C5 and carbon respectively show an increase in alpha value from 18-day foetuses to 7- day post natal animals. The alpha value then decreases until the animals are 28-days old and then increases to the age of 84- days. These results show that the reticulo-endothelial system increases in activity from the youngest foetus tested until the animal is about one week old. This activity falls slightly during the next three weeks to rise again as the rat reaches adulthood.

g. Conclusions

The clearance studies have demonstrated that foetal rats possess a functional phagocytic system, and that during

TABLE 4

Phagocytic indices (K and α) and weight indices for foetal and natal rats at various ages using <u>Salmonella typhimurium</u> C5

AGE (days)		Dose (orgs.)	Weight (gm.)			$\frac{W}{W.l.s.}$	K	α
			Body	Liver	Spleen			
Foetal Age	16	5×10^7	1.12	0.11	-	10.1	0.026	3.0
	18	5×10^7	2.55	0.23	0.001	11.0	0.034	3.6
	20	5×10^7	3.31	0.33	0.002	10.0	0.052	3.7
	22	5×10^7	4.85	0.41	0.007	11.6	0.055	4.4
Natal Age	4	5×10^7	7.2	0.35	0.02	19.5	0.055	7.4
	7	5×10^7	11.2	0.46	0.04	24.0	0.058	9.3
	10	10^8	16.5	0.71	0.05	21.7	0.052	8.1
	14	10^8	21.7	0.99	0.06	21.1	0.058	8.2
	28	2×10^8	68.7	4.04	0.46	15.3	0.066	6.2
	56	4×10^8	119.6	6.37	0.57	17.1	0.090	7.6
	84	6×10^8	180.1	8.64	0.68	19.3	0.18	10.9

TABLE 5

Phagocytic indices (K and α) and weight indices for foetal and natal rats at various aged using carbon.

Age (days)		Dose m.g.	Weight (gm.)			$\frac{W}{W.l.s.}$	K	α
			Body	Liver	Spleen			
Foetal Age	16	0.4	1.12	0.11	-	10.1	0.016	2.6
	18	0.4	2.55	0.23	0.001	11.0	0.022	3.1
	20	0.4	3.31	0.33	0.002	10.0	0.028	3.0
	22	0.4	4.85	0.41	0.007	11.6	0.048	4.2
Natal Age	7	0.9	11.2	0.46	0.02	24.0	0.058	9.3
	14	1.8	21.7	0.99	0.04	21.1	0.051	7.8
	28	5.4	68.7	4.04	0.46	15.3	0.062	6.1
	56	9.6	119.6	6.37	0.57	17.1	0.056	6.5
	84	14.4	180.1	8.64	0.68	19.3	0.056	7.4

the period studied its capacity to remove intravenously introduced particles from the circulating blood more than doubles. During the same period there is a fourfold increase in body and liver weight, but the ratio between the weight of the body and the weight of the liver remains constant. The increasing rate of phagocytosis does not appear to be directly related to an increasing liver weight for if it were the alpha value would remain constant. It could however be due to either a disproportionate increase in the number of phagocytic cells or to an increasing efficiency of the phagocytic cells themselves. The experiments indicate that from the beginning these cells in the liver which are known to have important functions in natal animals have the ability to recognise and phagocytose foreign particles. This implies, in terms of our present understanding, that the foetal rat possesses a mechanism that is usually associated with immunological competence. It also supports the concept that the reticulo-endothelial system is developed early in foetal life as a necessary physiological system involved primarily in catabolism and anabolism and in normal conditions accepts the secondary duty of host defence should it be called upon to do so after birth.

CHAPTER IV

THE DISTRIBUTION AND BACTERICIDAL ABILITY OF THE FIXED MACROPHAGES OF THE FOETAL RATS

1. Introduction

The fixed macrophages of the liver have been shown in innumerable studies in natal animals to be chiefly responsible for clearing micro-organisms and other particulate matter from the circulating blood, and to have therefore an important part in host defence against blood-borne infecting organisms. Biozzi *et al.* (1953) using carbon, and Benacerraf *et al.* (1955) using heat denatured human serum proteins complex with radioactive iodine (I^{131}), demonstrated that the uptake by livers and spleens of various animals species accounted for approximately 90 per cent of phagocytosed material. The ratio of the amounts removed by the liver and spleen respectively was found by these workers to vary with the dose. The liver accounted for the major proportion of uptake with small doses of rapidly cleared particles, while under the same circumstances, the spleen took up only trace amounts. With large doses (Benacerraf, 1958) and with less efficiently phagocytosed particles such as gelatin suspended colloidal chromium phosphate (Dobson, 1957) the proportion of injected particles recovered from the liver decreased, while the amounts found in

the spleen and bone marrow increased. Using isolated rat livers Wardlaw and Howard (1959) demonstrated a considerable difference in uptake of 13 species of bacteria from bacterial suspensions perfused through the livers. The percentage removal varied from 36 per cent to 87 per cent, thus demonstrating a considerable selectivity in the process of phagocytosis by the liver. The clearance from the blood of many types of micro-organisms has been shown to be dependent to a considerable degree on the presence of 'natural' or specific antibodies. In addition to this phagocytosis enhancing effect of antibodies it was suggested by Jenkin and Rowley in 1961 in the light of their work in 1959 that the events following ingestion of micro-organisms might also be dependent on the presence of these factors. Rapid destruction of micro-organisms is initially dependent on rapid phagocytosis, but it is not a necessary consequence, as was demonstrated by Biozzi et al. (1960) in regard to Mycobacterium tuberculosis. Mackaness (1961) further demonstrated this point by using a wild avirulent and a derived virulent strain of Listeria monocytogenes and showed their rate of uptake by macrophages in the mouse peritoneal aavity to be of a similar order but demonstrated an enormous difference in survival rates. The virulence of these organisms and others such as Salmonella typhimurium appears to depend on their ability to exist in vivo as facultative intra-

cellular parasites. Acquired immunity to Listeria monocytogenes was shown by Mackaness to be due to an apparent change in the intrinsic properties of the host macrophages and not to humoral factors. Complications therefore arise in attempting to define these parameters. Conditions for growth of a number of organisms inside a cell would seem to be suboptimal. Apparently there is either a balance established between growth and death of the organisms or there is growth followed by release of organisms from the cell. There is as a consequence parasitisation of other cells and the cycle repeats itself. In addition to this there is the possibility of multiplication in the extracellular phase.

Rowley and Whitby (1959) have demonstrated with in vivo experiments using mouse peritoneal macrophages and immune mouse serum that once the various species of Gram negative organisms used were phagocytosed they were equally rapidly destroyed irrespective of their virulence for the mouse. It seems evident from these studies that while opsonins are required for phagocytosis their role in determining intracellular survival of facultative intracellulr organisms seems to vary from one extreme to the other - from virulent Listeria monocytogenes which is phagocytosed but not killed to virulent Salmonella typhimurium which is rapidly phagocytosed and killed. If, however, Salmonella typhimurium exerts its pathogenic effect from an intracellular location it must on the one hand

be sufficiently opsonised to be phagocytosed and yet not carry enough opsonin with it to produce its intracellular death. Jenkin and Rowley (1961) have emphasised that it is probably that the intracellular fate of this organism and therefore its potential to produce disease is dependent on the titre of serum factors at the time of infection.

In this aspect of the study an attempt was therefore made to determine the organ location of the phagocytic cells of the reticulo-endothelial system of the foetal rats and to determine the bactericidal ability of these cells in the livers of foetal and neonatal rats.

2. Organ distribution of intravenously injected isotopically labelled *Salmonella typhimurium* C5

In these experiments rat foetuses aged 14-, 18-, and 22-days were injected with 5×10^7 isotopically labelled (P^{32}) *Salmonella typhimurium* C5. As the isotopically labelled suspensions of bacteria were used for up to five days the precaution was taken of washing and resuspending the organisms before each experiment. This was done because it was found in the initial experiments that as much as 15 per cent of the radioactivity injected could not be accounted for in recovery experiments. It was subsequently found that this was due to the loss of dialysable phosphate which could be removed from the bacterial suspension by washing. Unless this precaution was taken small quantities of label could be detected in the maternal circulation. Micro-organisms, on the other hand

were never detected in the maternal circulation.

The blood clearance was followed for 120 minutes and at each of the determined times a conceptus (or two conceptuses if the litter size allowed duplication) was removed from its attachment to the uterine wall and dissected into its major organs. These were digested with sodium hydroxide and the resultant material assayed for radioactivity. The results of this assay were expressed as a percentage of the total number of counts injected.

In these experiments the animals were not perfused and consequently a proportion of the radioactive counts recovered from each sample can be attributed to contained blood. For a similar reason in calculating the total recovery of radioactivity the amount estimated to be in the blood at any one time was not included in the figure for the total.

Table 6 shows the average blood volume of 14- 16-, 18- and 22- day foetal rats as calculated by the dilution of the radioactive label at zero time after injecting a known number of radioactive counts. It was a necessary step in the calculations to estimate the blood volume (Table 6) so that the total radioactive counts in the blood could be ascertained at any given time.

TABLE 6

Blood volume of foetal rats as calculated by dilution of radioactive label (P^{32}) compared with body and conceptus weight.

Foetal Age (Days	Blood Volume (ml.)	Body Weight (gm.)	Conceptus Weight (gm.)
14	0.10	0.54	0.89
16	0.17	1.12	1.57
18	0.26	2.55	3.10
20	0.38	3.31	3.87
22	0.53	4.85	5.54

The average weights of the conceptuses and the average weights of the foetuses at these ages are included in Table 6 to show the closer correlation between blood volume and body weight. The close correlation between blood volume and body weight in natal animals is well known.

Table 7 is an example of a balance sheet accounting for the distribution of radioactive label, and hence presumably micro-organisms (because of the intrinsic location of the P^{32}), at various times after their intravenous injection. It was seen from experiments in which the blood, organs and carcasses of 14-, 18-, and 22- day foetal rats were assayed for radioactivity following the intravenous injection of 5×10^7 isotopically labelled Salmonella typhimurium C5 that the clearance of these organisms was almost entirely due to the activity of the liver. It was also evident

TABLE 7

Percentage distribution of isotopically (P^{32}) labelled *Salmonella typhimurium* C5 in the organs of developing rat foetuses.

Time (mins)	Blood			Liver			Placenta			Lung			Gut and kidney			Brain			Heart			Foetal Carcass			Total Recovery		
	Age (Days)			Age (Days)			Age (Days)			Age (Days)			Age (Days)			Age (Days)			Age (Days)			Age (Days)			Age (Days)		
	14	18	22	14	18	22	14	18	22	14	18	22	14	18	22	14	18	22	14	18	22	14	18	22	14	18	22
1	89	66	65	5	15	22	13	31	39	22	3	6	2	3	3	2	1	1	1	1	1	54	47	27	103	84	96
5	68	69	46	7	5	22	26	35	36	5	2	4	1	2	4	2	1	1	1	1	1	59	56	29	98	110	113
10	59	66	25	12	30	62	20	16	11	12	4	4	2	3	3	3	2	1	1	1	1	50	44	15	88	95	98
20	27	42	9	21	55	69	25	1	7	3	2	4	2	4	3	3	2	1	2	1	1	43	27	15	83	95	98
60	13	11	4	20	54	67	28	10	7	2	2	4	3	3	3	4	1	1	1	1	1	53	30	17	106	99	111
120	12	10	4	34	49	64	20	10	7	2	4	4	5	2	5	3	1	1	1	1	1	36	34	17	130	109	101

in these experiments that the efficiency of clearance increased with age especially as the foetuses neared parturition and that this was due to an increasing capacity of the liver. The amount of radioactivity in the other organs, including the placenta, was considered to be due to the residual blood in these organs or to contamination during dissection. The total recovery of radioactivity was considered to be within the limits of experimental error.

3. Bacterial survival in the fixed macrophages of the livers of foetal and neonatal rats

Because of the results of the organ distribution studies described in the preceding section of this Chapter it was considered that a study of the bactericidal ability of the cells of the reticulo-endothelial system of foetal rats could be limited to a study of this ability in the phagocytic cells of foetal liver. Nevertheless it was decided to include the placentae in the investigation mainly because of the relatively large quantity of radioactivity retained in this organ, particularly in younger foetuses (Table 7), although as has been explaned, it was considered that this was due to retained blood. Initially the organism used in this study was *Salmonella typhimurium* C5. Approximately 10^4 of these organisms were injected intravenously into foetal rats aged 16-, 18-, 20-, and 22- days. At determined times a blood sample was aspirated from each foetus and the liver and placenta homogenised in adequate volumes of

sterile isotonic saline. The blood and a sample of the homogenate were plated out and the colonies counted after incubation. The total number of organisms in the blood, liver and placenta was then calculated and these results plotted as the $\log_{10}$ concentration of organisms against time.

It was evident from these results that foetuses at each of the ages examined treated the micro-organisms in a similar way. It was also shown that the numbers of organisms recovered from the placenta was a reflection of the number of organisms in the circulating blood, a fact which supports the contention advanced in the previous section. Fig. 13 shows a typical bacterial survival curve for Salmonella typhimurium C5 in 22- day foetal rats. It shows the relationship between the number of organisms in the circulating blood and in the liver over a period of 120 minutes and illustrates the disappearance of organisms from the blood and their progressive accumulation in the liver. It also suggests that in this time period foetal phagocytic cells are unable to kill the Salmonellae which they have removed from the circulation. In fact there is some indication that organisms actually commence to multiply. As it had been shown early in this present study that adult rats were not susceptible to this particular Salmonella it was presumed that the cells of the reticulo-endothelial

system were bactericidal towards it. In an attempt to discover the age at which a bactericidal mechanism becomes established, 10- day neonatal rats and 21- day weanling rats were subjected to bacterial survival studies using approximately two-fold and four-fold the foetal dose of organisms respectively. The results of these experiments are shown in Fig. 14 and Fig. 15 from which it can be seen by comparison with Fig. 13 that there was an indication of bacteriostasis at least in the 10-day neonates and every indication of killing in the 21- day weanling rats. It therefore appeared that the bactericidal ability of the phagocytic cells of the liver developed to a demonstrable level during the latter part of the suckling period.

In the first section of this Chapter the opinion expressed by Jenkin and Rowley (1961) that opsonins act not only by affecting the process of phagocytoses but also by contributing to the intracellular destruction of micro-organisms if at the requisite titre suggested that the foetal rats' inability to kill the test organism might result from an insufficiently high titre opsonins. The foetal rat has adequate opsonins however for its cells of the reticulo-endothelial system to phagocytose at their maximum level for Salmonella typhimurium C5 because it was shown in studies described in Chapter III that the addition of opsonin did not enhance the clearance of this organism. In an endeavour to discover whether optimal opsonisation would alter the absence of bactericidal

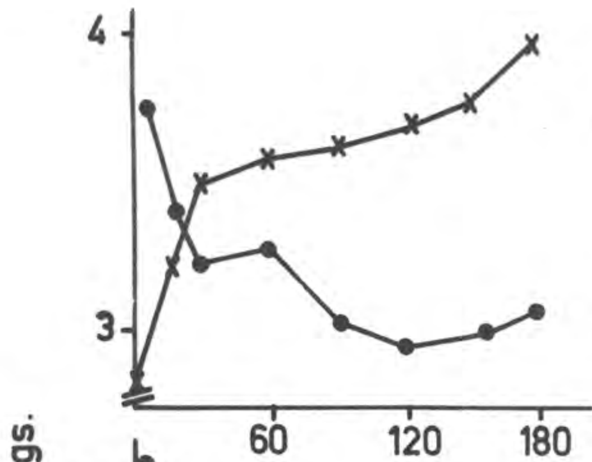


Fig.13 Survival curve for S. typhimurium C5 in 22-day foetal rats

x——x liver
•——• blood

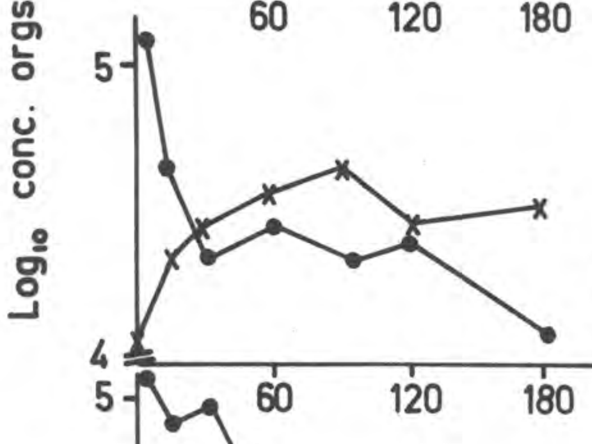


Fig.14 Survival curve for opsonised S. typhimurium C5 in 10-day neonatal rats

x——x liver
•——• blood

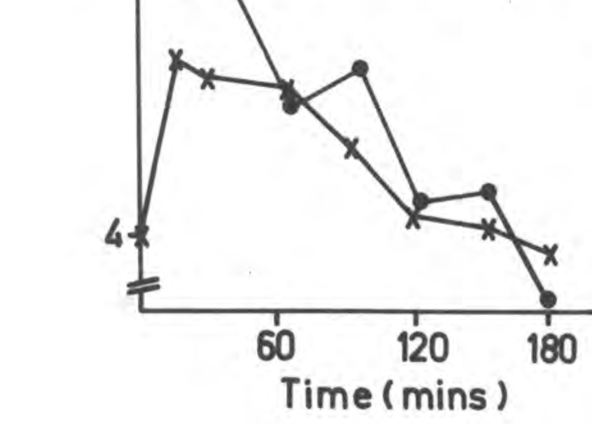


Fig.15 Survival curve for S. typhimurium C5 in 21-day weanling rats

x——x liver
•——• blood

properties of these cells approximately 10^4 opsonised Salmonella typhimurium C5 were injected into a series of 22- day foetal rats for the purpose of a bacterial survival study. The results of this study are shown in Fig. 17 which illustrates in comparison with Fig. 16 that this opsonisation procedure did not produce a bactericidal intracellular environment and that the organisms behaved in a similar way to the unopsonised ones. The clearance studies in Chapter III had also demonstrated that the rough Escherichia coli strains used were removed from the circulating blood with extraordinary rapidity and it was therefore questioned as to whether there were bactericidal mechanisms present in the cells of the reticulo-endothelial system for these organisms. Consequently approximately 10^4 Escherichia coli (Lilly) were injected into a series of 22- day foetal rats and bacterial survival experiments undertaken. Fig. 18 illustrates the results of these experiments and shows that for this rapidly cleared organism the foetal cells of the reticulo-endothelial system are also deficient in their bactericidal ability.

4. Conclusions

The studies described in this Chapter have again demonstrated that the phagocytic aspect of the reticulo-eddothelial system of foetal rats is a developing system. The organ distribution studies provide evidence for this by demonstrating both an increasing rapidity of uptake and an increase in the actual number of organisms cleared from the circulation as the age of the foetal rats increases. The observation of

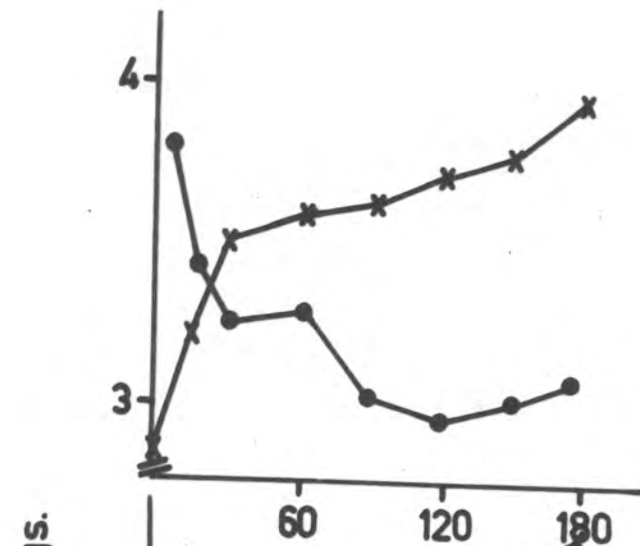


Fig.16 Survival curve for S.typhimurium C5 in 22-day foetal rats

x——x liver
•——• blood

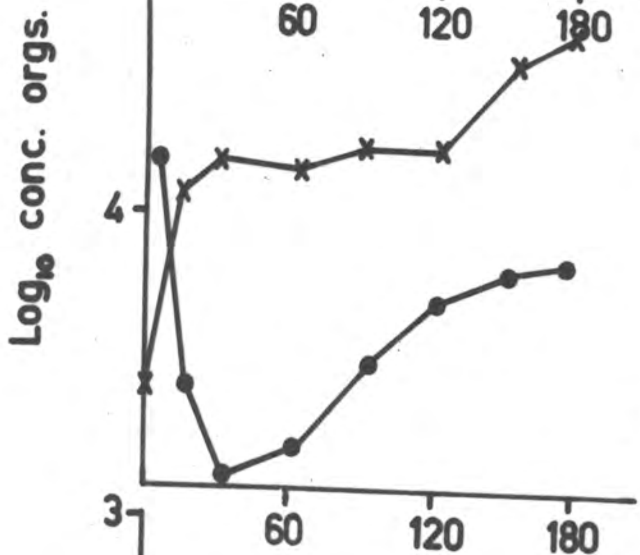


Fig.17 Survival curve for opsonised S:typhimurium C5 in 22-day foetal rats

x——x liver
•——• blood

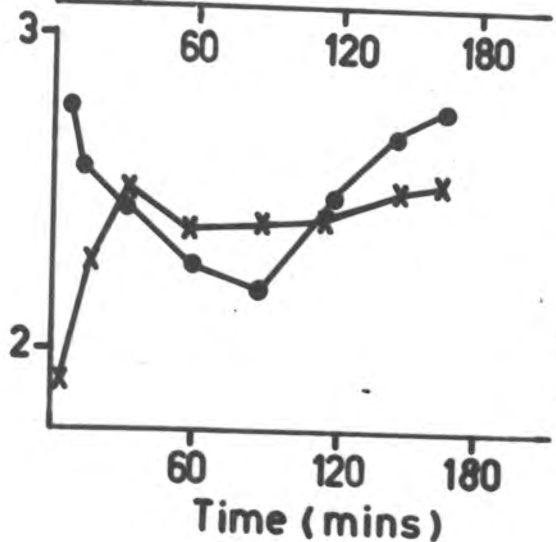


Fig.18 Survival curve for E.coli Lilly in 22-day foetal rats

x——x liver
•——• blood

Karthigasu and Jenkin (1963) that the embryonic membranes of chicks at an early stage of incubation accounted for a considerable percentage of the material phagocytosed was not paralleled in this study. The placenta and its membranes were treated together and were considered to be only passively involved in the radioactivity which they were found to contain.

An important purpose in performing these organ distribution studies was to show that it was in fact the liver and therefore most probably the cells of the reticulo-endothelial system of this organ which were responsible for the initial rapid clearance of micro-organisms and that it was not due to trapping in small vessels of other organs.

The bactericidal properties of the cells of the reticulo-endothelial system of the foetal livers as investigated in this study do not however show the same developing trend during foetal life but rather it appears that this aspect of development of the host defence mechanisms of the rat develops during neonatal life to be relatively effective by about the age of weaning. The phagocytic cells of foetal rats while able to phagocytose quite efficiently are apparently unable either to kill the bacteria tested or to prevent them from multiplying. The provision of extra rat opsonins does not change this situation and suggests that there are other factors ingoved which develop during the first weeks of natal life.

CHAPTER V

THE HISTOLOGY OF CLEARANCE BY THE FIXED MACROPHAGES OF FOETAL AND NATAL RATS

1. Introduction

Histological investigations of the cells and organs involved in clearing particles from the circulating blood have been numerous. By the end of the nineteenth century workers such as Hoffman and V. Recklinghausen (1867) and Kupffer (1878, 1899) had presented a picture of these cells in the liver. The accuracy of their descriptions made with the technical limitations of the time is remarkable in the light of present day refined techniques of election microscopy which have added little, except of minute detail to these original descriptions. The early investigators using colloidal metals such as gold, and various particulate dyes demonstrated the voracious capacity of the cells of the reticulo-endothelial system of the livers of natal animals to phagocytose this injected material. They demonstrated the ideal anatomical situations of these cells for constantly monitoring the circulating blood and clearing it of foreign particles.

In the first thirty years of this century this research was continued by numerous workers who attempted to quantitate by histological and chemical methods the uptake by various organs of introduced particles, (Maximow, (1906), Ranvier, (1890), Aschoff et al.(1924), Foot, (1921 etc.), Wislocki, (1924),

Drinker and Shaw, (1921), Cappell, (1929) and Pfull, (1926)).

From the time of the early investigations it was claimed by workers such as Kupffer that these phagocytic cells were part of the endothelial lining of the blood sinusoids of the liver and that they formed a syncitium, a view which has been held for many years, (Fawcett, 1955). While these cells have wide ramifications (Parks, 1956), present day studies with the electron microscope (Jezequel, 1962) indicate that they are separate cells forming part of the endothelial lining of the blood sinusoids.

Because of their avid phagocytic ability, their ideal anatomical site, and their great number (the liver contains more phagocytes than any other organ, Florey, 1962), the fixed macrophages of the liver have been accepted as the most important group of cells in the reticulo-endothelial system. It has already been emphasised that cells such as these are considered to have a more fundamental function than that which is often ascribed to them. The chain of events which follow erythrophagocytosis by cells of this system for example might well act as a model for the way in which the cells of the reticulo-endothelial system fulfil a basic physiological function by modifying phagocytosed substances as a step in their utilisation in other cells. The work of Vannotti (1957) has indicated the part played by the fixed macrophages of the liver in the reutilisation of the body's jealously guarded iron store and Bessis (1962), in an

electronmicrographic study has confirmed that the catabolism of the haemoglobin of phagocytosed red cells into molecules of ferritin takes place in these cells. It is in this form that the iron is stored in the body or passed on to erythroblasts by the reticular cells of the erythroblastic islets of bone marrow for haemoglobin synthesis.

At an immunological level Fishman (1959) has demonstrated that the passage of an antigen through macrophages is a necessary step in in vitro antibody production. More recently workers at the Walter and Eliza Hall Institute (1963) have shown in in vivo studies that following a local injection of a heavily labelled potent antigen the macrophages of the regional lymph nodes retain the labelled material while presumably handing on to the plasma cells the information necessary for antibody synthesis. The evidence therefore suggests that the physiology of iron metabolism might be a similar kind of procedure to that adopted for the handling of antigenic material by macrophages and the subsequent production of antibody by plasma cells. The possibility that antibody formation involves the participation of a team of cells has been reviewed by Wissler et al. (1957).

The introduction of electronmicrography has permitted a greater understanding of the process of phagocytosis by the hepatic macrophages. Parks and Chignoine (1956) demonstrated by this means the rapidity with which injected colloidal gold became associated with the liver phagocytes in mice and also that these particles enter the cells either by a

spheroidal or cleft-like depression of the cell membrane which later closes at the cell surface to produce an intracellular inclusion.

Despite the vast literature which exists concerning the histology of phagocytosis in natal animals no previous reference can be found to a comprehensive study in foetal mammals.

Following the demonstration in this study that various organs of foetal rats were capable of collecting injected particles and so clearing their circulating blood of foreign material it was decided to study clearance by histological methods. A variety of particles were used and these included viable bacteria and insoluble suspensoids. They were used alone and in various combinations.

2. Light microscopy

a. Organ distribution of injected particles in foetal rats

Because of the clearly defined way in which carbon can be demonstrated in histological sections and because its distribution over the time period studied appeared to be similar to that of other particles it has been used in this study as the model for the pattern of clearance by the cells of the reticulo-endothelial system of foetal rats.

Organs were removed from the animals used in the carbon clearance studies - described in Chapter III. They were fixed in formal saline and subsequently sectioned, mounted and stained with either haematoxylin and eosin or saturated aqueous picric acid.

Organs from foetal rats aged 14-, 16-, 18-, 20-, and 22- days were examined histologically 30 minutes after the intravenous injection of 0.4 mg. of gelatin suspended carbon with a particles size less than 500 Angstroms. The clearance studies in Chapter III have shown that the clearance of carbon from the circulating blood was virtually complete after this period of time. The organs examined were liver, spleen, gut, pancreas, lungs, heart, kidney, adrenal gland, brain, bone marrow, placenta and foetal membranes.

While the rate of uptake of carbon was obviously slower, and the number of phagocytic cells less in the younger foetuses the organ distribution was similar in the foetuses examined. A description of the distribution of carbon in 20- day foetal rats will serve therefore to give the general picture of the organ distribution of carbon in foetal rats above the age of 14- days.

It should be emphasised that the particles size of carbon used in these investigations precluded the possibility of seeing individual particles by light microscopy and that these particles only became visible on agglomeration. This meant, therefore, that as far as phagocytosis was concerned it was only cells which had phagocytosed a quantity of carbon that could be identified as constituent cells of the reticulo-endothelial system.

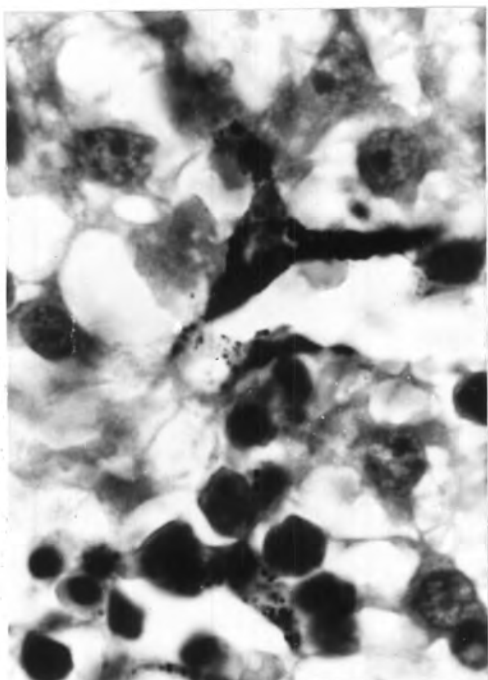
Three organs, the liver, spleen and adrenal gland were observed macroscopically to have collected carbon, and the

histological aspect of clearance in these organs will be described first.

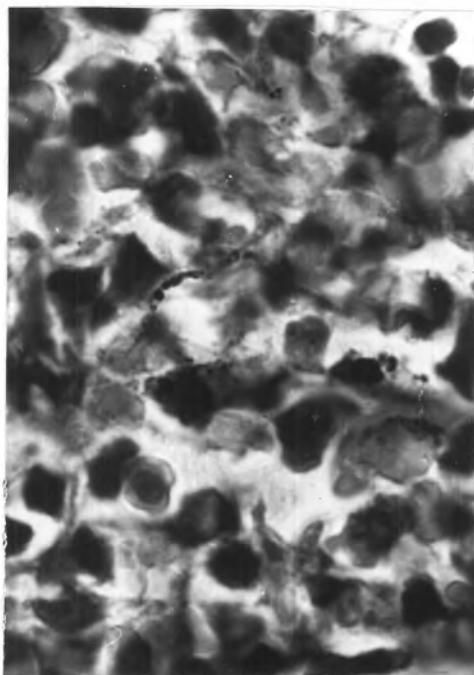
Livers of foetal rats, in common with livers of other foetal animals, have a haemopoietic function as well as functions attributable to natal livers. The presence of haemocytoblasts and their resultant series of cells gives the foetal liver a much less compact architecture and a considerably more diverse cell population than that of natal livers.

Amongst both the hepatic parenchymal cells and the haemopoietic tissue, blood sinusoids with a diameter two to three times that of an erythrocyte have extensive ramifications. The sinusoids are lined by sessile vascular endothelium interspersed with dendritic phagocytic cells - the 'sternzellen' of Kupffer (Fig. 19). The processes of these cells appear to extend both intra- and extra- vascularly and the latter extensions appear to pass between the parenchymal and haemopoietic cells. It is the dendritic cells which exhibit a voracious appetite for foreign particles. As far as could be determined it was considered that the majority of these cells were involved in the phagocytosis of carbon at any one time.

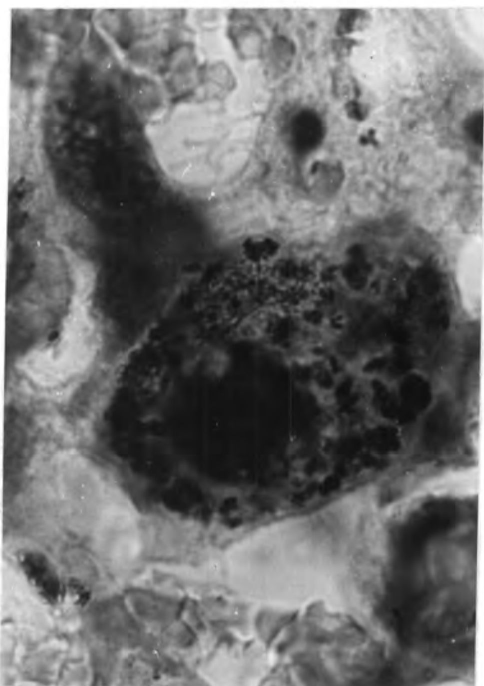
The spleens of these foetal animals also have a haemopoietic function and do not have a marked division into red and white pulp. Nevertheless it can be seen following the phagocytosis of carbon that this organ contains phagocytic cells spread more or less evenly throughout its substance.



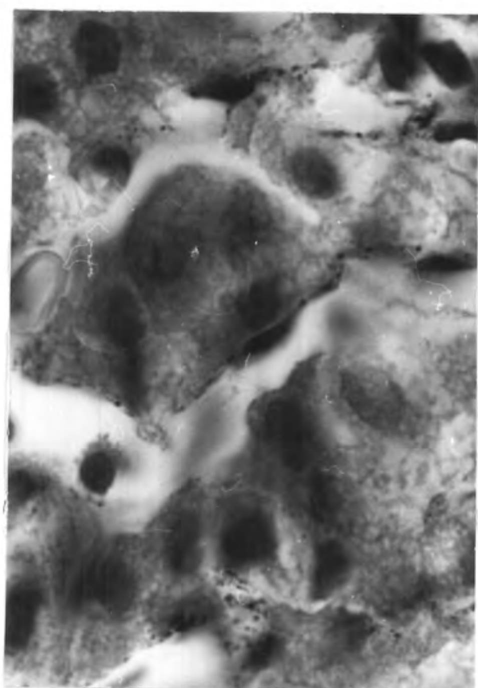
Liver



Spleen



Placenta



Adrenal

Fig. 19. Photomicrographs of organs from a 20-day foetal rat 30 mins after 0.4 mg. I.V. carbon, demonstrating the cells in these organs which phagocytosed carbon. H.& E. x 1300

(Fig. 19). They appear to be considerably less dendritic and smaller than their counterparts in the liver. Because of the smaller organ size and apparently smaller number of phagocytic cells the spleen was considered to account for a relatively small amount of the material cleared from the blood.

The adrenal gland was the third organ which had, from a macroscopic point of view, retained some of the circulating carbon particles. Histological sections showed that this carbon was limited to the foetal cortex and was associated with cells bearing a close similarity, both of situation and morphology, to the phagocytic cells in the liver. The foetal cortex, which constitutes the bulk of the foetal gland, consists of large granular cells tending to be arranged in cords and having a rich capillary blood supply. It is along these vessels that the phagocytic cells are situated (Fig. 19). They appear to be more concentrated towards the periphery of the gland which is the site from which the true cortex infiltrates the foetal cortex and eventually takes its place in neonatal life. It is considered that the contribution which this organ makes towards blood clearance is also relatively insignificant.

The other organs in which carbon could be detected associated with cells were the placenta and lungs. The cells in these two organs involved in the phagocytosis of carbon were however very rare. In the lungs small amounts of carbon were evident in cells whose type was difficult to

ascertain. It is probable that some of the carbon seen in this organ was trapped in anatomical backwaters.

The picture seen infrequently in the placenta showed considerable phagocytic activity of an accasional large cell closely associated with the syncitium (Fig. 19). These were probably trophoblastic giant cells in the cytoplasm of which the carbon appeared to remain in a more diffuse state than the rapid aggregation seen in the hepatic sinusoidal macrophages. These cells in the placenta were rounded and did not have the characteristic processes seen for examples in the phagocytic cells of the liver.

The other organs examined, gut, pancreas, heart, kidney, brain, bone marrow, and foetal membranes, did not contain, as far as could be determined, any cells which appeared to have phagocytosed carbon.

b. Comparison of distribution of injected particles in foetal and adult rat livers.

The marked variation in architecture between foetal and adult livers rather than a variation in cell type seems to account for the obvious difference in distribution of phagocytosed particles. The foetal liver has been shown to be less compact and apparently of no well organised internal structure (Fig. 20). The adult liver on the other hand is well known to have a highly regular structure with the parenchymal cells of each polyhedral lobule arranged in cords radiating from the central vein to the periphery(Fig. 21).

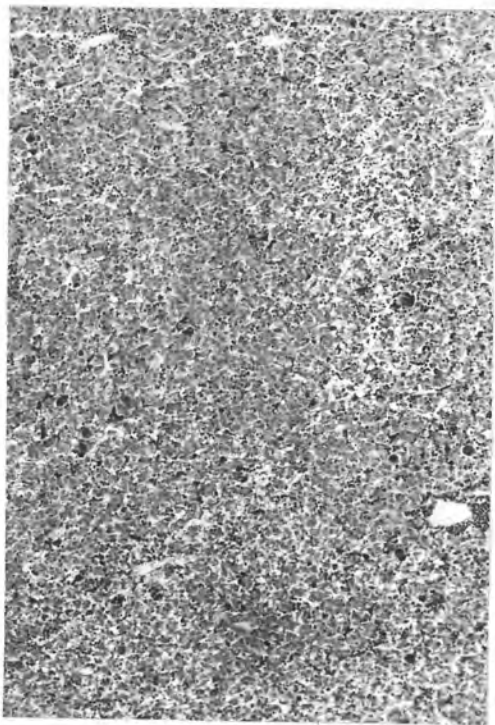


Fig.20. Photomicrograph of a 20-day foetal rat liver, 30 mins. after I.V. carbon H. & E. x 95

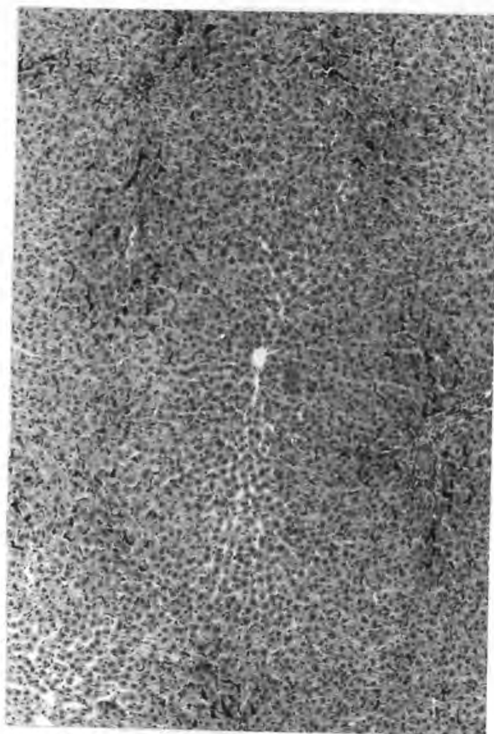


Fig. 21. Photomicrograph of an adult rat liver 30 mins. after I.V. carbon H. & E. x 95

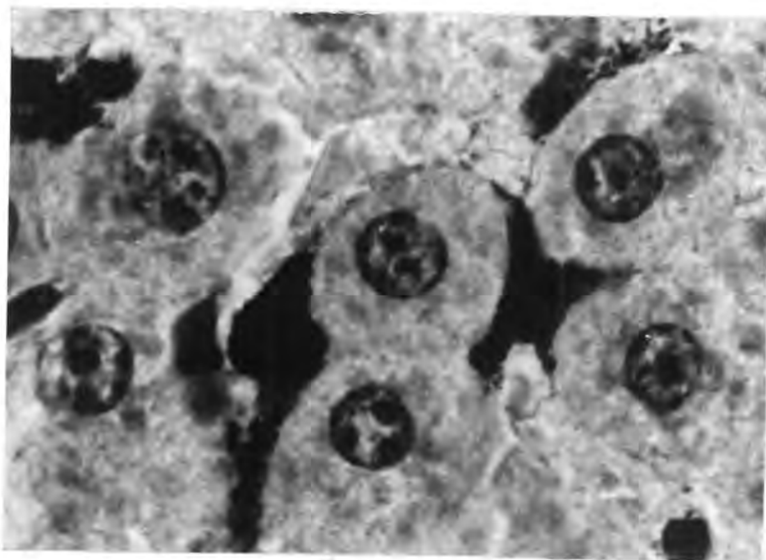


Fig. 22. Photomicrograph of an adult rat liver after I.V. carbon, demonstrating the relationship of the hepatic phagocytes to the parenchymal cells, H. & E. x 2000

Around the periphery of each lobule are portal canals and between the cords of the parenchymal cells there are sinusoids containing blood that drains inwards to the intralobular vein. The hepatic phagocytes are arranged, along these sinusoids. It was presumed that the phagocytic cells at the periphery of each lobule were the ones which phagocytosed the bulk of the carbon because these were the first to encounter particles in the blood flowing from the portal canal to the intralobular vein. In contrast to this the relative lack of organisation in the foetal liver meant that here the phagocytic cells all had an opportunity to take up carbon and consequently it was seen in these sections that the majority of the foetal hepatic phagocytic cells had phagocytosed carbon.

While the foetal phagocytes often appeared to have more extensive processes than the adult phagocytes this appearance could possibly be attributed to the less compact architecture of foetal livers and the ease thereby with which these dendritic processes could be demonstrated.

It was frequently observied in adult livers, however, that the carbon-filled phagocytes were apparently not only enclosing the cords of cells but also passing between the individual parenchymal cells (Fig. 22).

c. Time sequence of carbon clearance by the liver in foetal and adult rats

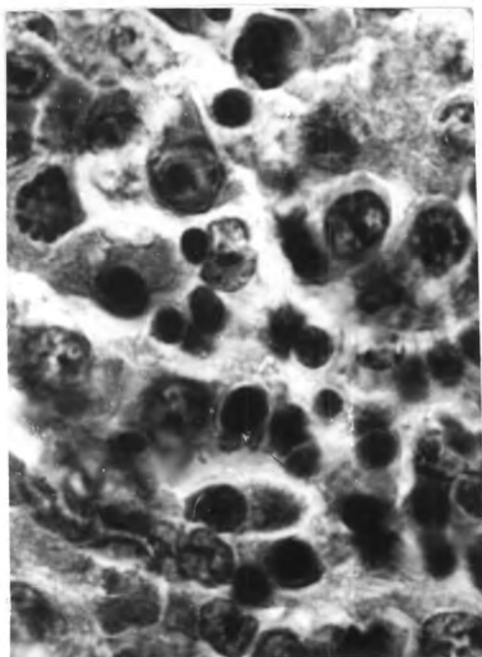
Livers were removed from foetal rats aged 14-, 18-, 20-, and 22- days, and from adult rats at time 1,2,4,6, 8,10, 15,

30, 60, 90, 120 and 180 minutes. The sequence of events which were seen to occur as the time after injection increased gave a most striking picture of the process of phagocytosis. The first evidence of the clearance of carbon was obvious histologically almost immediately after the carbon had been injected intravenously.

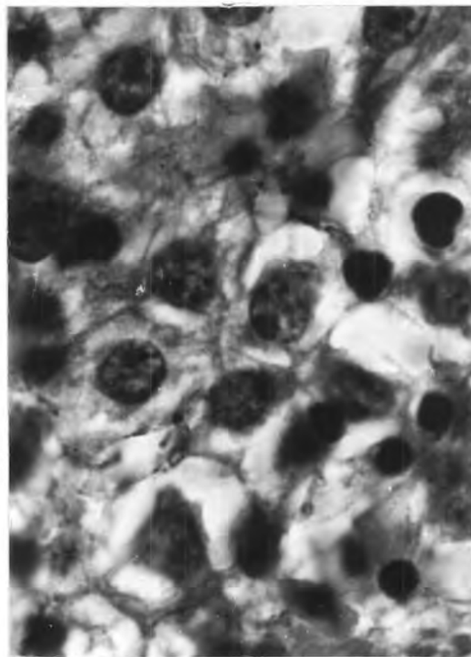
The changes in the phagocytic cells of the foetal livers are illustrated in Figs. 23 and 24 using a 20-day series as an example of the pattern observed in all the ages examined. The particles of carbon were apparently distributed over the surface of the phagocytes and their processes in the one minute samples. While the clearance of particles from the blood continued, those particles which had presumably been taken into cells were collected in the bodies of the cells so that the nuclei of the phagocytes were obscured from view.

The reticular pattern produced by the initial association of the particles with the processes of the cells was seen to diminish slowly until in the 30 minute sample this pattern had disappeared. At this stage most of the carbon in the phagocytic cells was seen to be in a perinuclear location. This coincided with the time at which clearance was considered to be near completion according to blood assay studies and from this time until the experiments were terminated at 180 minutes the histological picture remained the same.

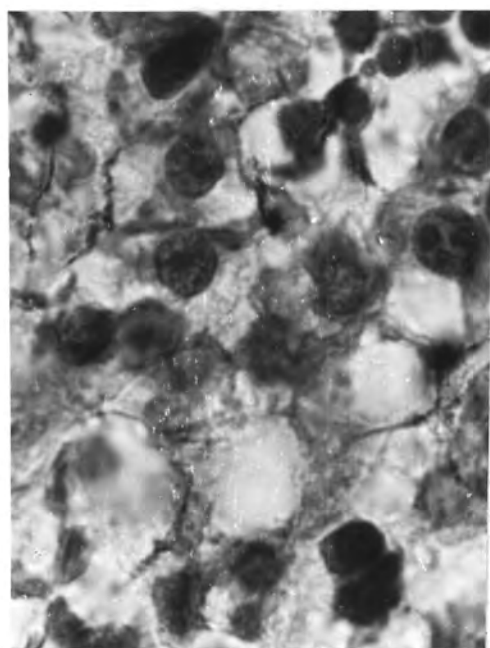
The rate at which the carbon was picked up by the processes of the cells and then moved into the body of the



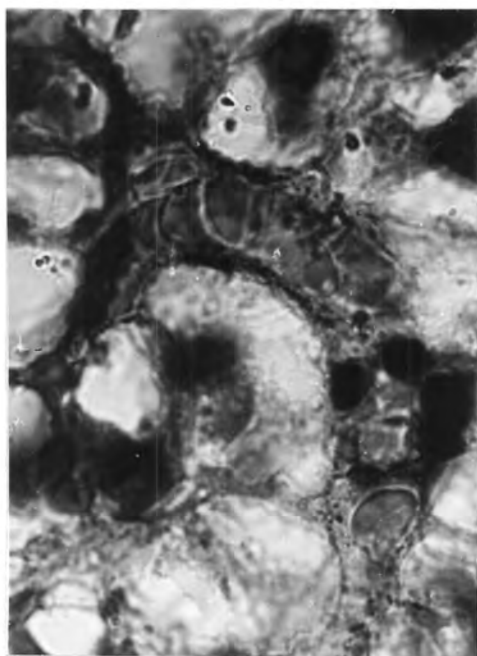
Normal Liver



1 min. after I.V. carbon

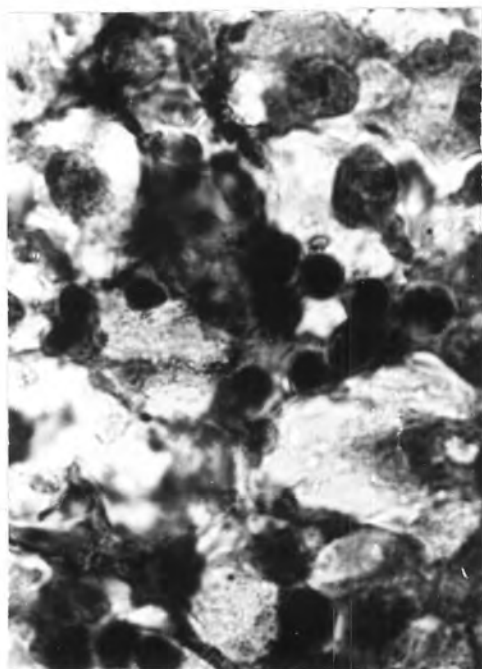


2 min. after I.V. carbon

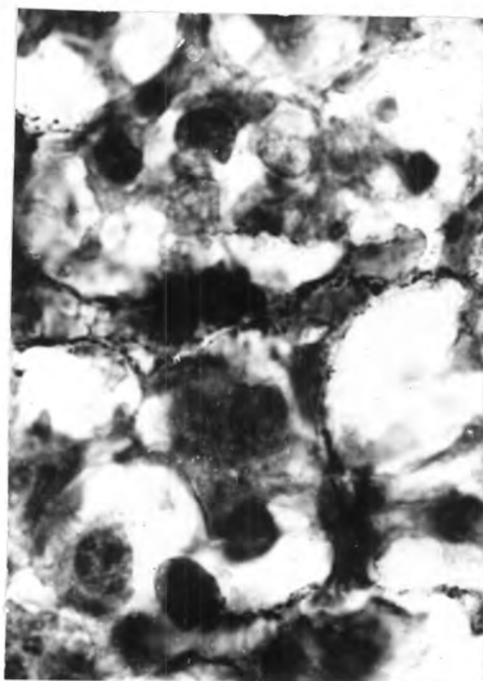


4 min. after I.V. carbon

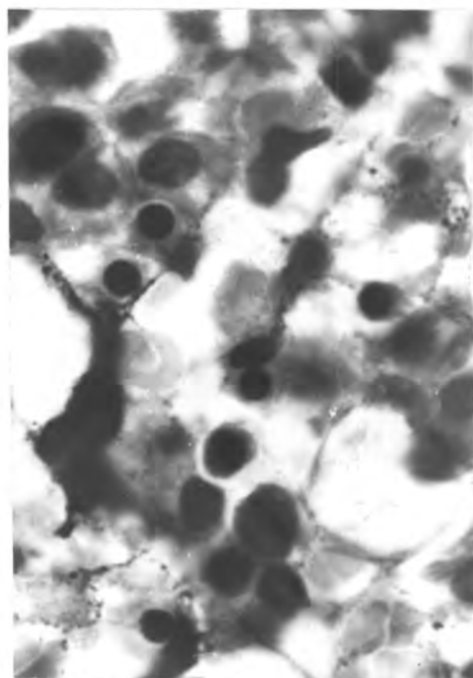
Fig. 23. Photomicrographs of livers from 20-day foetal rats at time intervals after the I.V. injection of 0.4 mg. carbon. The appearance of the reticular pattern due to the collection of carbon by the hepatic phagocytes is evident H. & E. x 1300



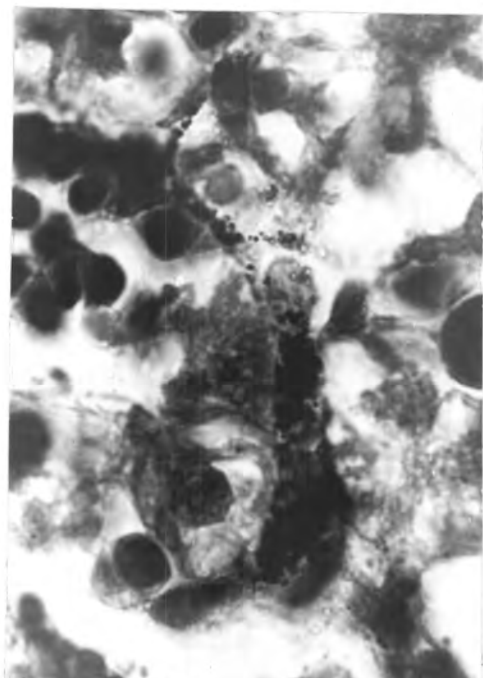
6 mins.after I.V. carbon



8 mins.after I.V. carbon



15 mins. after I.V. carbon

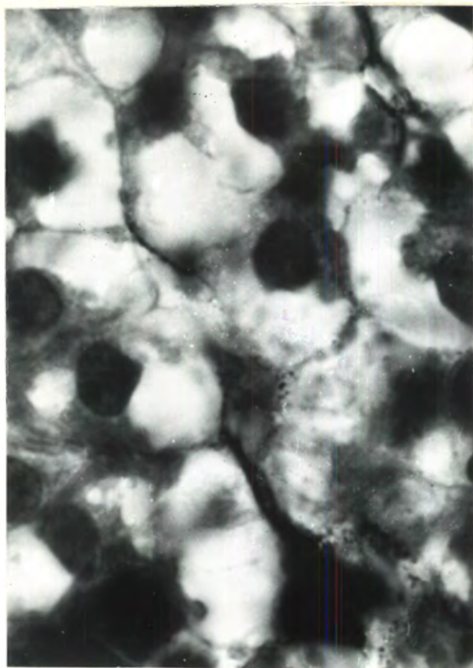


30 mins. after I.V. carbon

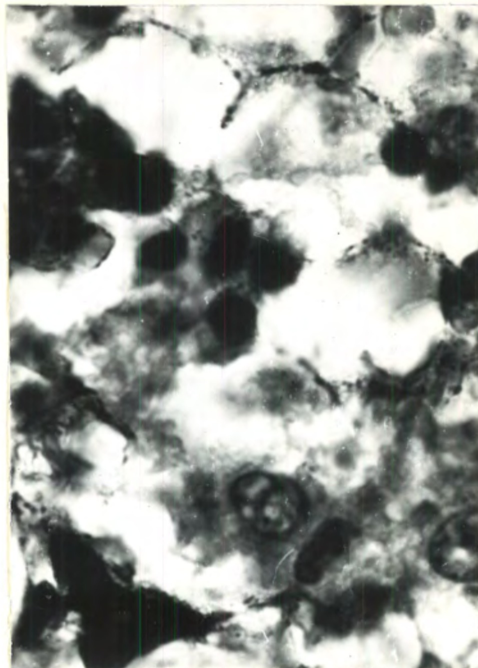
Fig. 24. Photomicrographs of livers from 20-day foetal rats at time intervals after the I.V. injection of 0.4 mg. carbon. The progressive collection of carbon into a perinuclear location in the hepatic phagocytes is apparent. H. & E. x 1300.

cell varied with the age of the foetus, being obviously faster as the age increased. The picture seen in the adult livers was somewhat the same as that seen in the foetal livers but exhibited a very much more rapid change. It was probably the greater efficiency of clearance which made the process of phagocytosis less obvious in the adults than in the slow motion version seen in the foetal livers, and made it more difficult to detect by this method the wide ramifications of the adult phagocytes.

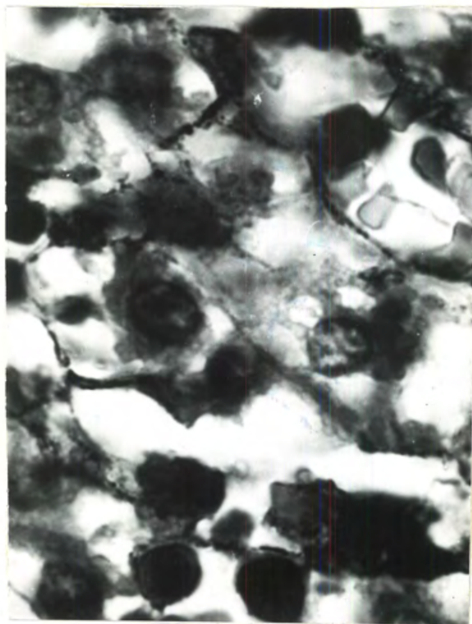
A question was posed as to whether the particles associated with a phagocytic cells were moved to their final location in the cell by some intracytoplasmic flow from the processes inwards or whether it was due to the processes of the cell being retracted and the cell "rounding-up" after phagocytosing its quota of foreign material. In an attempt to answer this question a second dose of carbon was injected into a series of foetal and adult rats - one 10 minutes after the initial dose and the other 30 minutes after the initial dose - and the animals sacrificed according to the time scale used in the original experiments. It was anticipated that if the processes had retracted, the histological picture following the second dose of particles would fail to show them. Histological examination did show however a picture similar to that after a single injection, except that the body of the cells were already loaded with carbon (Fig. 25). This suggested, therefore, that the processes of the phagocytic cells remained extended and that the particulate material



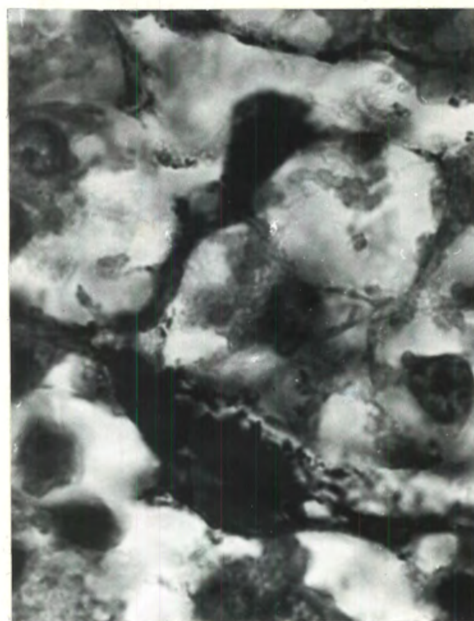
1 min. after 2nd dose of carbon



2 mins. after 2nd dose of carbon.



4 mins. after 2nd dose



8 mins. after 2nd dose

Fig. 25. Photomicrographs of livers from 20-day foetal rats after a second dose of carbon, taking 10 mins. after the initial injection as zero time. H. & E. x 1300.

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was shifted by some other mechanism operating within the cell.

d. Distribution of mixtures of particles in the livers of foetal and adult rats.

Various mixtures of dyes and carbon and the two solutions used for the electronmicrographic study to be described in the next section were injected intravenously and the organs examined at times up to 125 minutes.

It was observed that those particles which could be seen by light microscopy were to be found mixed together in the same phagocytic cell. Cells which had phagocytosed one type of particle were uncommon and even when they were seen it was impossible to say categorically that only one type of particle had been phagocytosed by the one cell.

The findings in the livers of both foetal and adult animals were apparently quite parallel and indicated that not only could an individual cell phagocytose different types of particles but that it could also phagocytose particles of different size.

3. Electron microscopy.

a. Introduction

While it seemed reasonably conclusive that the phagocytic cells in the animals examined were in fact engulfing the various test particles (see Chapter II) it was decided to gain further evidence for this, if possible, by electron micrography. It was also considered that this method of study might add to the picture of the morphology of the hepatic phagocytes of the rat and make clearer the process

of phagocytosis by these cells.

The particles selected for this study were considered to be identifiable by electron micrographic techniques and represented a range of sizes from 100 Angstroms to 3 microns. All of the particles proved to be readily identified except in the case of ferritin which could not be distinguised from the endogenous ferritin already present in the tissues.

b. Phagocytosis by hepatic macrophages in foetal and natal rats.

In this investigation mixtures of particles (see Chapter II) were injected intravenously into 20- day foetal and 84-day natal rats which were sacrificed at times up to 125 minutes. The livers of these animals were prepared for examination by electron microscopy. The results of this investigation demonstrated quite clearly the phagocytic ability of the foetal hepatic macrophages and the relationship which these cells have to the hepatic parenchymal cells. It was apparent that the rate of uptake by the adult cells was more rapid and that these cells were equipped to destroy the phagocytosed bacteria whereas those in foetal animals appeared to be unable to do so.

The anatomical relationship between the macrophages and the parenchymal cells was well shown in the adult material. It could be seen that the macrophages were closely applied to the hepatic cells and were observed, as illustrated in Fig. 26 to extend between them to neighbouring sinusoids. The macrophages were also shown (Fig. 27) to extend across sinusoids to parenchymal cells on the other side.

These findings demonstrated to an even greater degree than was shown by light microscopy the ideal situation which the macrophages have for removing unwanted material from the circulating blood.

The intimate relationship which was shown to exist between macrophages and the villous border of the adjacent parenchymal cells demonstrated an ideal relationship for transfer between these cells. Because of the less compact nature of foetal livers and because of the considerable proportion of haemopoietic tissue, it was not possible to demonstrate such well-defined anatomical relationships between the macrophages and parenchymal cells of foetal livers. Nevertheless foetal hepatic macrophages were observed to have similar characteristics to the natal macrophages. They were large prominent cells interspersed amongst sessile endothelial cells which lined the blood sinusoids.

A cross section of a typical sinusoid lined by sessile endothelial cells in a foetal liver is shown in Fig. 28. In this figure injected carbon and polystyrene latex can be seen in the lumen of the sinusoid.

Fig. 29 illustrates the presence of injected <u>Escherichia coli</u> (Lilly), carbon and thorotrast in the lumen of a foetal liver sinusoid. It demonstrates what was probably a shrinkage artefact of the bacterial cytoplasm from its cell wall. This appearance was seen throughout the study. Fig. 30 shows an early stage of phagocytosis with a pseudopod of an hepatic

macrophage commencing to phagocytose a micro-organism. Two other points of interest are well illustrated in this electron micrograph. They are firstly the difference in size and number of mitochondonia in the macrophage and the hepatic parenchymal cell - those in the latter being much more numerous and several times larger than in the former. The second point of interest is a relative absence of ergastoplasm in macrophages and its abundance in the parenchymal cells.

Figs. 31 and 32 show two stages in the phagocytosis of a bacterial cell, and Fig. 33 demonstrates the intracellular location of a number of bacteria. A membrane enclosed vacuole containing three bacteria, carbon and thorotrast can be seen in this macrophage; the other bacteria are enclosed in separate vacuoles and a small vacuole can be seen which contains carbon. An unexpected finding is illustrated in Fig. 34. This shows the presence of bacteria, carbon and thorotrast in a vacuole with a continuous membrane in a liver parenchymal cell. The lead stained glycogen in the upper right hand portion of the electron micrograph is a good indication of the cells identity. Fig. 35 shows the collection of polystyrene latex and carbon into several vacuoles in a foetal hepatic phagocyte 16 minutes after injection. This electron micrograph also illustrates the presence of micro-villi at the cell surface which is a common characteristic of these cells.

The presence of bacteria was also noted in the parenchymal cells of the 84- day livers. The rapidity with which the

bacteria were seen to be destroyed in the parenchymal cells is perhaps indicative of the devastating array of enzymes to which these organisms were probably subjected. Figs. 36 and 37 demonstrate the late results of degradation of bacteria in hepatic phagocytes (Fig. 36) and liver parenchymal cells (Fig. 37). The marked difference in the way in which these cells treated bacteria was made clear in these photographs. In contradistinction to the parenchymal cells, the macrophages appeared to act on the bacteria in a much less severe way to produce 'myelin figures' which are electron micrographically typical of phospholipids.

4. Conclusions

The histological investigations described in this chapter have shown that the cells responsible for the clearance of foreign particles from the blood appear similar in foetal and natal rats. There are differences however of anatomy and also of the mechanisms available for bacterial degradation. The latter demonstrates most probably the functional immaturity in this respect at least of the foetal hepatic phagocytes. It supports the findings described in Chapter IV in which it was shown that these cells were apparently unable to kill the test organisms until the rat reached the age of weaning.

These studies have demonstrated that introduced particulate matter is in fact removed from the circulating blood by phagocytosis and that this function in quantitative terms is due almost exclusively to the hepatic macrophages of the liver in foetal rats. These results support those desxribed

in Chapter IV where it was shown that the foetal liver soon collected most of the radioactivity cleared from the circulating blood after the intravenous injection if isotopically labelâed bacteria.

That individual macrophages could phagocytose particles which varied considerably in size was demonstrated in this study. These results also indicate the presence of a variety of opsonins in concentrations sufficient to permit phagocytosis by the hepatic macrophages of foetal livers. The histological picture of slower clearance in terms of slower association of the test particles with the foetal macrophages of the youngest foetuses examined could reflect more a deficiency of opsonins than a lack of development of the cells themselves. The finding of phagocytic cells in the periphery of the adrenal cortex of foetal rats appears to lend support to the previously discussed primary function of the reticulo-endothelial system. The reason that phagocytosed carbon is found in this situation in foetal rats and not in adult animals might be due to the change from 'foetal cortex' to 'true cortex' which occurs towards the end of foetal life and in early natal life. This change-over entails degeneration of the foetal cortex and infiltration from its surface towards the medulla by the true cortex. Phagocytes are probably in this situation to deal with the effete material and take up for the same reason blood-borne foreign particles.

The electron micrographic evidence showed quite conclusively that there were functional foetal phagocytes which however appeared to lack the ability to break down bacteria. On the other hand the apparently gentle degradation of the bacteria in adult macrophages tends to support the contention that these cells not only phagocytose and catabolise complex compounds but that they do this in such a way that the catabolic products can be reutilised if required.

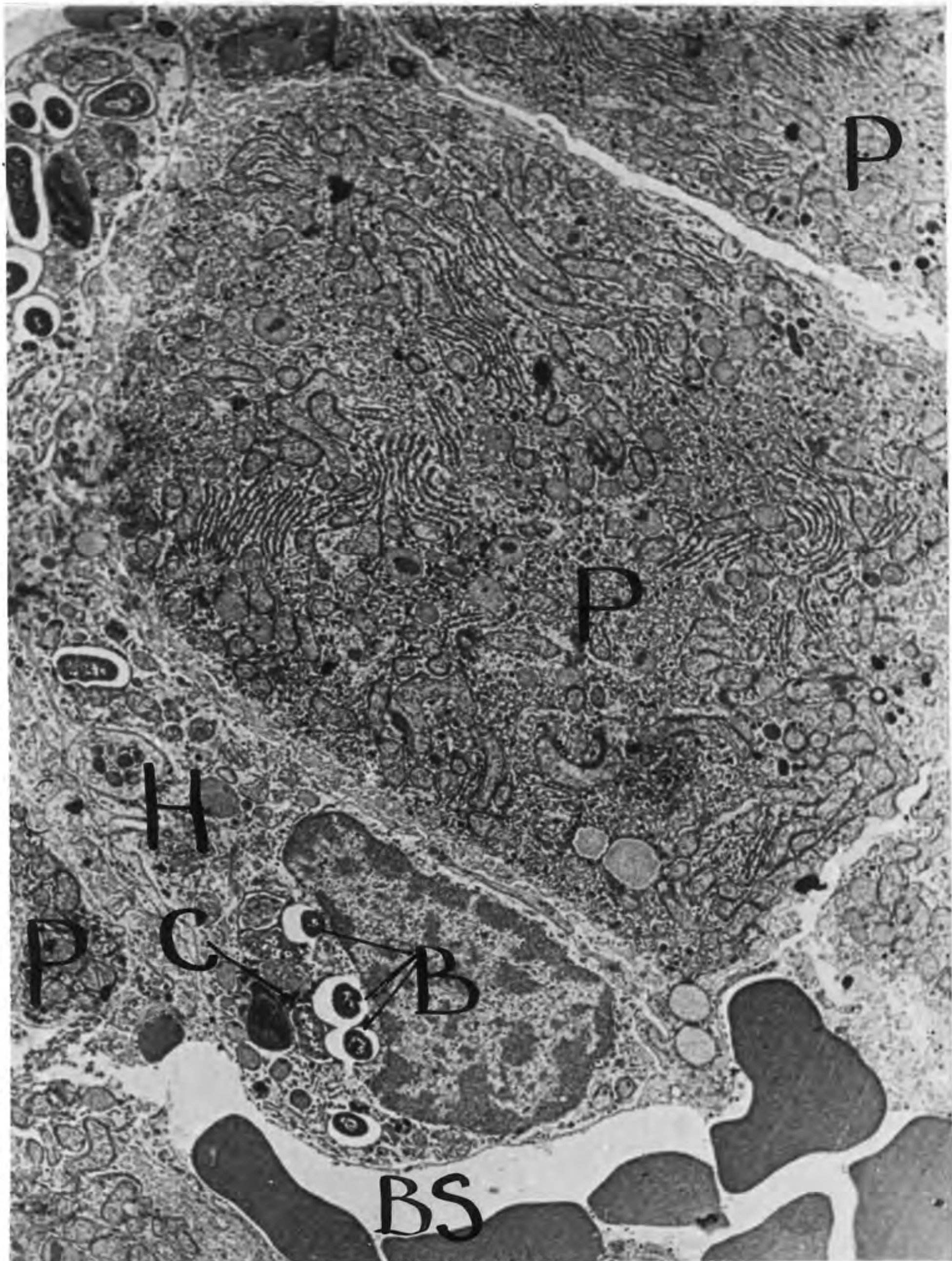


Fig. 26. Electron micrograph of a three months old rat liver 25 mins. after an I.V. injection of suspension B (See Chap.II). An hepatic phagocyte (H) can be seen extending between parenchymal cells (P) from one blood sinusoid (BS) to another in the extreme upper left corner. Bacteria (B) and carbon (C) can be seen inside the hepatic phagocyte. x 5000.

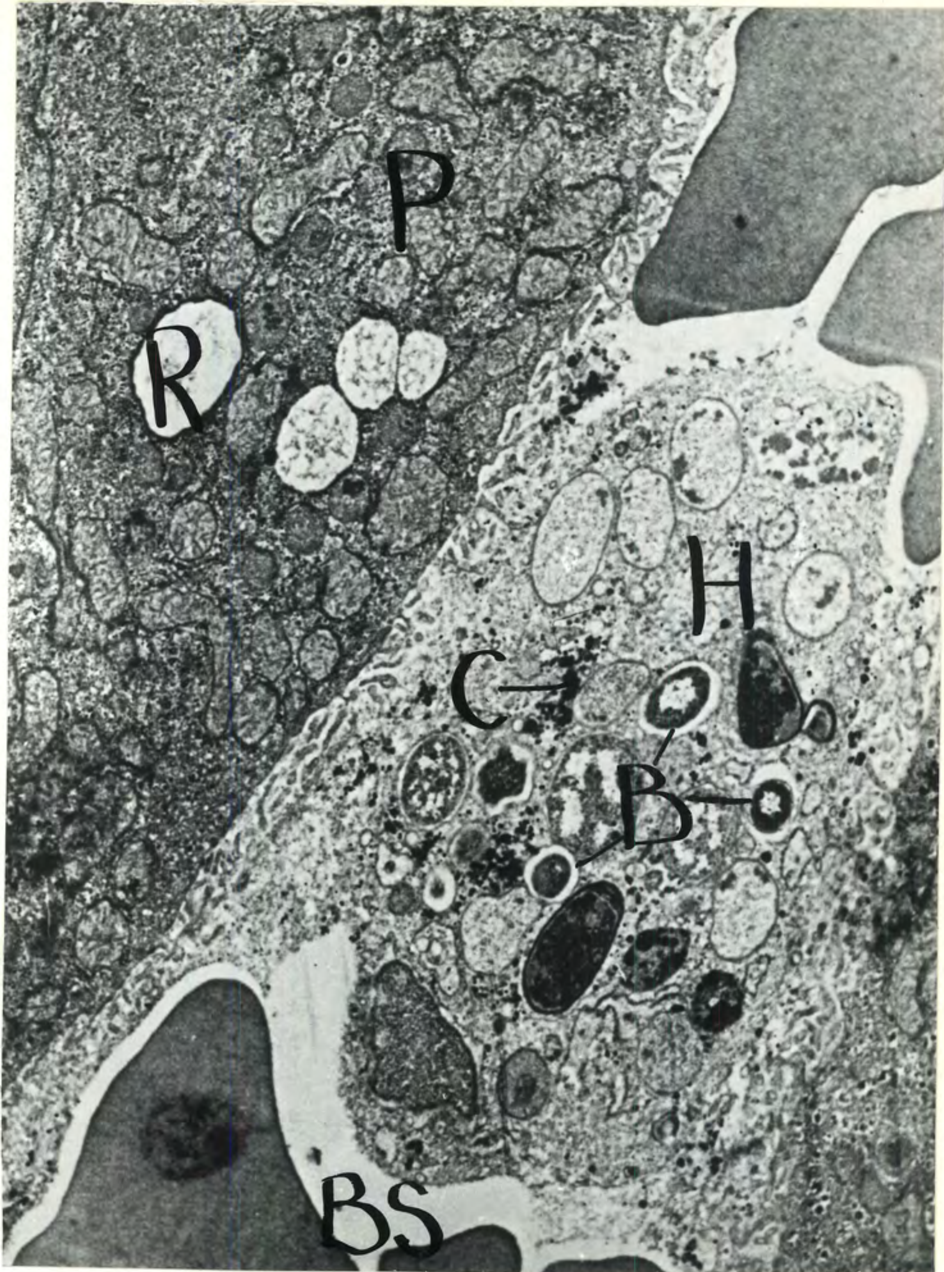


Fig. 27. Electron micrograph of a 3 month old rat liver 25 minutes after I.V. injection of suspension B (See Chap.II). An hepatic phagocyte (H) can be seen bridging a blood sinusoid (BS) and can be seen to be continuous with the sessile endothelial lining of the sinusoid. Bacteria (B) and Carbon (C) can be seen in the hepatic phagocyte and the remnants of bacteria (R) can be seen in a parenchymal cell (P).

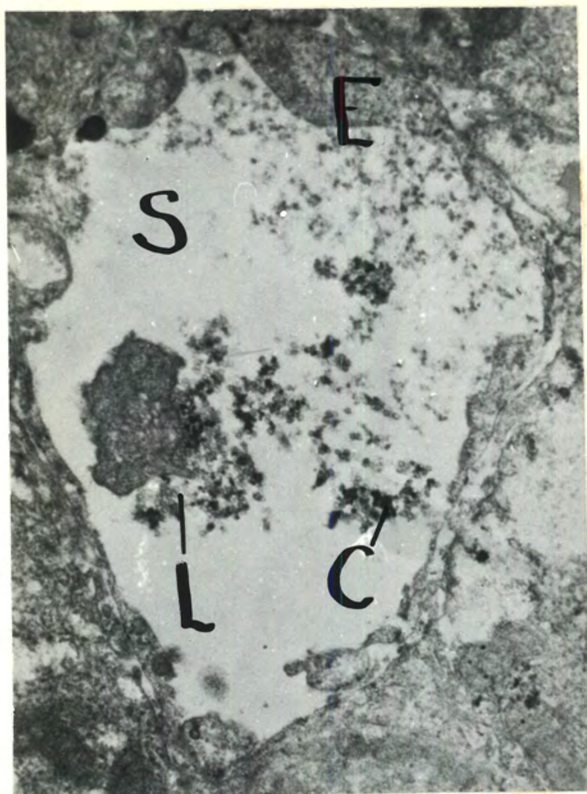


Fig. 28 Electron micrograph of an hepatic sinusoid (S) in a 20-day foetal rat demonstrating the sessile endothelial lining (E) and polystyrene latex (L) and carbon (C) in the lumen. One min. after I.V. injection. x 10,000

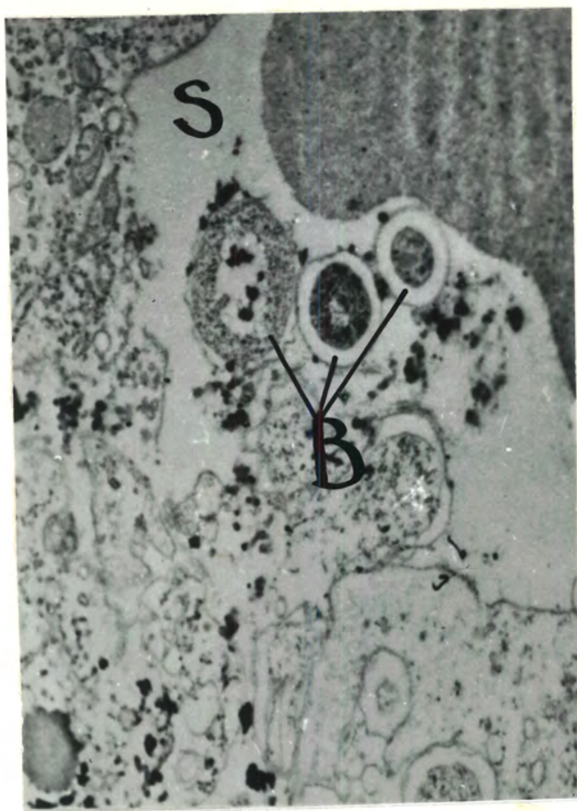


Fig. 29 Electronmicrograph of the liver of a 20-day foetal rat showing injected bacteria (B) lying in the lumen of a sinusoid (S) x 15,000

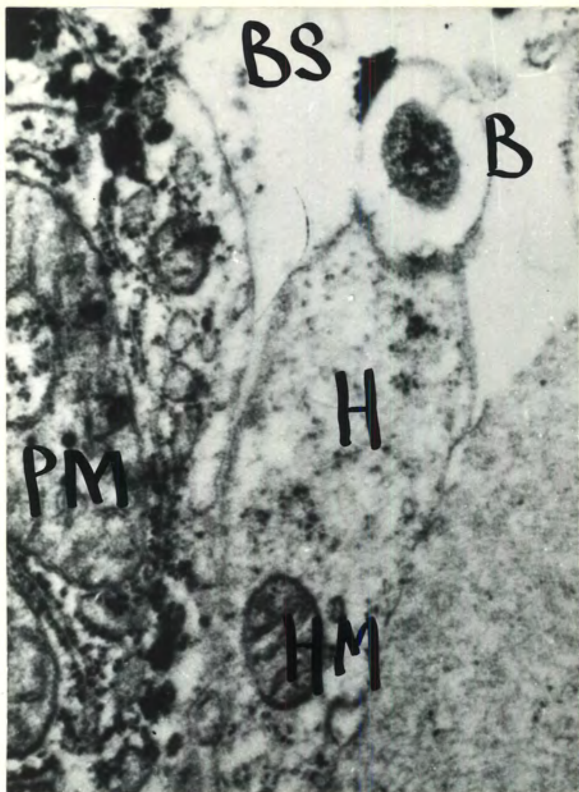


Fig. 30 Electronmicrograph of a 20-day foetal liver showing an early stage of phagocytosis of a bacteria (B) by an hepatic phagocyte (H) B.S: sinusoid,
H.M: phagocyte mitochondria
P.M: parenchymal cell mitochondria
x 25,000

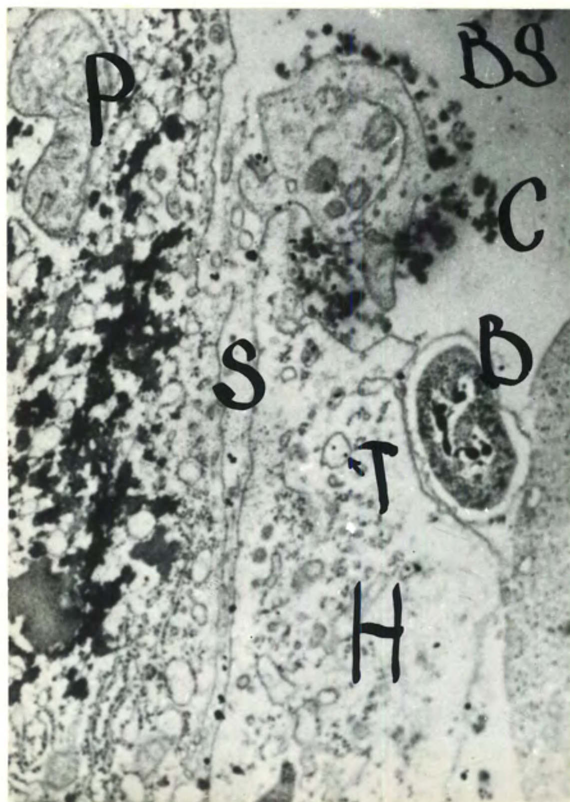


Fig. 31 Electronmicrograph of a 20-day foetal liver showing an early stage of phagocytosis of a bactera (B) by an hepatic phagocyte (H)
B.S: sinusoid C; carbon
P; parenchymal cell
T; thorotrast
S; space of Disse
x 20,000

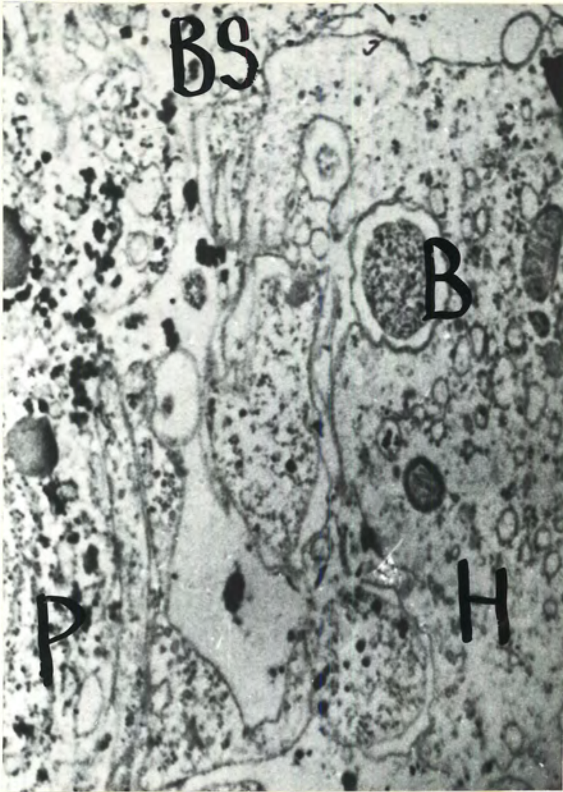


Fig. 32 Electron micrograph of a 20-day foetal liver showing a stage in the phagocytosis of a bacteria (B) by an hepatic phagocyte (H)
P; parenchymal cell
B.S; sinusoid
x 15,000

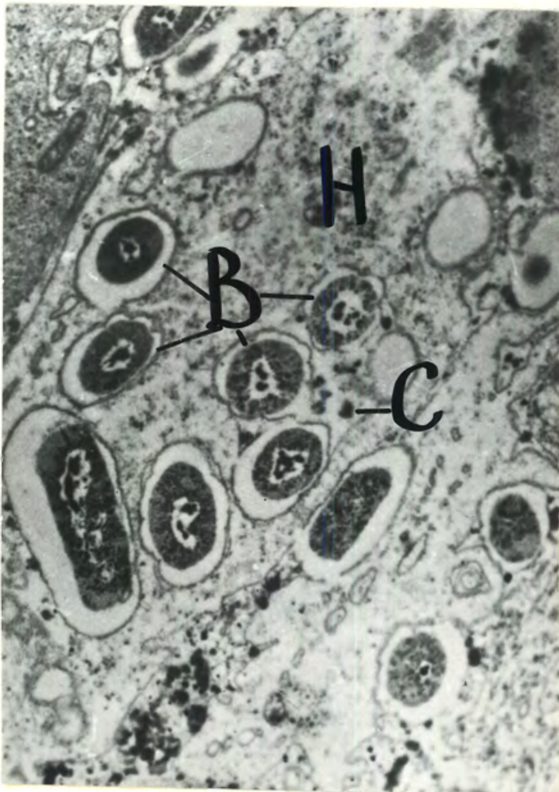


Fig. 33 Electron micrograph of a 20-day foetal liver showing bacteria (B) and carbon (c) inside vacuoles in an hepatic phagocyte (H)
x 20,000

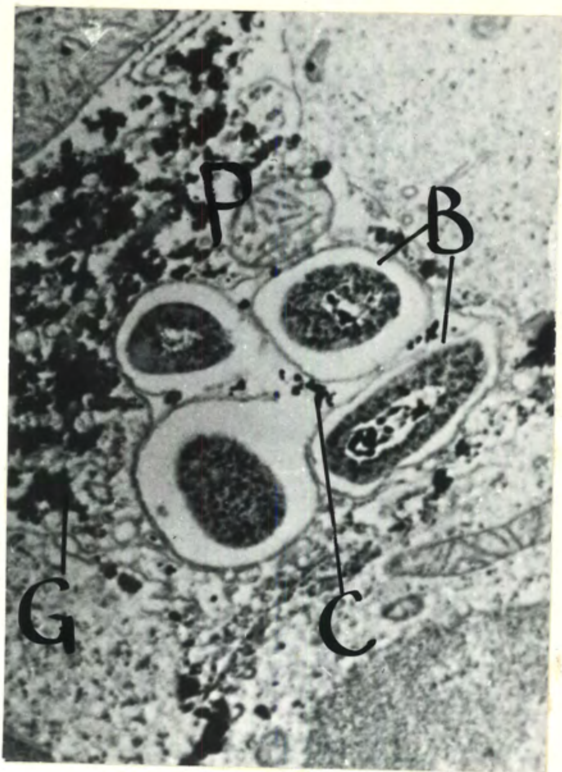


Fig. 34 Electronmicrograph of a 20-day foetal liver showing bacteria (B) and carbon (C) inside vacuoles in an hepatic parenchymal cell (P)
G : glycogen x 20000

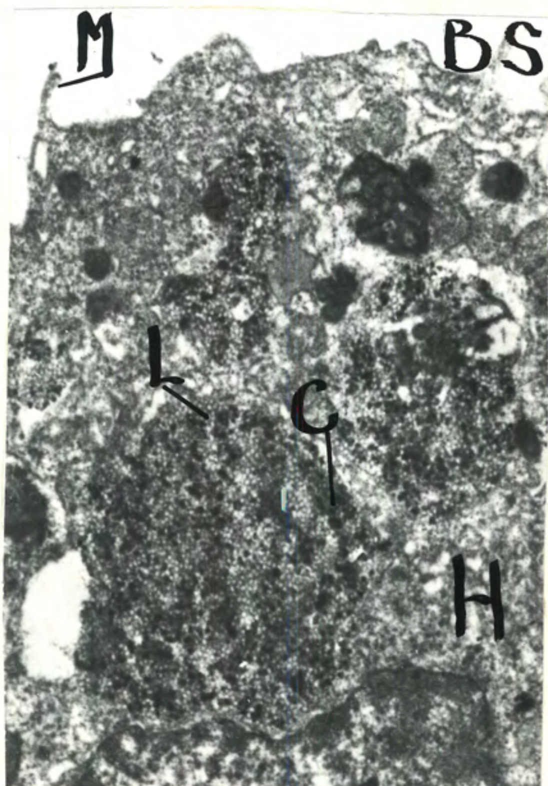


Fig. 35 Electronmicrograph of a 20-day foetal liver showing polystyrene latex (L) and carbon (C) in vacuoles in an hepatic phagocyte (H)
B.S: sinusoid
M: micro-villi
x 10000

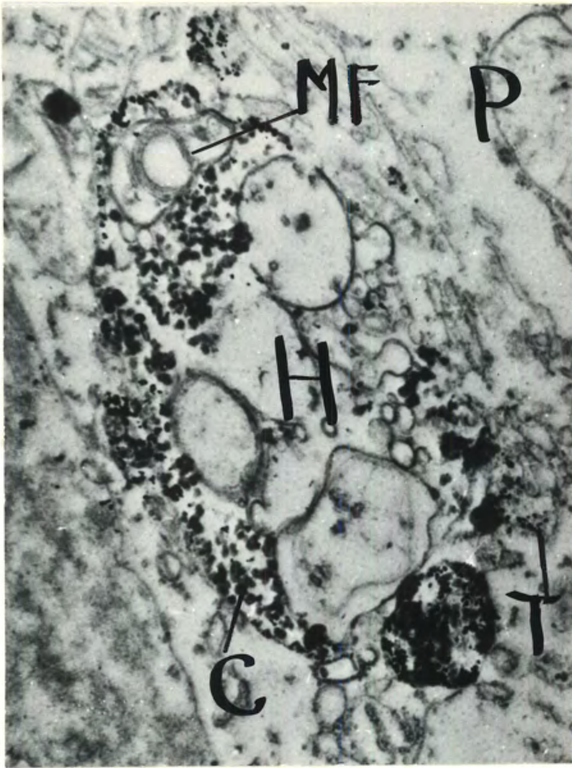


Fig. 36 Electronmicrograph of an adult rat liver showing carbon, thorotrast and what was considered to be the remnants of bacteria in the form of 'myelin' figures' (MF) in a vacuole in an hepatic phagocyte (H) P: parenchymal cell x 15,000

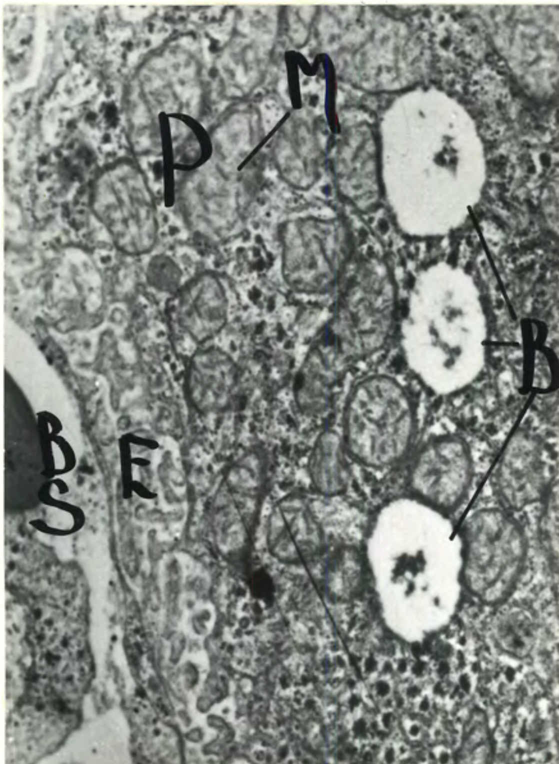


Fig. 37 Electronmicrograph of an adult rat liver showing the remnants of three bacteria (B) in vacuoles in an hepatic parenchymal cell (P)
M: mitochondria
BS: sinusoid
E: endothelium
x 10,000

CHAPTER VI

SERUM STUDIES IN FOETAL AND NATAL RATS

1. Introduction

It has already been emphasised that phagocytic cells and serum factors appear to be interdependent in host defence against infecting micro-organisms. Numerous workers have demonstrated in a variety of ways that specific antibody enhances phagocytosis and that so-called 'natural' antibodies in normal sera have this property to a lesser extent. Substances which facilitate or even permit phagocytosis were called 'opsonins' by Wright and Douglas in 1903 and it is the study of 'natural' opsonins to *Salmonella typhimurium* C5 in serum from rats of various ages with which this chapter is mainly concerned.

Rowley and Jenkin (1962) and Turner and Rowley (1962) have investigated pig serum opsonins to this strain of *Salmonella* and the latter workers have supported the initial work by demonstrating that these opsonins are not the classical gamma-globulin-type antibodies produced by specific immunisation but are beta-macroglobulins with a sedimentation coefficient of $S_{20}W19$. For the remainder of this chapter classical gamma-globulins will be referred to as 7S antibodies and the macroglobulins as 19S antibodies. A view has been expressed by these workers and by Kunkel (1960) that natural opsonins to particulate antigens such as red blood cells and bacteria belong to the macroglobulin class of antibody while

antibodies produced in response to immunisation with these antigens are commonly of the 7S type with an early 19S response.

Methods of assaying for the biological activity i.e. for the phagocytosis promoting effect of serum, serum fractions and embryonic fluids have been by the in vivo mouse clearance method described by Biozzi et al. (1953). This is a sensitive and highly reproducible method for detecting small quantities of opsonin but where an even more sensitige and equally reproducible method was required, clearances were done in chick embryos by the method described by Karthigasu and Jenkin (1963). Isotopically labelled Salmonella typhimurium C5 was the test organism used exclusively in these clearance studies. This particular organism is only very slowly cleared from the blood by the cells of the reticulo-endothelial system of Swiss white mice and chick embryos. The usually gave a K value of approximately 0.020 and 0.001, respectively. In each assay unopsonised organisms were used as a control.

Considerable difficulty has been experienced in usming the various protein fractions obtained in the variety of analytical methods employed in this study in order that from one method to the next there was some similarity in the fractions described by the same name. This difficulty was encountered in the literature as is illustrated by the following definitions of gamma-globulin. Tiselius (1937) defined gamma-globulins as that group of proteins showing cathodic or no migration on electrophoresis at pH 8.6,:

Peterson and Sober described gamma-globulin as the protein fraction which does not bind to D.E.A.E. cellulose in the presence of 0.01M buffer. Turner and Rowley, in 1963 in confirmation of the work of Fahey and Horbett (1959), claimed that immuno-electrophoretic evidence showed that gamma-globulin sould be eluted by 0.1M buffers and that this fraction migrated both towards the cathode and the anode. In addition to this there was evidence from the present study to show the heterogeneity of the various fractions obtained. It has been considered prudent therefore to use a simple somewhat general system of nomenclature similar to that suggested by Smithies (1959). Fig. 38 shows a diagrammatic representation of this system in relation to the common patterns seen in this study of starch-gel and paper-strip electrophoretic analyses of rat serum.

2. Vertical starch-gel electrophoresis

Serum from 16-, 18-, 20- and 22- day rat foetuses and from 1-, 7-, 14-, 28-, 56-, 84- day and adult natal rats were electrophoresed in starch-gel, using a standard technique with constant conditions of voltage, pH and ambient temperature. A control of 84- day rat serum was run on each gel to allow comparison from gel to gel. The results of this procedure are illustrated in Figs. 39 and 40 which are photographs of the stained gel surface showing the patterns obtained at each age tested. While it was evident that there was an overall similarity in electrophoretic pattern from foetal to adult serum there were specific differences. It was seen for

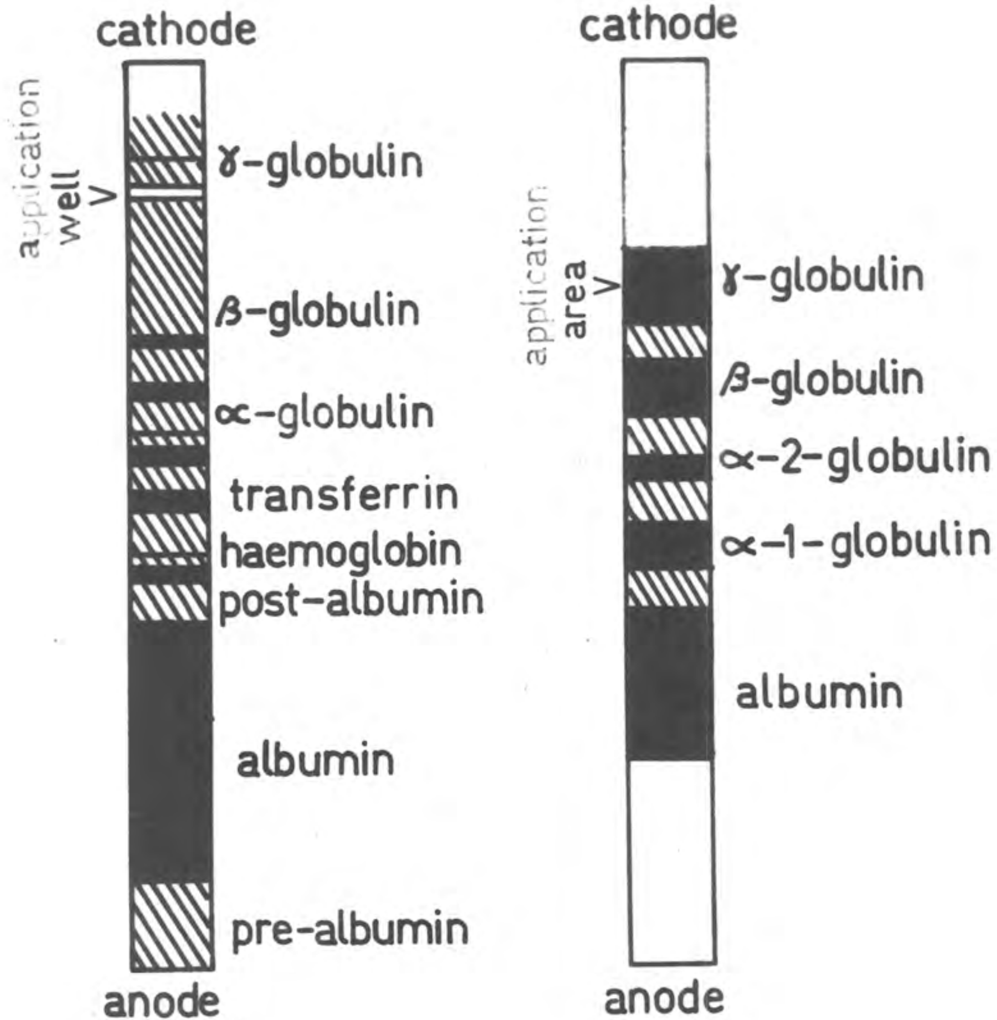


Fig.38 Diagrams of a stained starch gel (left) and a paper strip (right) following the electrophoresis of adult rat serum

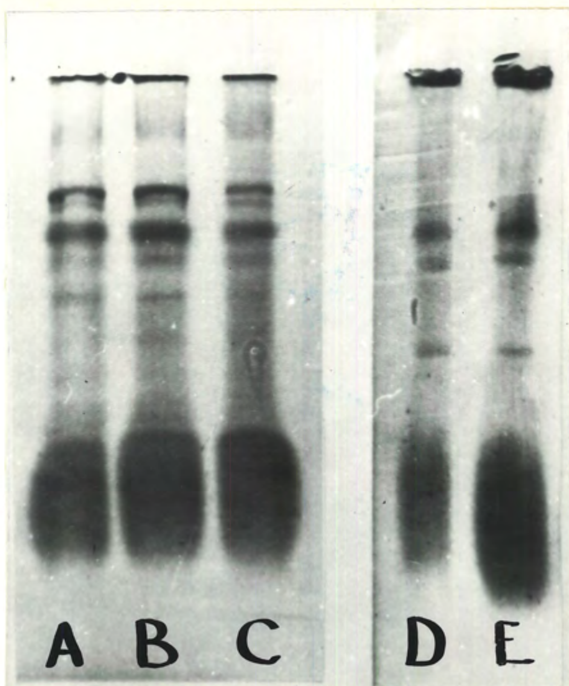


Fig. 39 Vertical starch gel electrophoresis
A; 18-day-foetal serum
B; 20-day foetal serum
C; 22-day foetal serum
D; 18-day embryonic fluid
E; 20-day embryonic fluid

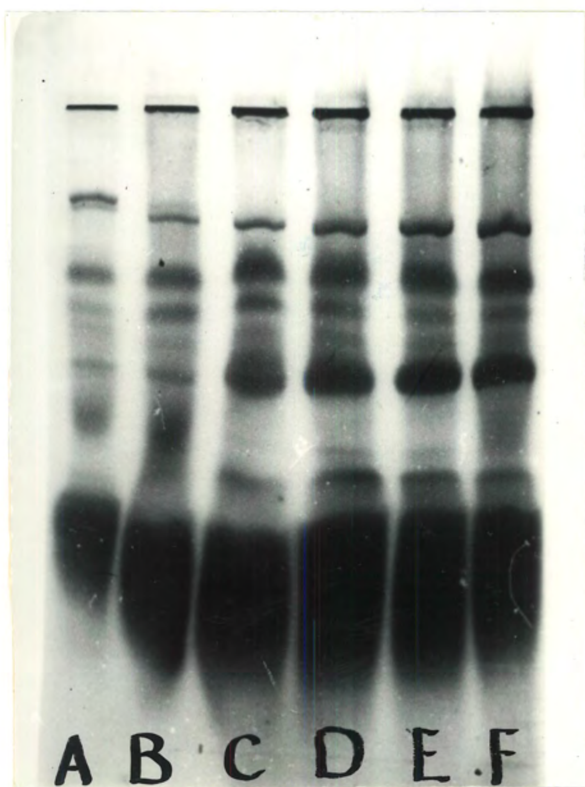


Fig. 40 Vertical starch gel electrophoresis
A; 1-day natal serum
B; 7-day natal serum
C; 14-day natal serum
D; 28-day natal serum
E; 56-day natal serum
F; 84-day natal serum

instance that the cathode migrating gamma-globulin was deficient in the foetal samples and increased in concentration until adulthood in the natal samples. A well defined band was present in this region in the natal samples a short distance from the application well. A change occurred in the mid beta-globulins from the foetal to the 28- day natal rat serum. The double band evident in this region in the neonatal serum appeared to reverse its intensity until there was only a single band from the age of 28- days. The transferrin band was seen to intensify over this neonatal period and the more intense heamoglobin band present in these same serum samples reflected the difficulty in collecting completely non-heamolysed samples from these small animals. An increase in intensity of the post-albumin band up to 28- days was noted, as well as an increasing quantity of albumin and pre-albumin.

It was noted that the walls of the application wells deteriorated during electrophoresis, the effect being more pronounced as the age of the animals increased. This was attributed to the hydrolytic activity of serum amylase and the results of assays for this enzyme are described below.

In a similar way, but with 0.2 ml. samples in enlarged application wells, embryonic fluid from 16-, 18- and 20- day foetuses was electro-phoresed. The electrophoretic patterns showed a faint but similar distribution to the major bands of the serum proteins which migrated towards the anode (Fig. 39). Destruction of the starch of the wells was extensive, particularly on the cathode side of the well.

3. Paper-strip electrophoresis

Serum from 16-, 18-, 20- and 22- day foetal rats and from 3-, 7-, 14-, 28-, 56- and 84- day natal rats were electrophoresed under standardised conditions on paper strips with 0.075 ionic strength pH 8.6 veronal buffer. The stained strips were then analysed by absorption in an "Analytrol" and the tracings so obtained were sectioned according to the major peaks and the percentage composition of the protein fractions calculated.

These results tabulated together with the fraction concentrations in mg./ml. are shown in Table 8. It was seen that the gamma-globulin constituted in general terms a similar proportion of both foetal and natal rat serum until the animals were 28- days old and from then on there was a sharp increase. The beta-globulins comprised a fairly constant proportion throughout all the age groups while the alpha-2-globulins showed (with one exception) a high level during foetal life and then a constant but lower level was maintained until 84- days of age when the proportion decreased. The alpha-1-globulins, on the other hand constituted a constant small proportion during foetal life, increased for seven days after birth and then decreased for the next few weeks increasing again considerably in the older animals. However these fluctuations in alpha-1-globulin proportions might simply reflect incomplete separation of these proteins from albumin. The albumin fraction appears to retain a fairly constant level in the ages examined.

TABLE 8

Protein analysis of foetal and natal rat serum with percentage distribution and concentration in mg/ml.

	AGE (days)	PROTEIN FRACTIONS										
		gamma-glob.		beta-glob.		alpha-2-glob.		alpha-1-glob.		albumin		TOTAL
		mg/ml.	%	mg/ml.	%	mg./ml.	%	mg./ml.	%	mg/ml.	%	mg./ml.
FOETAL	16	1.1	9.5	2.5	22.0	3.3	28.4	0.5	4.6	4.8	41.1	11.5
	18	2.3	9.0	6.1	24.2	3.2	13.0	1.1	3.9	11.7	46.8	25.0
	20	2.5	7.5	7.6	22.9	8.0	24.3	1.4	4.2	13.9	42.0	33.0
	22	2.3	6.7	7.4	21.4	9.8	28.3	2.1	6.1	12.9	37.5	34.5
NATAL	3	2.0	5.0	7.6	19.0	5.8	14.6	5.4	13.5	19.2	48.1	40.0
	7	3.5	8.1	7.8	18.0	6.5	15.0	5.8	13.5	14.5	33.5	43.2
	14	4.6	9.4	11.3	23.1	6.4	13.1	4.4	9.0	21.8	44.4	49.0
	28	4.2	7.6	12.5	22.4	7.1	12.8	5.1	9.2	26.7	48.0	55.6
	56	9.1	14.7	15.1	24.6	7.7	12.5	9.1	14.9	20.8	34.0	61.2
	84	10.1	15.2	12.1	18.3	5.8	8.7	12.5	18.9	25.6	38.7	66.1

Paper-strip electrophoretic analyses were also done on 14-, 16-, 18- and 21- day embryonic fluids by applying a 0.1 ml. sample to each paper strip. Fluid from 21- day foetuses was the most mature tested because the embryonic fluid diminishes in volume during the last day of foetal life until there remains only a small quantity of very viscous fluid at term. The results of these analyses are shown in Table 9. They demonstrate a striking similarity in the percentage distribution of the main protein components of embryonic fluid to that of rat serum. It can be seen that the percentage distribution of each fraction remained constant in the period between 14- days and 18- days except that the beta-globulins decreased and continued to decrease up to the 21st day. Between the 18th day and 21st day there was a disproportionate increase in the gamma-globulin level and a slight increase in the proportion of albumin.

4. Quantitative protein estimation by spectrophotemetry

Dilutions of 1:200 of serum samples and 1:20 dilutions of embryonic fluid in 0.15M saline were prepared and the optical density determined by U.V. absorption at a wave length of 280 millimicrons. By reference to the previously prepared standard curve the protein concentrations in mg./ml. were determined and are recorded in Tables 8 and 9. These values in conjunction with the results of paper-strip electrophoresis were used to estimate the protein content of each main protein fraction. The results of these calculations are also given in the Tables, and are presented graphically in Fig. 41

TABLE 9

Protein analysis of embryonic fluids with percentage distribution and concentration in mg./ml.

	AGE (days)	PROTEIN FRACTIONS										
		gamma-glob		beta-glob		alpha-2-glob		alpha-1-glob		albumin		TOTAL
		mg./ml.	%	mg/ml.	%	mg/ml.	%	mg/ml.	%	mg/ml.	%	mg/ml.
FOETAL	14	0.6	9.9	1.7	27.7	0.7	10.5	0.4	6.9	2.8	44.1	6.3
	16	0.6	10.3	1.5	25.8	0.7	12.6	0.3	5.1	2.7	46.6	5.8
	18	0.6	9.3	1.2	18.4	0.7	11.0	0.4	5.6	3.1	48.7	6.3
	21	1.5	14.3	1.8	17.2	1.1	11.0	0.5	5.1	5.5	53.0	10.3

which illustrates the changing pattern of serum protein concentrations as the rats matured. Table 8 and Fig. 41 illustrate that the total serum proteins show a six-fold increase in concentration over the age range studied and that the beta-globulins and albumins increase by a similar amount but that the gamma-globulin and alpha-1-globulin fractions show a 10-fold increase respectively while the alpha-2-globulins doubled in concentration. It can also be seen from Tables 8 and 9 that the total protein concentrations of the foetal serum ranged from between three to five times the concentration of protein in the embryonic fluids at the corresponding ages. These results appear to be in general agreement with the changing patterns seen on the starch-gel electrophoresis.

5. Quantitative estimation of serum amylase

The way in which the starch-gel was damaged around the application wells when rat serum and embryonic fluids were applied prompted an investigation into the amylase activity of these samples. The results obtained by iodine/starch titration are shown in Table 10 and illustrate the relatively enormous concentration of starch reducing activity in these samples.

6. Biological Assay of opsonins to *Salmonella typhimurium* C5

The degree to which the serum from 20- and 22- day foetal rats and 1-, 5-, 14-, 21-, 28-, 56- and 84- day natal rats enhanced the clearance of *Salmonella typhimurium* C5 after opsonisation with each serum sample was assayed by clearance

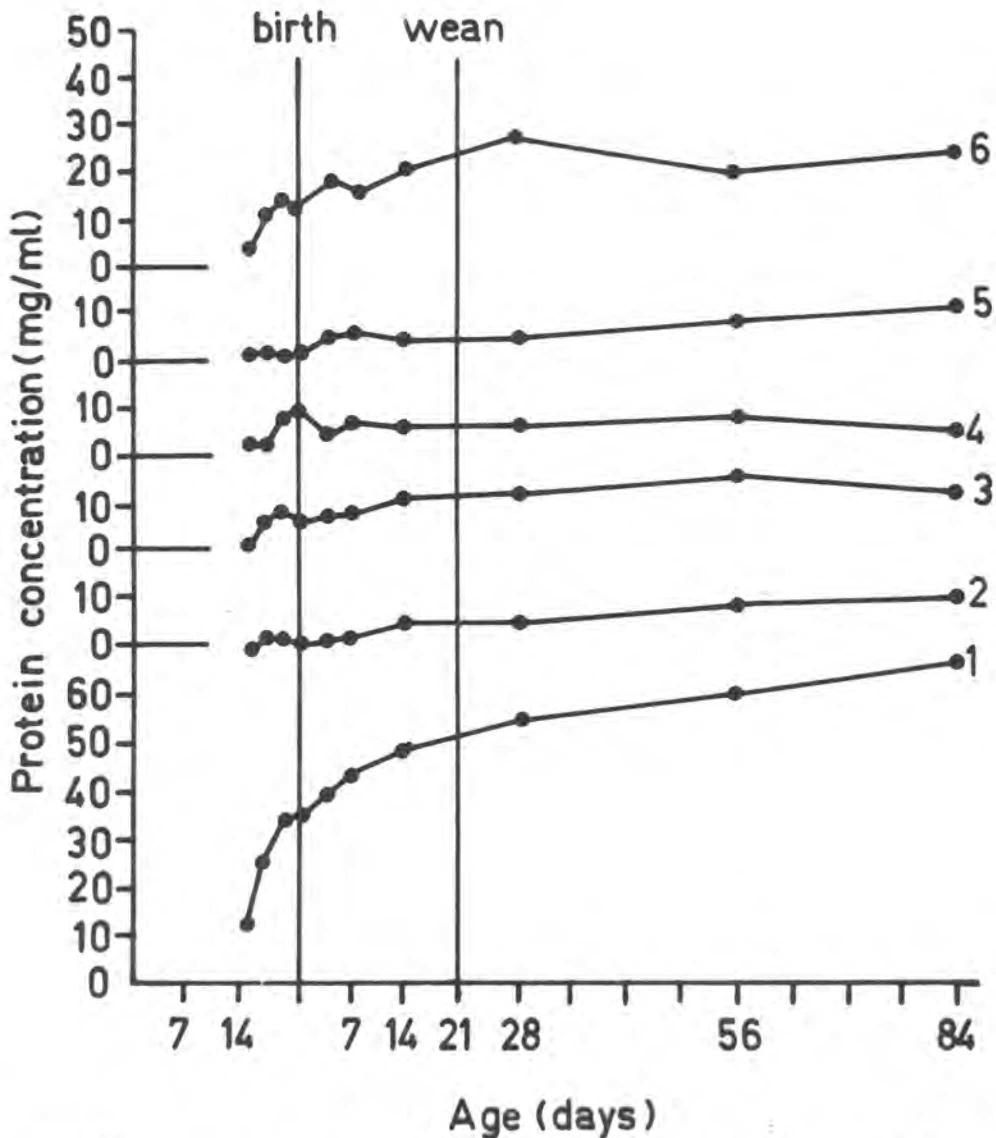


Fig.41 The concentration of the total serum proteins and the major protein fractions at various foetal and natal ages

TABLE 10

Amylase content of rat serum* and

embryonic fluid samples

Sample	Age (days)	Amylase Content units/100 ml.
Embryonic Fluid	18	2285
	20	4800
Foetal Serum	22	4430
Natal Serum	7	929
	28	1797
	56	2512
	84	2230

* Assays of human serum by the same method for example give a value with a range of from 60 to 200 units/100 ml.

studies in mice. Mice cleared this organism when unopsonised from their circulation at an average rate of K= 0.020. This amount therefore must be subtracted from the K values shown in Fig. 42 to obtain the actual increase in clearance rate due to the opsonins in each serum sample. This figure, which shows the K values obtained with each sample, illustrates the way in which the opsonising ability of rat serum changes with age, and demonstrates that following a constant level from late foetal to neonatal life a considerable increase in opsonins to the test organism occurs near the time of weaning.

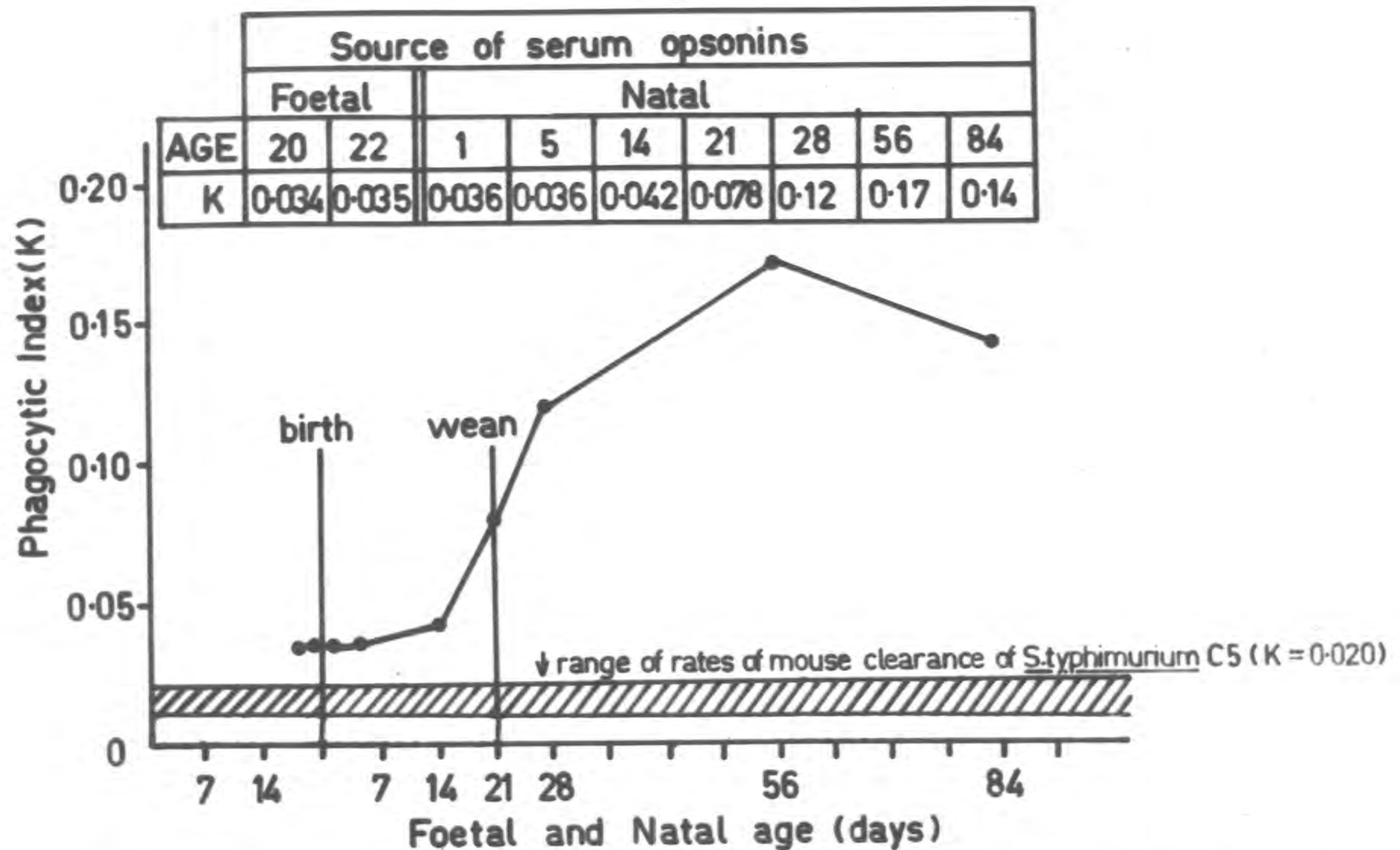


Fig42 Rates of clearance from the circulation of mice of <u>S. typhimurium</u> C5 opsonised with serum from rats of various ages

This increasing trend continued until the rats were 56- days old and then fell slightly during the next four weeks.

7. Titration of opsonic activity in adult rat serum

Serial dilutions were prepared from a sample of pooled adult rat serum. These dilutions were used to opsonise isotopically labelled Salmonella typhimurium C5 which was then used for clearance studies in mice. The results are presented in Fig. 43 which shows that the serum was diluted out to 1:8 without a reduction in the K value. Further doubling diltuions diminished the activity in a linear fashion until at a dilution of 1:128 there was no evidence of activity.

8. Bactericidal activity to Escherichia coli Lilly

As an extra test for the presence of antibody in both foetal and natal serum samples a bactericidal assay was performed. The organism used was Escherichia coli (Lilly) which had been shown to be sensitive to the bactericidal activity of serum from a variety of sources. To dilutions of 16-, 18-, 20- and 22- day foetal serum and 7-, 14-, 28-, 56- and 84- day natal serum were added 2×10^3 Escherichia coli (Lilly). These samples were incubated at 37^0C and at times 0, 10, 20, 40 and 60 minutes aliquots were plated and then incubated and counted. The results for each sample were expressed as percentages of the zero time count and are shown as a histogram in Fig. 44. It can be seen that there is an increase in bactericidal activity from the 16- day foetal serum sample to the 7- day neonatal sample and that from this latter age to 84- days the samples quickly reduced the number of viable bacteria to a level which could not be counted.

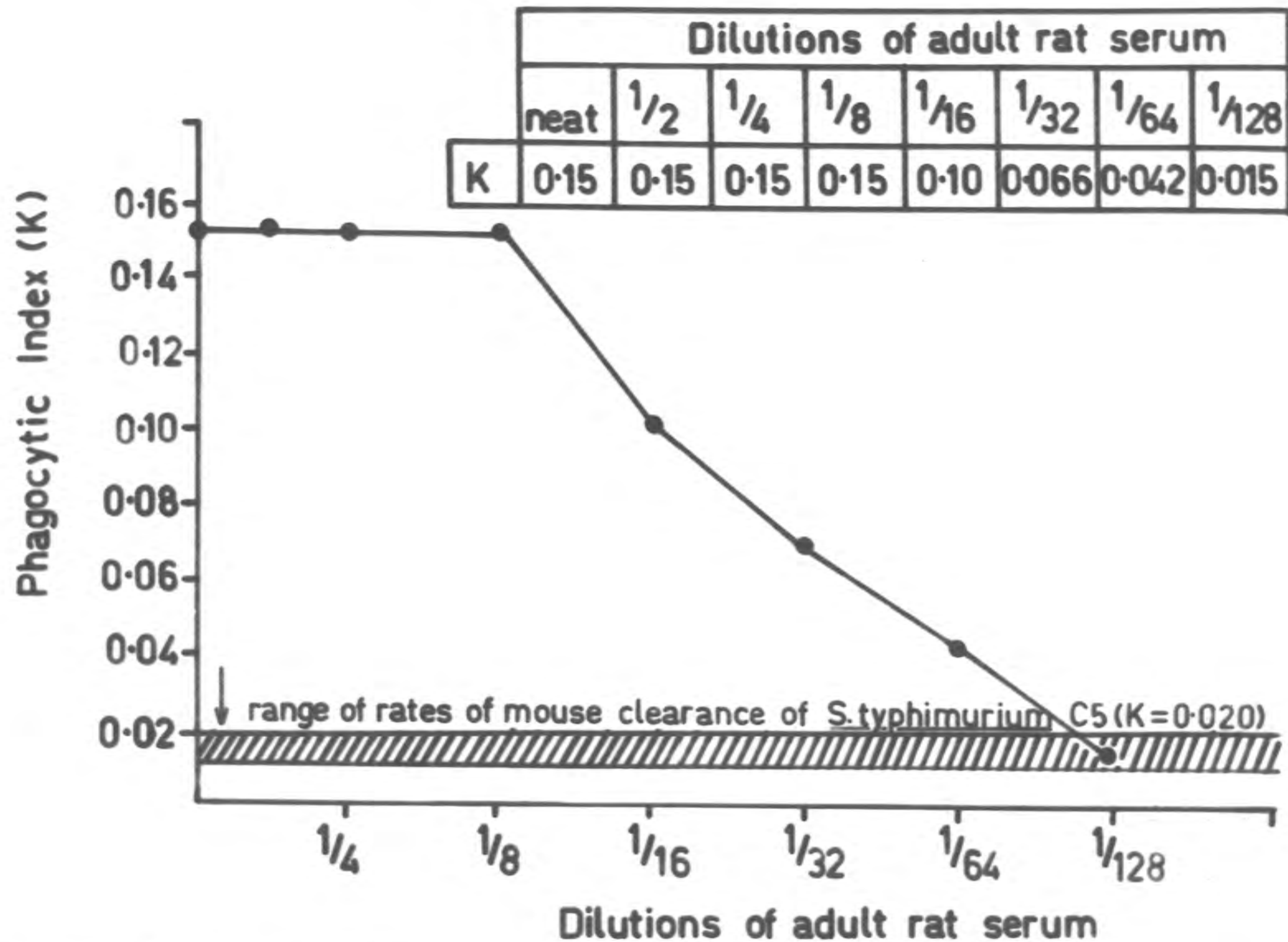


Fig.43 The opsonic activity towards S.typhimurium C5 of serial dilutions of adult rat serum

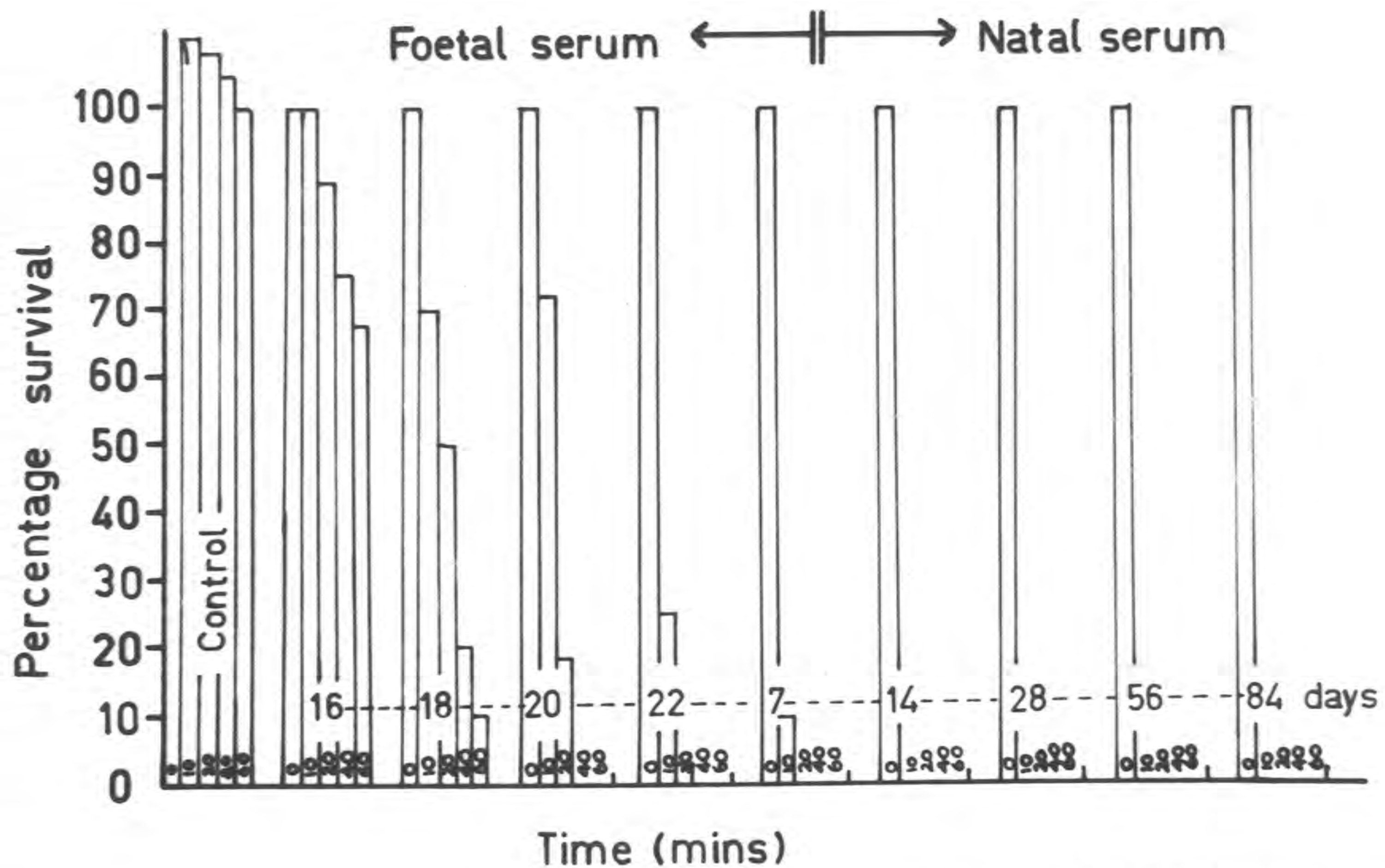


Fig. 44 Percentage survival of Escherichia coli (Lilly) at times 0, 10, 20, 40 and 60 minutes when incubated with 1:5 dilutions of foetal and natal serum samples

9. Characterisation of opsonins to *Salmonella typhimurium* C5

a. Preparative starch-gel electrophoresis

A stach-gel on which 1.2 ml. (80 mg.) of adult rat serum had been electrophoresed was divided horizontally into eight strips to include in each strip a major protein band. The protein was extracted from each strip of gel and the fractions subsequently prepared for biological assay by opsonising *Salmonella typhimurium* C5 for mouse clearance studies. Protein recovery by these extraction methods was 25 per cent which was somewhat lower than that reported by other workers (Turner and Rowley, 1963). Opsonic activity was found in the Fraction 1 which contained the fast moving gamma-globulins and Fraction 6 which contained alpha-globulins or post-albumins. The K values for these fractions, which were concentrated by freeze-drying to a volume of 1 ml. were 0.034 and 0.080 respectively. This represents 2 per cent and 5 per cent of the applied opsonic activity. While these recoveries of activity seem low in comparison with the values reported by Turner and Rowley (1963) it should be emphasised that these workers included the starch of the application wells in their slow moving beta-globulin fractions and therefore in all probability the macroglobin antibodies. These have been found from subsequent investigations to account for most of the opsonic activity of rat serum. All of the fractions were analysed by starch-gel electrophoresis,

the patterns of which are reproduced diagrammatically in Fig. 45. This demonstrated the recovery and the relative heterogeneity of the fractions, especially of fraction 7 which contained the greatest opsonic activity. Fraction 1 was not demonstrable by this method.

b. Reduction by sulphydryl reagents

It has been previously reported by Benedict et al. (1963) that sulphydryl uncoupling reagents such as 2-mercaptoethanol can reduce macroglobulin antibodies with a resultant loss in their biological activity. Gamma-globulins are also reduced by these reagents but this does not necessarily result in loss of activity. It was because of the suggestion by Rowley and Jenkin (1962) that natural opsonins in pig serum were probably macroglobulins that a sample of pooled adult rat serum was reduced with 2-mercaptoethanol as one step in attempting to characterize its active principles. A sample of pig serum was reduced at the same time to check the efficacy of the reduction, since Turner (1963) had previously determined that sulphydryl reduction of normal pig serum would almost eliminate its biological activity towards Salmonella typhimurium C5.

After reduction the samples were dialysed against a large volume of 0.15 M NaCl for 24 hours at 4^{0}C to remove the 2-mercaptoethanol from the samples. While repolymerisation can occur to some extent when the mercaptoethanol

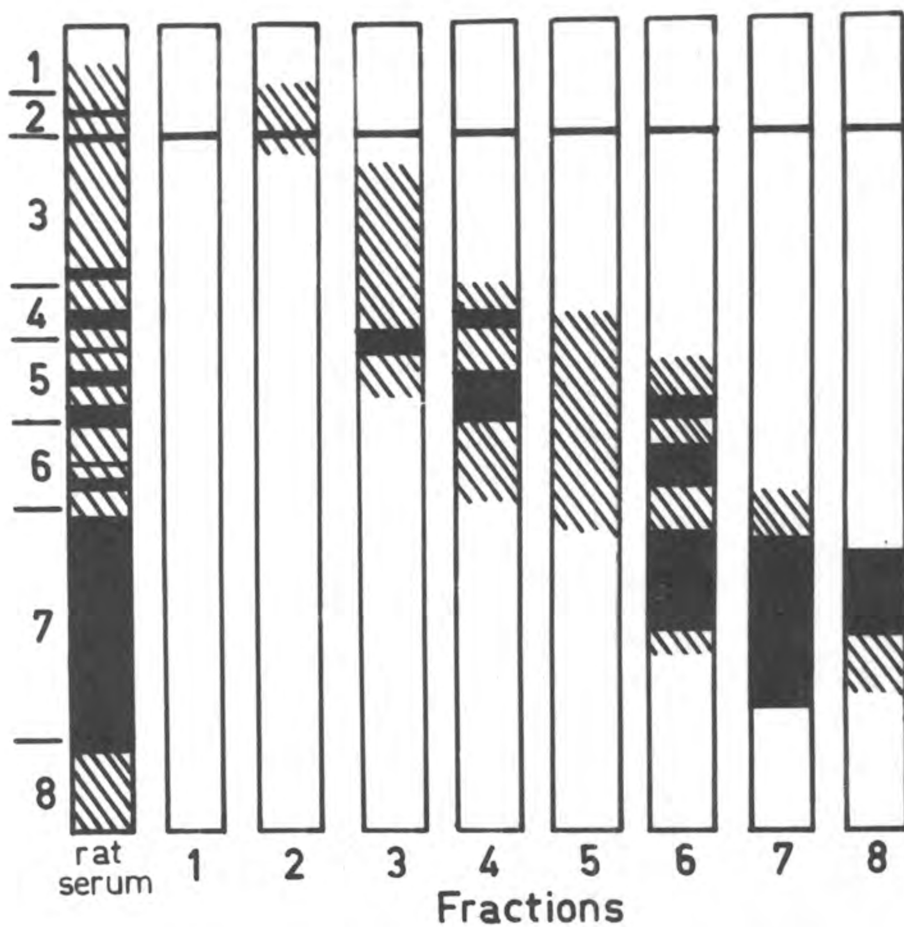


Fig.45 Diagramatic reproduction (actual size) of the fractions from the preparative starch gel electrophoresis

Fractions 1 and 6 active

Fraction 1 was not demonstrable

■ sharply defined densely stained zones

▧ diffusely stained zones

is removed, this does not result in return of biological activity (Benedict et al., 1963).

These reduced serum samples at 1:2 dilution were then tested for opsonic activity. The loss in the activity of the pig serum was as expected and the K value for the rat serum was K= 0.090. This indicated after reference to the previous titration of opsonic activity (see supra) that approximately 80 to 90 per cent of the activity had been destroyed by the sulphydryl reagent and was presumably attributable to macroglobulins.

c. Separation of adult rat serum by density gradient ultracentrifugation.

In an effort to study the biological activity towards *Salmonella typhimurium* C5 of both the macroglobulin and lower molecular weight antibodies in rat serum, 1 ml. (65 mg.) of adult rat serum was separated into these two parts by density gradient ultracentrifugation. Fig. 46 shows the separation pattern obtained by this method and illustrates the division of serum proteins into two groups. The macroglobulin fraction - Fraction 1 - consisted of 8 per cent of the applied protein. The remainder was pooled in two fractions on the basis of a small but constant trough which appeared on the crest of the larger peak which represented the bulk of protein. These three fractions were concentrated by pressure dialysis and assayed for opsonic activity and protein content. The results of

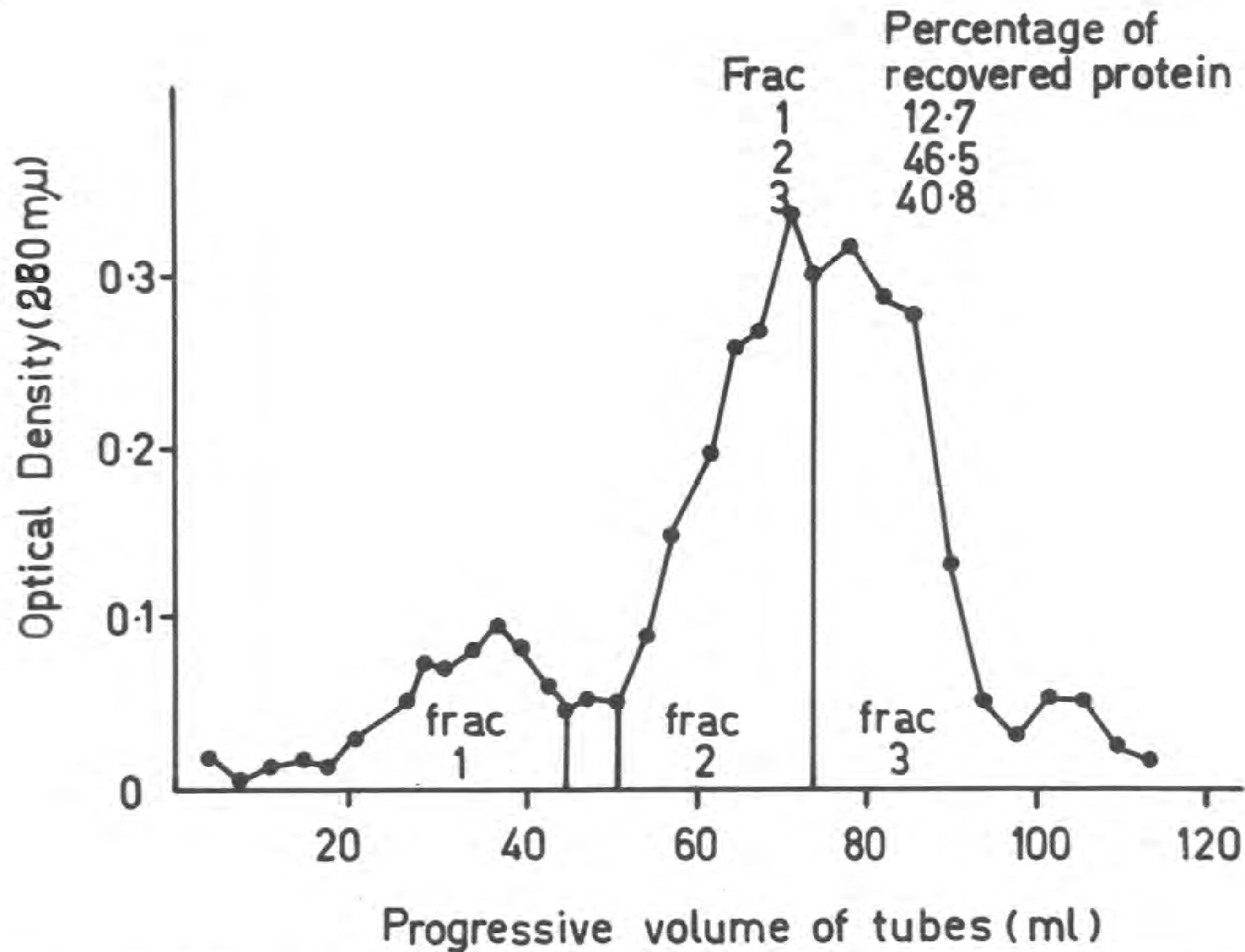


Fig.46 Separation of adult rat serum by density gradient ultracentrifugation

these assays are shown in Table 11 from which it can be seen that 69 per cent of the applied protein and 27 per cent of the applied activity was recovered.

TABLE II

Separation by density gradient ultracentrifugation of 1 ml. of adult rat serum containing 65 mg. of protein and 112 units of opsonic activity to *Salmonella typhimurium* C5

Fraction	Volume of concentrated fraction (ml.)	Protein content (mg.) recovered	Percentage of applied protein recovered	Opsonic units of activity recovered	Percentage of opsonic activity	
					Based on total activity applied	Based on total activity recovered
1	5.12	5.1	7.8	22	19.6	73.4
2	5.35	21.2	32.6	4	3.6	13.3
3	3.41	18.6	28.6	4	3.6	13.3
Total	11.88	44.9	69.0	30	26.8	100

The bulk of the activity occured in Fraction 1, the macroglobulin fraction, and 11 per cent of the recovered protein therefore accounts for 73 per cent of the total activity. Fractions 1 and 2 contain 27 per cent of the activity in 87 per cent of the recovered protein. The fractions containing the small molecular weight proteins suffered the main loss of protein in this and dubsequent

experiments where pressure dialysis was used to concentrate the fractions. In an attempt to minimise this loss, the fractions were concentrated to a minimum volume of approximately 4 ml.

The method of quantitation of the macroglobulins used in this study was based on the assumption that both the macroglobulins and the remainder of the serum proteins had identical extinction coefficients. This assumption has probably introduced minor errors, for Turner (unpublished data) has recently shown a difference in extinction coefficient between the macroglobulin and remainder of serum proteins in pig serum when isolated by density gradient ultracentrifugation.

These results therefore support the evidence obtained by mercaptoethanol reduction when it was estimated that 80 per cent to 90 per cent of the opsonic activity was lost by reducing the macroglobulins. Starch-gel electrophoresis of the fraction and immuno-electrophoresis of the fractions against specific rabbit antiserum to rat serum demonstrated the relative heterogeneity of these three fractions.

<u>Separation of foetal rat serum by density gradient ultracentrifugation</u>.

In a similar way to the above 3 ml. (105 ml.) of 22- day foetal rat serum was separated by density gradieht ultracentrifugation. Fig. 47 shows the fractionation pattern obtained by this method. The material was divided into

Frac	Percentage of recovered protein
1	9·9
2	90·1

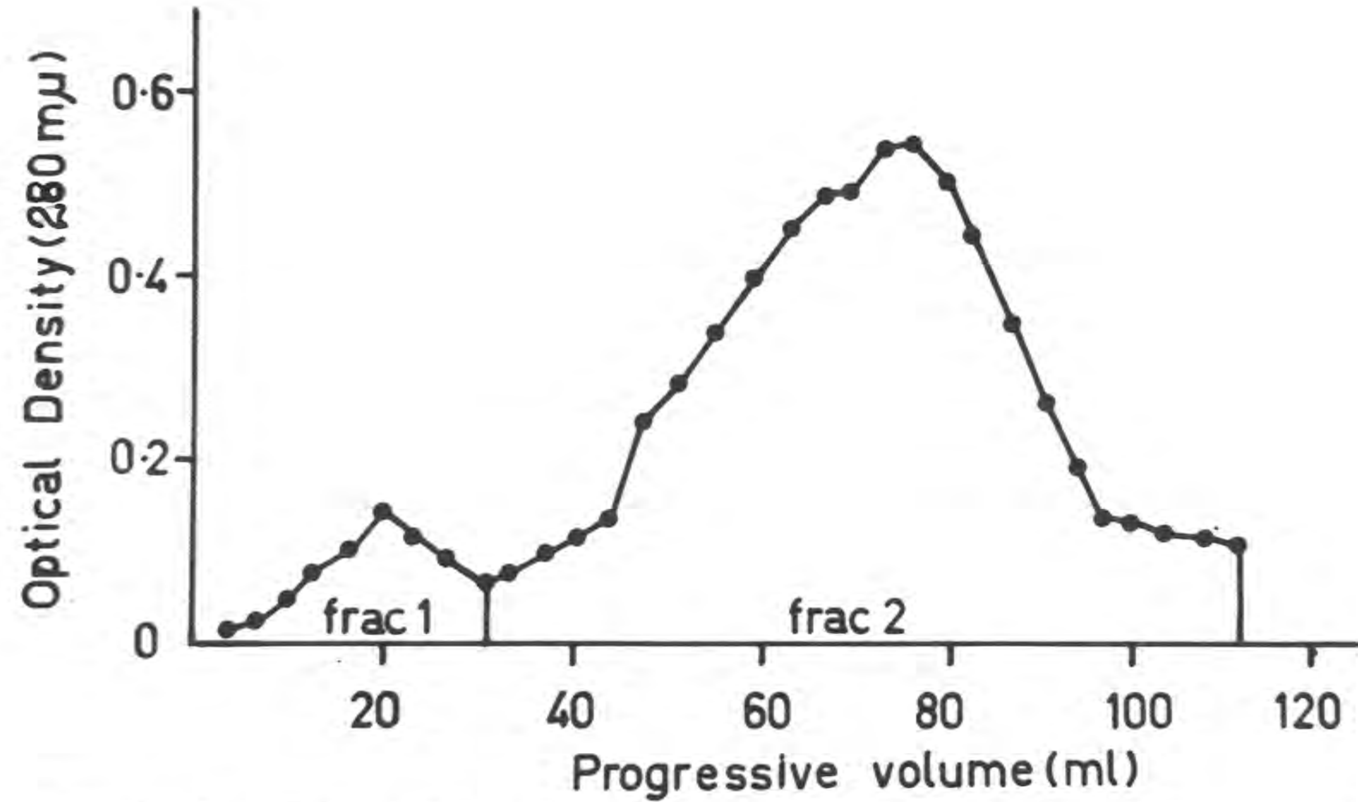


Fig.47 Separation of foetal rat serum by density gradient ultracentrifugation

two fractions on the basis of this pattern and Fraction 1 was considered to contain the macroglobulin portion. The large fraction volumes were concentrated by pressure dialysis and the samples so obtained were assayed for protein content and biological activity. These samples were assayed for opsonic activity by chick embryo clearance studies. It had been shown previously that foetal rat serum only exhibited a marginal increase in clearance rate when used to opsonise Salmonella typhimurium C5 and assayed by mouse clearance. Fig. 47 shows the results of the assays and reveals the similarity in macro-globulin content of both foetal and adult rat serum.

TABLE 12

Separation by density gradient ultracentfifugation of 3 ml. (105 mg.) foetal rat serum.

Fraction	Volume of concentration fraction (ml.)	Protein content (mg.) recovered	Percentage of applied protein recovered	Units of opsonic activity recovered	Percentage of opsonic activity recovered
1	3.8	11.0	10.5	8	30.8
2	3.6	61.2	58.3	18	69.2
Total	7.4	69.2	68.8	26	100

The table showed that 69 per cent of the applied protein was recovered and that 31 per cent of the biological activity was in Fraction 1 which constituted 10.5 per cent

of the applied protein. The largest proportion of the activity (69 per cent) was in Fraction 2 which contained 58 per cent of the applied protein. These results contrast with those for adult rat serum in which most of the activity was found in the macroglobulin fraction. Starch-gel and immuno-electrophoresis of the frmctions demonstrated their relative heterogeneity.

d. Gel filtration chromatography of adult rat serum

The indications from mercaptoethanol reduction and separation by density gradient ultracentrifugation were that the major proportion of rat serum opsonins were macroglobulins. These methods however were analytical rather than preparative and to obtain adequate amounts of protein for further investigation it was decided to employ gel filtration. This method was used to separate the serum into a fraction with a molecular weight greater than 200,000 and a fraction with a molecular weight smaller than this. For this purpose a column of poly-dextran gel (Sephadex G 200) which acts as a molecular sieve was employed. A typical elution pattern is shown in Fig. 48. It demonstrates that the serum was divided by this method into three well defined peaks. The macro-globulins were eluted in the first small peak and represent 10 per cent of the protein content of the eluate. The tube contents were pooled on the basis of this pattern to give three fractions. These fractions were assayed

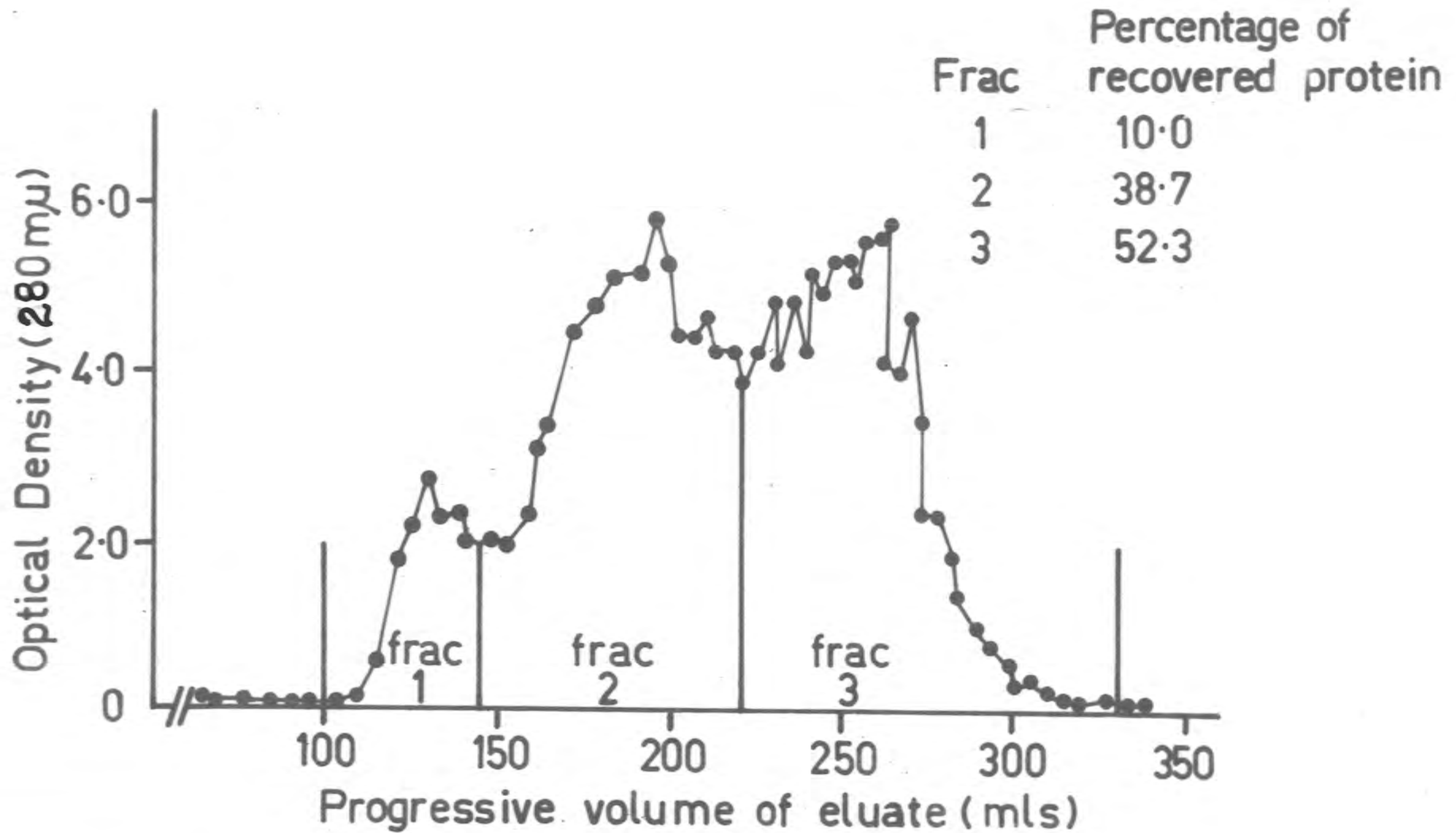


Fig.48 Separation of adult rat serum by gel filtration

for opsonic activity and for protein content. Table 13 shows the results of this study in which 14 ml. (910 mg.) of adult rat serum was separated by gel filtration. These fractions were concentrated by pressure dialysis to the volume shown in Table. 13.

TABLE 13

Separation by gel filtration of 14 ml. of adult rat serum containing 910 mg. and 1568 units of opsonic activity to Salmonella typhimurium C5

Fraction	Volume of concentrated fraction (ml.)	Protein content (mg.) recovered	Percentage of applied protein recovered	Opsonic units of activity recovered	Percentage of opsonic activity:	
					Based on total activity applied	Based on total activity recovered
1	4.5	121	13.0	720	45.9	54
2	5.2	380	41.8	220	14.0	16.5
3	13.1	322	35.4	399	25.5	29.5
Total	22.8	823	90.2	1399	85.4	100

This fractionation procedure resulted in a recovery of 90.2 per cent of the applied activity. The first fraction separated contained 54 per cent of this activity and was considered, in the light of previous work, to be the macroglobulin fraction. There was also considerable activity in Fractions 2 and 3 which contained 17 per cent and 30 per cent of the activity, respectively.

In an endeavour to investigate more fully the characteristics of Fraction 1 an aliquot of 1 ml. (84 mg.) of this

fraction concentrate was subjected to density gradient ultra-centrifugation. Fig. 49 shows the separation pattern obtained and demonstrates that this material could be divided into three well defined peaks. These were used as a basis for pooling the contents of the tubes into three fractions which constituted 57 per cent, 19 per cent and 24 per cent of the recovered protein, respectively. These fractions were concentrated by pressure dialysis and assayed for opsonic activity and protein content. The results are shown in Table 14 from which it can be seen that 65 per cent of the protein, containing 29 per cent of the activity, was recovered, and that 72 per cent of this activity was in the first fraction. Much smaller amounts of activity, 9 per cent and 19 per cent were found in Fractions 2 and 3 respectively.

TABLE 14

Separation by density gradient ultra centrifugation of 1 ml. of Fraction 1 from the gel filtration of adult rat serum containing 160 units of opsonic activity to Salmonella typhimurium C5

Fraction	Volume of concentrated Fractions (ml.)	Protein content (mg.) recovered	Percentage of applied protein recovered	Units of opsonic activity recovered	Percentage of opsonic activity:	
					Based on total activity applied	Based on total activity recovered
1	6.5	31.0	36.9	34	21.3	72.3
2	2.3	10.3	12.3	4	2.5	8.5
3	3.4	13.2	15.7	9	5.6	19.2
Total	12.2	54.5	64.9	47	29.4	100

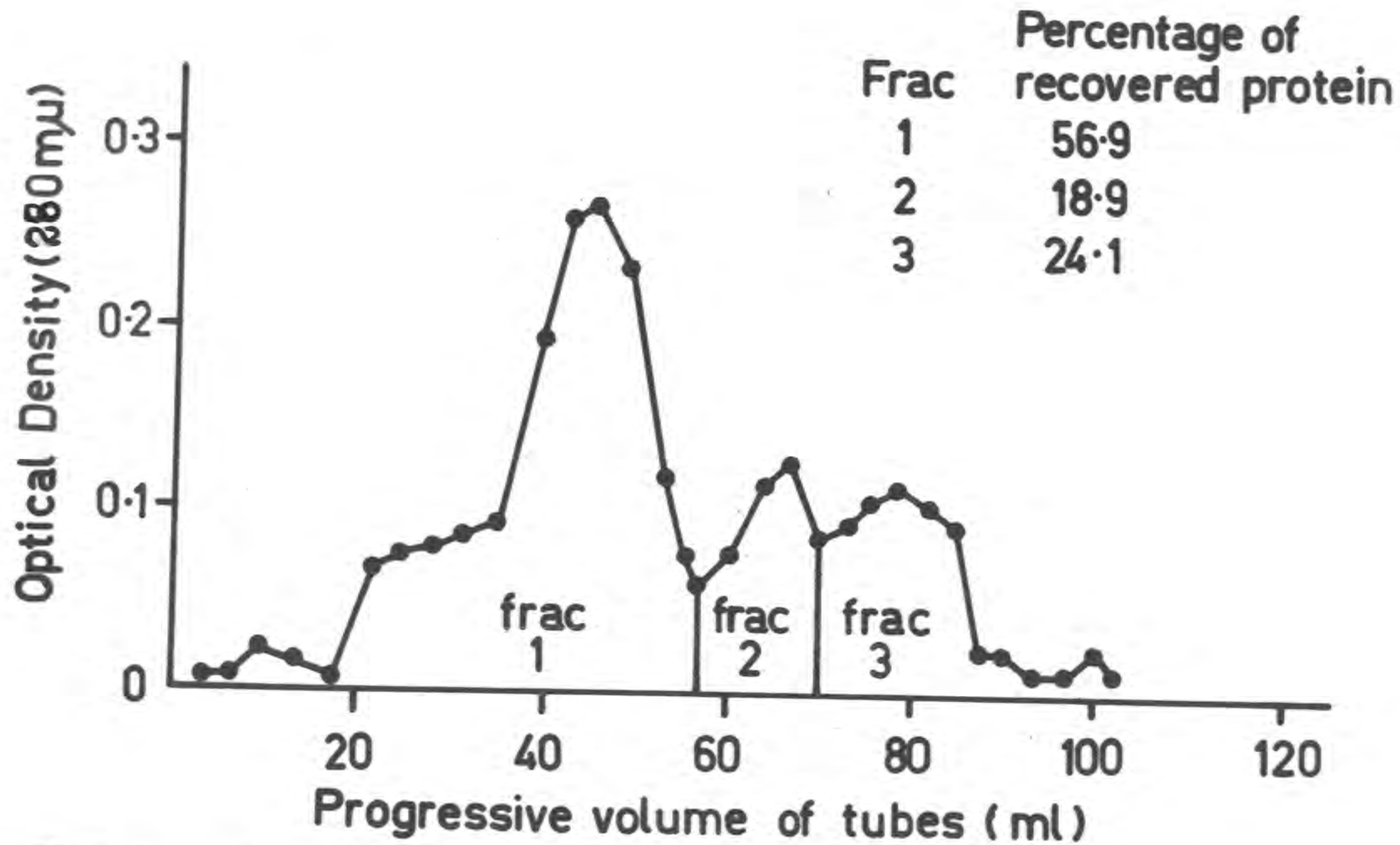


Fig.49 Separation by density gradient ultracentrifugation of fraction 1 from the gel filtration in fig.48

This density gradient ultracentrifugation of the macroglobulin fraction from the gel filtration showed that while the major part of the activity was in the bottom of the tubes a significant amount of activity was spread throughout the contents of the tubes. Starch-gel electrophoresis of the fractions and immuno-electrophoresis against specific rabbit antiserum to rat serum showed that both the initial gel filtration separation and the density gradient ultracentrifugation of Fraction I from this separation had produced relatively heterogeneous fractions.

e. Separation by ion exchange chromatography with D.E.A.E. cellulose

While the previously described experiments had shown that the macroglobulin fraction of adult rat serum contained most of the opsonins to *Salmonella typhimurium* C5 they also showed firstly that this fraction was heterogeneous and secondly that the remainder of the serum contained demonstrable opsonic activity. In an endeavour to further characterise the opsonins, particularly those other than the macroglobulin antibodies, 4 ml. (260 mg.) of adult rat serum was fractioned by D.E.A.E. cellulose column chromatography. Fig. 50 shows the resultant elution pattern obtained by this means using a volume of 750 ml. of phosphate buffer with a gradient of 0.01 M to 0.40 M. Eight fractions were obtained on the basis of the major

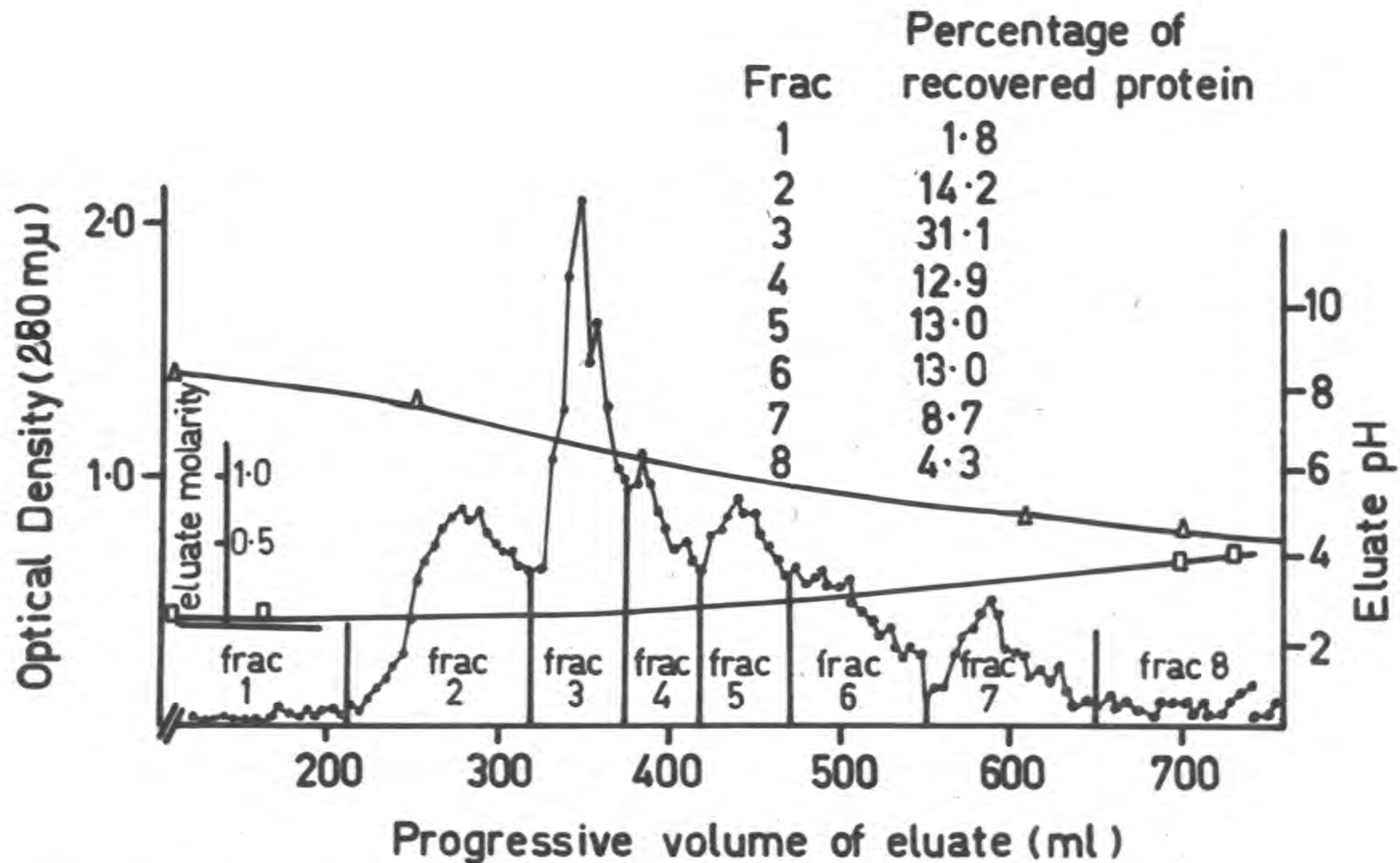


Fig.50 Separation of adult rat serum by D.E.A.E. column chromatography
•— eluate optical density △—△ eluate pH □—□ eluate molarity

TABLE 15

Separation by D.E.A.E. cellulose column chromatography of 4 ml. of adult rat serum containing 260 mg. of protein and 448 units of opsonic activity to *Salmonella typhimurium* C5

Fraction	Volume of concentrated Fraction (ml.)	Protein content (mg.) recovered	Percentage of applied protein recovered	Opsonic units of activity recovered	Percentage of opsonic activity	
					Based on total activity applied	Based on total activity recovered
1	3.8	3.1	1.2	2	0.04	2.9
2	2.5	34.5	13.3	6	1.3	8.7
3	3.6	68.8	26.5	0	0	0
4	3.8	16.5	6.3	3	0.07	4.3
5	3.7	26.4	10.2	1	0.02	1.5
6	5.9	25.0	9.6	36	8.0	52.2
7	3.8	16.1	6.2	19	4.2	27.5
8	5.0	4.0	1.5	2	0.04	2.9
Total	32.1	194.4	74.8	69	13.67	100

peaks of this elution pattern. These were concentrated by pressure dialysis and assayed for protein content and biological activity. The results of these assays are shown in Table 15 which serves to illustrate that most of the activity was eluted over the molarity range of 0.12 M to 0.32 M. By inference from other investigations

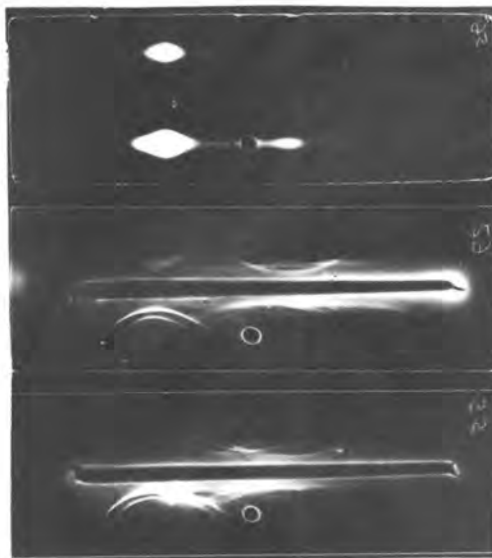
these fractions (Fractions 6 and 7) were considered to contain the 19S macroglobulin antibodies. A small amount of activity was also found in the other fractions with the exception of Fraction 3. Apart from Fractions 6 and 7 the gamma-globulin fraction (Fraction 2) was seen to contain most activity.

It can be seen from Table 15 that Fractions 6 and 7 contained 16 per cent of the applied protein recovered; the nett overall recovery of protein being 75 per cent. The biological activity of these two fractions accounted for 80 per cent of the recovered activity and was completely destroyed by treatment with mercaptoethanol.

Starch-gel electrophoresis showed that all of the fractions were heterogeneous. Immuno-electrophoresis of each fraction against rabbit anti-rat serum, while also demonstrating this heterogeneity, illustrated more clearly than the results of the starch-gel electrophoresis the way in which the fractions had been separated.

f. Immuno-electrophoresis

In addition to testing the fractions from the various separation procedures described above 20- day pooled foetal rat serum and 20- day pooled embryonic fluid, normal pooled adult rat serum and serum from rats hyperimmune to Salmonella typhimurium C5 were subjected to electrophoresis against specific rabbit antiserum to adult rat serum. The results are shown in Fig. 51 which



A Foetal serum control

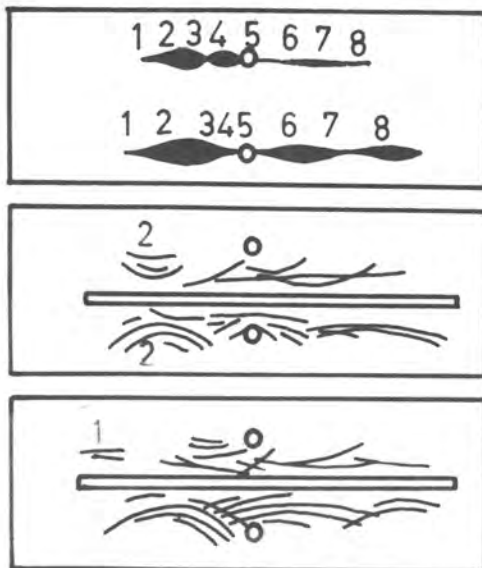
B Adult serum control

C 20–day embryonic fluid

D Adult serum

E 20–day foetal serum

F Adult serum



A

B

C

D

E

F

1 pre–albumin
2 albumin
3 post-albumin
4 α-globulin
5 α/β globulin
6 β-globulin
7 β-2M–macroglobulin
8 γ-globulin

Fig.51 Immuno–electrophoretic analysis of embryonic fluid and rat serum

graphically demonstrate the immunological variations between the samples. Adult rat serum produced some fourteen distinguishable precipitin bands. The outstanding features of this pattern were the four bands which occurred in the albumin region (albumin A, B, C and D) and the gamma-globulin bands which extended from their electrophoretically defined zone to the pre-albumin zone. In each of the alpha/beta-globulin and post-albumin zones there was one well defined band, and in the beta zone there were two well defined beta-2A bands and one beta-2M band.

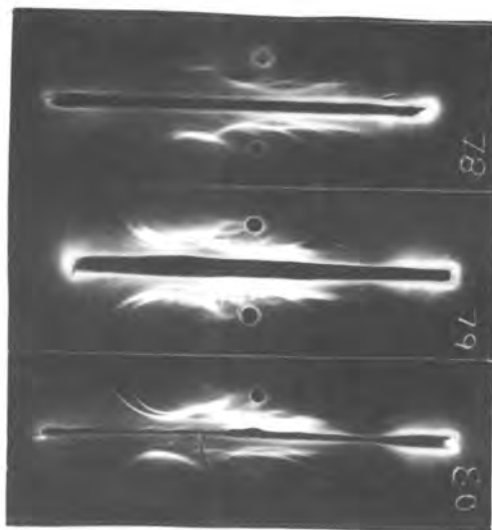
A sample of the hyperimmune rat serum, while being essentially similar to the normal serum, showed an intensification of the bands in the gamma-globulin and beta-globulin zones. The samples of foetal rat serum and embryonic fluid showed a marked similarity to each other with the exception of the albumin zone. They were broadly similar to the adult serum but with a striking difference in the albumin region and an overall reduction in the number of precipitin bands especially in the beta-globulin region. The four dense bands of albumin in the adult serum were absent in the foetal serum which, however, exhibited a faint diffuse pre-albumin band and a diffuse 'post-albumin' zone containing three bands. The foetal sample contained bands corresponding to alpha- beta- and gamma-globulins and the alpha/beta-globulin mixture of adult serum.

A pre-albumin zone was absent in the embryonic fluid and there was only one band in the pos t-albumin zone. There were three bands in the albumin zone which apparently corresponded to albumin B and C of adult serum with an ibterposed band which was designated albumin B_1. The pattern otherwise was essentially the same as for foetal serum.

To obtain an indication of the way in which the foetal immuno-electrophonetic pattern changed to the adult pattern, 20-day foetal rat serum, and 7-, 14-, 28-, and 84- day natal rat serum was electrophoresed against rabbit anti-rat serum. Fig. 53 shows the result of these experiments and demonstrates an increasing complexity of serum proteins with increasing age. This was particularly apparent in the albumin anzd in the alpha-globulin regions.

Foetal serum has been shown to contain a similar proportion of albumin to that present in adult serum. In foetal serum however there are no precipitin bands corresponding to the position of albumin, although albumin was present in the control (Figs. 51, 52). Thi s was taken to indicate that foetal albumin was antigenically different from adult albumin.

In the alpha-globulin region of foetal serum several precipitin bands occurred which, because of their lateral displacement, were suggestive of a collection of macro-globulins. While these bands were present in the foetal



A 20-day foetal serum

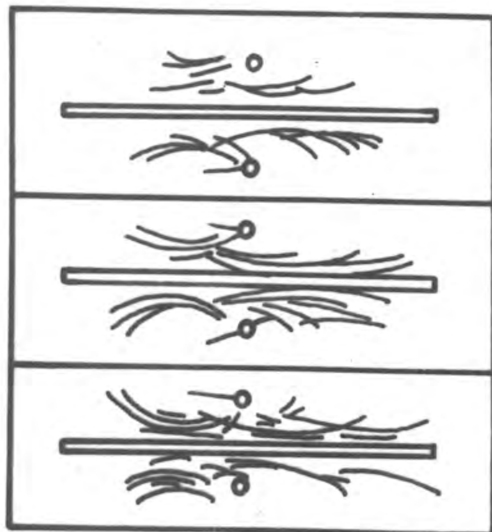
B 7-day natal serum

C 14-day natal serum

D 28-day natal serum

E 84-day natal serum

F 1:3 dilution of 84-day natal serum



A

B

C

D

E

F

see key on fig. 51

Fig.52 Immuno-electrophoretic analysis of rat serum from animals of various ages

and 7- day neonatal serum samples they were not so apparent in serum from rats older than this. This may however be due to masking by the intense albumin bands present in samples from the older animals.

10. Conclusions

A picture emerges from this aspect of the study of a developing humoral system which has its origin in foetal life. The source of the humoral factors which are responsible for bactericidal activity and opsonic activity of foetal and neonatal serum was not investigated in this study.

The variation from adult serum seen in the starch-gel and immuno-electrophoretic pattern of foetal and neonatal serum is an indication of a changing serum protein distribution as the animal matures to about the time of weaning. At about this time an adult-type pattern is established. The plateau in opsonic activity and the general similarity in distribution of proteins in the foetal and neonatal serum as seen from the starch-gel electrophoresis is suggestive however of a dependence on maternal transfer. This observation is supported by the similarity of serum protein distribution in the embryonic fluids, the way by which it has been shown (Brambell *et al.*, 1951) that a proportion of maternal transfer occurs in the rabbit.

The opsonic activity in normal adult rat serum has been shown to exist mainly in the macroglobulins and it was assumed that these opsonins were the so-called 'natural' antibodies. There was however a significant amount of opsonic activity in both the gamma-globulins and the post-albumins which was considered to result from either or all of the following situations. Firstly the activity in the non-macroglobulin fraction could result from the presence of other antibodies directed against similar antigens and produced by a recent or concurrent antigenic stimulus. Secondly that this opsonic activity was produced by denaturation of the macroglobulins during fractionation procedures. These two explanations seem unlikely in view of similar results obtained from a variety of methods and with different batches of serum. The third situation which seems the most likely is that the initial stimulus which caused the production of the specific 19S macroglobulin also stimulated the continued production of 7S and postalbumin antibodies. The presence of active macroglobulins in foetal serum, even though the order of activity was very low, is of importance in suggesting that the foetus is in fact producing some of its own immunologically active proteins, since it is well documented that macroglobulins do not readily pass the placental barrier in a variety of

animals (Valquist 1958, Kunkel 1960). The presence of proportionately more activity in Fraction 2 of foetal serum supports the contention that much of foetal humoral immunity has been provided by maternal transfer, for it is known that 7S antibodies are most readily and even specifically transferred to the foetuses of some animals (Valquist 1958).

C H A P T ERR V I I

DISCUSSION

Ebert and DeLanney when reviewing the ontogenesis of the immune response in 1959 commented that, "we have failed to reach even the first level of understanding the mechanisms whereby the capacity for an immune response is established during development". This statement highlighted the way in whâch the developmental aspects of immunology have been dismissed with the tacic assumption that foetal animals are immunologically incompetent. That this has been an unwarranted and misleading assumption has been demonstrated by several studies concurrent with the present one which have shown in various ways that foetal animals have the capacity to be immunologically responsive even if not in the full blown adult sense. What is more these studies have, in their own way, demonstrated a broadening of immunological horizons as embryonic life proceeds. The work of Karthigasu and Jenkin (1963) and of Silverstein et al. (1963) has for example shown a developing phagocytic system in chicks on the one hand and on the other an increasing capacity to respond by antibody formation to a wider range of antigens in foetal lambs of increasing age.

Burnet (1959) has stressed the survival value of the immune mechanism and considers that this highly complex phenomenon has evolved for the singular purpose of host defence against pathogenic micro-organisms.

Presumably after having found in a number of mammalian species that neonates were relatively immunologically incompetent in a variety of ways it was considered that immunological mechanisms developed from about this time. In mammals at least the dependence on maternal transfer for foetal host defence has probably been accepted as reason enough for presuming that foetal mammals do not require the protection of an immune mechanism. The lack of cells and organs which are intimately connected with an animal's response to foreign antigens has also been acсepted as demonstrating foetal and neonatal immunological inadequacy (Good *et al.* 1959). Dixon and Weigle (1957) have presented evidence that this is not so much a deficiency in the cells but a deficiency in the neonal environment which prevents the expression of their immunological competence.

Against this background then the results of recent studies with foetal animals might provide a tangible link between primitive phagocytic systems and host defence mechanisms. If functional phagocytic cells and the production of antibody are accepted as the hallmarks of immunological competence at two levels then it appears that foetal animals should be accepted as having this status. If in fact foetal and neonatal mammals have no need for a defence mechanism against micro-organisms because of maternal protection, it is necessary to explain the presence of quasi-immunological mechanisms in those foetal animals examined.

It has been established in this and other studies that these

mechanisms are deficient in comparison to those in adult animals and that they develop in magnitude and scope during foetal and neonatal life. The concept of a system of cells which recognise foreign material in what is probably a similar fashion to the discriminative way in which a unicellular organism recognises food needs, close examination. In the tissues of more complex animals, such as the sessile Ceolenterates, there are to be found large amoeboid cells which appear not only to transport food, but also to dispose of waste material. This implies a further advance, in the phylogenetic sense, in recognition processes if these cells can now recognise "not wanted" as well as "wanted". Rather than supposing therefore that immune mechanisms evolved de novo because of their considerable survival value it seems reasonable to argue along similar lines to those suggested by Boyden (1962) who has suggested that these mechanisms of host defence developed as a natural consequence from an already established recognition system. This system appears to expand its scope during foetal and neonatal life, and to become operative as a host defence mechanism after the young animals emerge from a protective maternal environment.

Boyden, by analogy with other highly specific biochemical phenomena, postulates that the method of recognition might be dependent on the affinity of complementary macromolecules. Large numbers of these occur, many with only fine structural differences, in any one species of animals. If then fine differences in macromolecular configuration mean the

difference between recognition or non-recognition the highly specific nature of the process finds a readly explanation.

This investigation of the development of the reticulo-endothelial system in foetal rats commenced with experiments designed to determine the functional capacity of this system in regard to its phagocytic ability. The rates at which intravenously injected viable and non-viable particles were removed from the circulating blood was determined by quantitative methods. It was shown with all the particles used that rough strains of bacteria were removed very quickly while smooth strains by comparison were removed slowly. It was demonstrated that in regard to <u>Salmonella typhimurium</u> C5 at least, the slow rate of clearance was not necessarily caused by a deficiency of serum opsonins to this organism. This suggested that some other limiting factor was operative, but it was difficult to understand what this was in immunological terms. It might have been that there was a lack of the requisite opsonins in adult rat serum for this smooth strain to be cleared in foetal rats.

It was demonstrated in this and other studies that the liver was the most active organ in removing blood-borne foreign particles. It is interesting to note that while in foetal rats the $\frac{W}{W.l.s.}$ index remained constant the phagocytic index (K) and the alpha value ($\frac{W}{W.l.s.} \sqrt[3]{K}$) increased. It appears therefore that the rapidly increasing weight of the developing foetal liver is not directly related to the increasing phagocytic index, but that this is most probably

due to an increasing number of, or an increasing efficiency of, the phagocytic cells themselves.

It was from these clearance studies that the first indication was noted of a rapidly developing system in foetal life which was followed by a plateau, or at least a decrease in the rate of development, from birth until the animals were approximately two weeks old. This projection of the near term foetal state into early natal life was noted in the titre of opsonins to Salmonella typhimurium C5, in the electrophoretic pattern of the serum and in the bactericidal ability of the hepatic phagocytes. This evidence suggests that the neonatal rat is in a state of immunological stasis (or at least retarded development) which seems to correspond to the period described by Billingham et al. (1956) during which tolerance could be induced - the tolerance-responsive period of an animals' life.

The change towards establishing adult-type patterns of the various parameters mentioned above coincides with a decrease in maternal transfer (Brambell et al.,1951) and from a teleological point of view coincides therefore with the time at which the young animals begin to need a protective mechanism of their own. It is conceivable therefore that the investigations in this study involving foetal rats have not been so much measuring a process which can be paralleled with an adult-type immunological (host defence) - type of response but rather a more basic function of a system which is, during foetal life, developing at a rapid rate to take part in the

enormous cellular activity which prevails at this time. It could be argued that this basic function becomes well established by parturition and that during neonatal life, while under maternal protection, the adaption of this system to host defence takes place. A relatively slow change over then occurs during the latter part of the suckling period from maternal to self protection. It is suggestive that the process of weaning, i.e. separation from maternal immunological influence, is in fact the stimulus required for this change-over. Support is available for this reasoning from the work of Davis and Osborn (1953) who have shown that premature infants weighing as little as 1300 gm. can produce diptheria antitoxin in response to immunisation procedures in four to eight weeks after birth. Normal infants immunised in the first few days of life also take four to eight weeks to produce antitoxin. Uhr (1960) considers that separation of the foetus from the mother is an event which permits the introduction of the young animals immune (host defence) response. Sterzl (1960), who has repeated similar experiments in pigs, is in agreement with this interpretation and suggests that not only is immunological inadequacy due to a delay in maturation, but that it is also due to a lack of previous contact with an antigen in amounts sufficient to elicit an immune response. The continued presence of maternal antibody could obviously diminish or eliminate antigens as they involved the neonate. Silverstein _et al_. (1963) have demonstrated that sufficient antigen will stimulate the production of antibody in foetal lambs.

It was demonstrated in this study that the cells of the reticulo-endothelial system of near term foetal animals and young neonates were similar in their phagocytic and bactericidal ability and that during foetal life these properties increased. Similar features were shown in *in vitro* investigations in regard to the bactericidal property of the serum from such animals. The significance of this latter property in relation to host defence is difficult to determine for it is commonly a matter of conjecture as to whether this mechanism expresses itself to any extent *in vivo*. In the case of foetal rats it was shown that while the foetal serum samples killed *Escherichia coli* (Lilly) in *in vitro* experiments with increasing efficiency as the age increased this was not the case in *in vivo* experiments. These experiments showed that for times in excess of those used for the *in vitro* experiments the animals were unable to kill the test organisms which in fact appeared to multiple in the *in vivo* situation.

The histological investigations demonstrated the existence of phagocytes in the situations commonly described in natal animals. The presence of phagocytes at the periphery of the foetal adrenal cortex and their ability to phagocytose carbon provided a link between a physiological and host defence type function.

It was however clearly demonstrated that the liver was by far the most active phagocytic organ. Because of less compact cellular architecture of the foetal liver the wide ramifications of the hepatic phagocytes were more easily

distinguished following the injection of carbon. The ramifications, or dendritic processes, of this type of cell were observed in adult tissue particularly at the electron-microscopic level. A factor involved in making the processes of the foetal hepatic phagocytes more easily detected in the foetal livers could also have been due to the slower movement of the particles into the body of each phagocyte following their association with the cell surface. It was also observed that in the youngest foetal animals the rate of association of the particles with the cells appeared much slower. This observation was supported by the slower clearance rate calculated for these young foetuses. The electromicrographs illustrated the avidity which the hepatic phagocytes have for removing introduced foreign particles from the blood and it is presumed that this avidity is also exhibited towards "not wanted" particles produced by the host itself. An indication was gained of a somewhat purposeful treatment of bacterial cells by the hepatic phagocytes, especially by comparison with the rapid dissolution observed when bacteria were seen to be in hepatic parenchymal cells and also that observed by Florey (1962) in polymorphonuclear leucocytes. That this "purposeful treatment" of digestible particles is an important function of macrophages in reutilisation and as a step in antibody production has been discussed.

The studies concerned with the serum of foetal and natal animals also displayed a change from foetal to neonatal life. It was determined that serum from a series of

neonatal rats in the first two weeks of life exhibited a similar level of opsonic activity. This was a similar pattern to that seen in the plateau in phagocytic index in neonatal animals and it is not inconceivable that these two phenomena were interdependent. Investigations were carried out which were directed towards characterising the "natural" antibodies present in rat serum. These opsonins enhanced the clearance of Salmonella typhimurium C5 in mice and chick embryos. The results obtained from the fractionation procedures of adult rat serum and the associated biological assays were similar to those reported by Turner and Rowley (1963) who examined pig serum in the same way.

Approximately 80 to 90 per cent of the opsonic activity of adult rat serum resides in the macroglobulin fraction which constitutes some 10 per cent of the total serum protein. There was, however, a significant amount of antibody which was shown to be other than macroglobulin. Some of this opsonic activity was demonstrated in the gamma-globulin fraction and a slightly larger proportion than this was considered to be in the post-albumin fraction.

The stimulus for, or the raison d'etre, of 'natural' antibody has caused considerable speculation and has led to investigations such as those of Sterzl et al. (1959) who have shown with germ free piglets that no natural antibody could be detected if there had been no antigenic stimulus. It would appear from investigations such as this that natural antibody is an acquired antibody the stimulus for

the production of which is gained early in an animal's life. The possibility does exist, however, that some natural antibodies might arise from autostimulation, since it is now well documented that certain host component cross-react with bacterial and viral antigens (Jenkin, 1963).

Opsonins were also detected in foetal rat serum and these were shown to be both 19S and 7S types. The proportion of macroglobulins in foetal serum was similar to that in adult rat serum, but there was proportionately far greater activity in the 7S fraction in the foetal serum. The presence of macroglobulins in foetal rat serum was suggestive of foetal production of macroglobulins. While the presence of proportionately greater quantities of opsonins in the 7S fraction on the other hand was attributed to maternal transfer.

The reasons for the clearly obvious changes due to age which were observed in the immuno-electrophoretic studies of foetal and natal rat serum have not been investigated in this study. Antigenic proteins of the same strain of animals have been observed previously (Kelleher and Villee, 1962) and it would appear that this study has also demonstrated the antigenic dissimilarity of some foetal and natal serum proteins, especially in the albumin region.

Because the origin of mammalian foetal serum proteins is obscure it has been difficult to interpret the results of these serum studies in relation to the contribution made to foetal serum by the mother or by the foetuses themselves. The intimate association of the mammalian mother and her

foetuses, the routes by which relatively free interchange from mother to foetus occurs and the source of foetal serum proteins are not well understood. The techniques developed and the information gained from this study should provide the means by which questions relating to the production of foetal proteins can be answered. By injecting C^{14} labelled amino acids into foetal animals which can then be kept isolated and alive for many hours it should be possible to determine what proportion of its serum protein it is manufacturing. As an extension of this it should also be possible to investigate the production of antibody by foetal animals.

In summary, therefore, it has been clearly demonstrated that foetal rats exhibit the presence of a functional reticulo-endothelial system which increases in its capacity as the animal progresses to birth. It has been argued that this system has a more fundamental function than that of host defence which is commonly ascribed to it. Future investigations based on the results of this study could elucidate problems concerned with the production of serum proteins, especially antibodies, by foetal mammals.

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ACKNOWLEDGEMENTS

I wish to express my considerable appreciation to Dr. Charles Jenkin and to Professor D. Rowley and Professor G.B. Mackaness for their guidance, help and constructive criticism throughout this study.

I am grateful to Dr. Keven Turner for his helpful advice and discussion in regard to the biochemical aspects of this study; to Dr. Bryan Radden for his assistance in preparing the histological slides, and to Dr. John Casley-Smith for his assistance in preparing the electron micrographs and in discussing their interpretation.

I am grateful to the University of Adelaide for the award of a Nuffield Research Fellowship during the period of this work.