Immunology, 1965, **9**, 61.

The Functional Development of the Reticulo-Endothelial System

II. THE HISTOLOGY OF BLOOD CLEARANCE BY THE FIXED MACROPHAGES OF FOETAL RATS

P. C. READE* AND J. R. CASLEY-SMITH†

Microbiology Department and Electron Microscope Unit, Departments of Botany, Microbiology and Zoology, University of Adelaide, South Australia

(*Received 5th October* 1964)

Summary. A histological study has been made of the distribution of particles injected intravenously into foetal rats. It was shown that the organ distribution was similar to that observed in adult rats and that the particles removed from the circulating blood were to be found inside the fixed macrophages.

INTRODUCTION

Numerous histological investigations of the cells and organs involved in clearing particles from the circulating blood of natal mammals have been reported at length since the middle of the last century. A variety of microorganisms and non-viable particles such as colloidal metals and particulate dyes have been injected into such animals. The voracity with which certain fixed cells remove particulate matter from the blood by phagocytosis has been demonstrated. It has been shown repeatedly that the liver is well endowed with these phagocytes and it is this organ which is considered to contain more of these cells of the reticuloendothelial system than any other (Florey, 1961).

Since the early descriptions by workers such as Kupffer (1878, 1899) it has been recognized that these fixed hepatic macrophages are in an ideal anatomical situation for constant monitoring of the circulating blood and clearing it of foreign particles. These early investigators working with the technical limitations of their time described these cells with considerable accuracy. It is only in recent years that the use of electron microscopy has corroborated these findings and added information of fine detail (Parks, 1956; Jezequel, 1962).

Despite the large volume of literature that exists concerning the histology of phagocytosis by the cells of the reticulo-endothelial system in natal animals, no previous reference can be found to a comprehensive study in foetal mammals.

Following the demonstration that various organs of foetal rats were capable of collecting injected particles and so clearing their circulating blood of foreign material (Reade and Jenkin, 1965) it was decided to study this clearance by histological methods. It was the purpose of this study to investigate the reasons for clearance in foetal rats and hence to determine whether this was due to mechanical trapping, surface adherence or phagocytosis.

* Nuffield Research Fellow, 1962–63.

† Senior Lecturer in Electron-Microscopy.

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 was used in this study as the model for the pattern of clearance of intravenously injected particles from the blood of foetal rats.

MATERIALS AND METHODS

Animals

Hooded Wistar rats at predetermined stages of pregnancy.

Particles used for clearance studies

(a) *Carbon.* A shellac-free preparation of Indian ink (Ink No. C11/1431a) prepared by Günther Wagner, Hanover, Germany, and treated according to the method described by Biozzi, Benacerraf and Halpern (1953) giving a particle size of less than 500 Å at a concentration of 32 mg/ml in 2 per cent gelatin at pH 7·4.

(b) *Thorium dioxide.* The material used was 'Thorotrast', a preparation of thorium dioxide (Testagar & Co. Inc., Detroit, Mich., U.S.A.).

(c) *Bacterial strain. Escherichia coli* (Lilly) known to contain a relatively low concentration of lipopolysaccharide (Wardlaw, 1963).

Dose of particles injected intravenously into each foetus

(a) Carbon: 0·4 mg.

(b) Mixture of particles: carbon 0·4 mg, Thorotrast 0·4 mg, *E. coli* (Lilly) 5×10^7 organisms.

Surgical procedures

These were performed on foetuses aged 14, 16, 18, 20 and 22 days from conception and were exteriorized and manipulated according to the method described previously by Reade and Jenkin (1965).

Material examined by light microscopy

The material was removed from the foetuses immediately after their separation from the uterine wall and placed without delay in 10 per cent buffered formol saline. After fixing for 24 hours the specimens were prepared for histological examination by routine techniques. Haematoxylin and eosin and saturated aqueous picric acid were used to stain the 7 μ thick sections.

Material examined by electron microscopy

This material was finely divided in the presence of 2 per cent OsO_4 in 0·15 M phosphate buffer and then fixed for 2 hours. The specimens were then handled according to routine methods and embedded in 'Araldite' (Ciba Ltd, Basle) and methacrylate and stained with uranyl acetate, phosphotungstic acid or lead hydroxide. The sections were examined in a Phillips E.M. 100 B electron microscope.

RESULTS

ORGAN DISTRIBUTION OF INJECTED PARTICLES IN FOETAL RATS

Thirty minutes after the intravenous injection of carbon the organs to be examined—liver, spleen, gut, pancreas, lungs, heart, kidney, adrenal gland, brain, bone, placenta and foetal membranes—were removed from the test foetuses and prepared for histological

examination. It was observed that the organ distribution both macroscopically and microscopically was similar in all of the foetuses examined.

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

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 considerably more diverse cell population than that of natal livers.

Blood sinusoids with a diameter two to three times that of an erythrocyte ramify extensively amongst both the hepatic parenchymal cells and the haemopoietic tissue. The sinusoids are lined by sessile vascular endothelium, interspersed with dendritic phagocytic cells—the 'Sternzellen' of Kupffer. 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 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. 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. 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 mechanically trapped.

Evidence of considerable phagocytic activity of an occasional large cell closely associated with the syncytium was seen in the placenta. These were probably trophoblastic giant cells in the cytoplasm of which the carbon appeared to remain in a more diffused 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 example 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.

TIME SEQUENCE OF CARBON CLEARANCE BY HEPATIC PHAGOCYTES OF FOETAL RATS

Livers were removed from foetal rats 1, 2, 4, 6, 8, 10, 15, 30, 60, 90, 120 and 180 minutes following the intravenous injection of carbon. The first evidence of cellular involvement in the clearance of this carbon from the circulating blood was obvious in the 1 minute specimens.

The changes in the phagocytic cells of the foetal livers are illustrated in Figs. 1 and 2 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 samples taken at 1 minute. 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 cell varied with the age of the foetus, being obviously faster as the age increased.

A question was posed as to whether the particles associated with a phagocytic cell 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 rats, one 10 minutes and the other 30 minutes after the initial dose, and the animals killed 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 was already loaded with carbon. This suggested, therefore, that the processes of the phagocytic cells remained extended and that the particulate material was shifted by some other mechanism operating within the cells.

DISTRIBUTION OF A MIXTURE OF PARTICLES IN THE LIVERS OF FOETAL RATS

It was observed that those particles which were large enough to be detected by light microscopy, i.e. bacteria and carbon could be seen frequently to be mixed together in the same hepatic phagocyte. Cells that had phagocytosed one type of particle were uncommon but when they were seen it was impossible to say categorically that only one type of particle had been phagocytosed by the one cell.

THE RELATIONSHIP OF INJECTED PARTICLES TO THE HEPATIC PHAGOCYTES OF FOETAL RATS AS DETERMINED BY ELECTRON MICROSCOPY

The mixture of particles was intravenously injected into 20-day foetal rats which were killed at times up to 125 minutes. The livers of these animals were prepared for examination by electron microscopy.

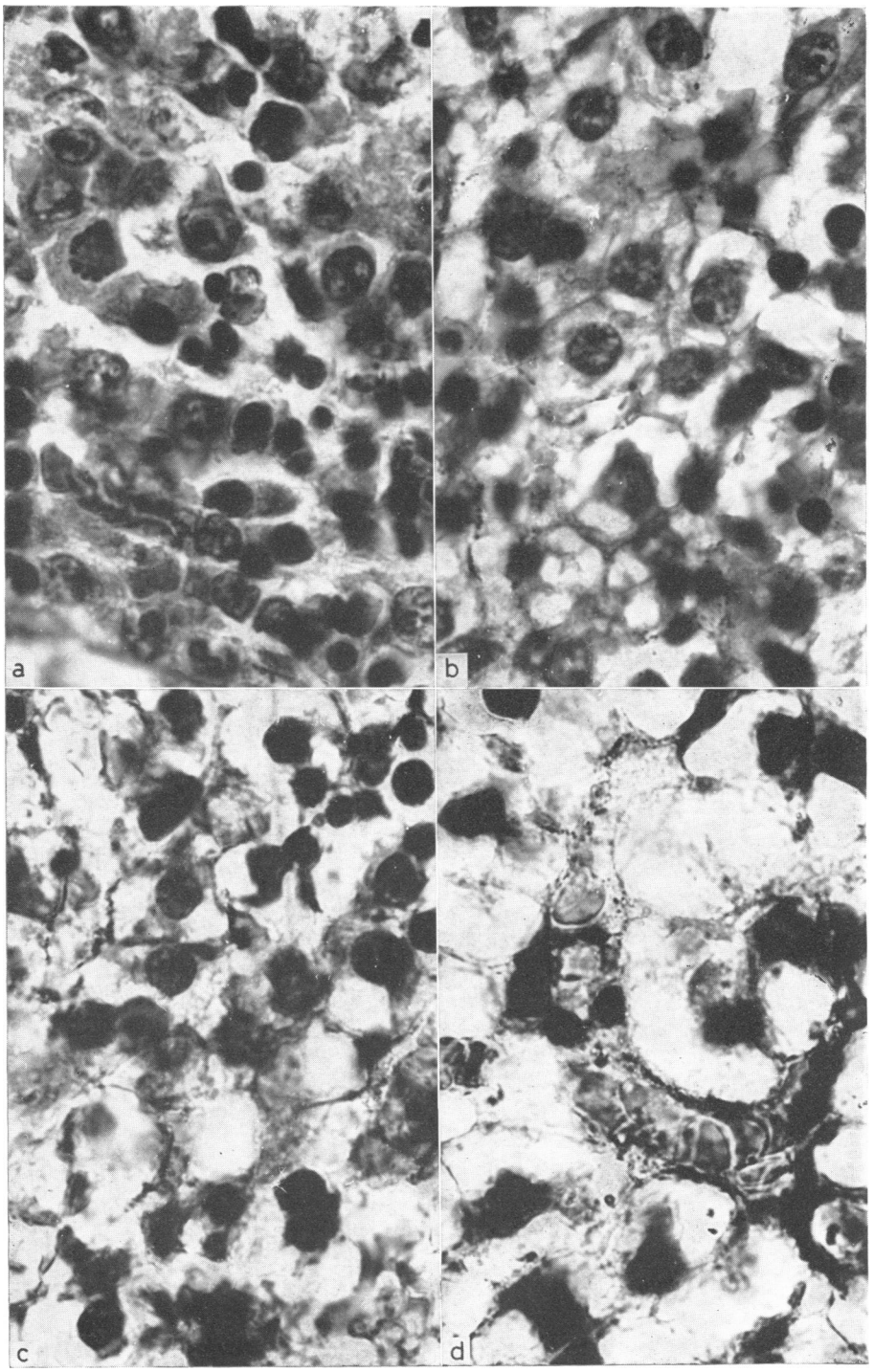

FIG. 1. Photomicrographs of livers from 20-day foetal rats at time intervals after the intravenous injection of 0·4 mg carbon. (a) Normal liver; (b) 1 minute after i.v. carbon; (c) 2 minutes after i.v. carbon; (d) 4 minutes after i.v. carbon. Haematoxylin and eosin, ×650.

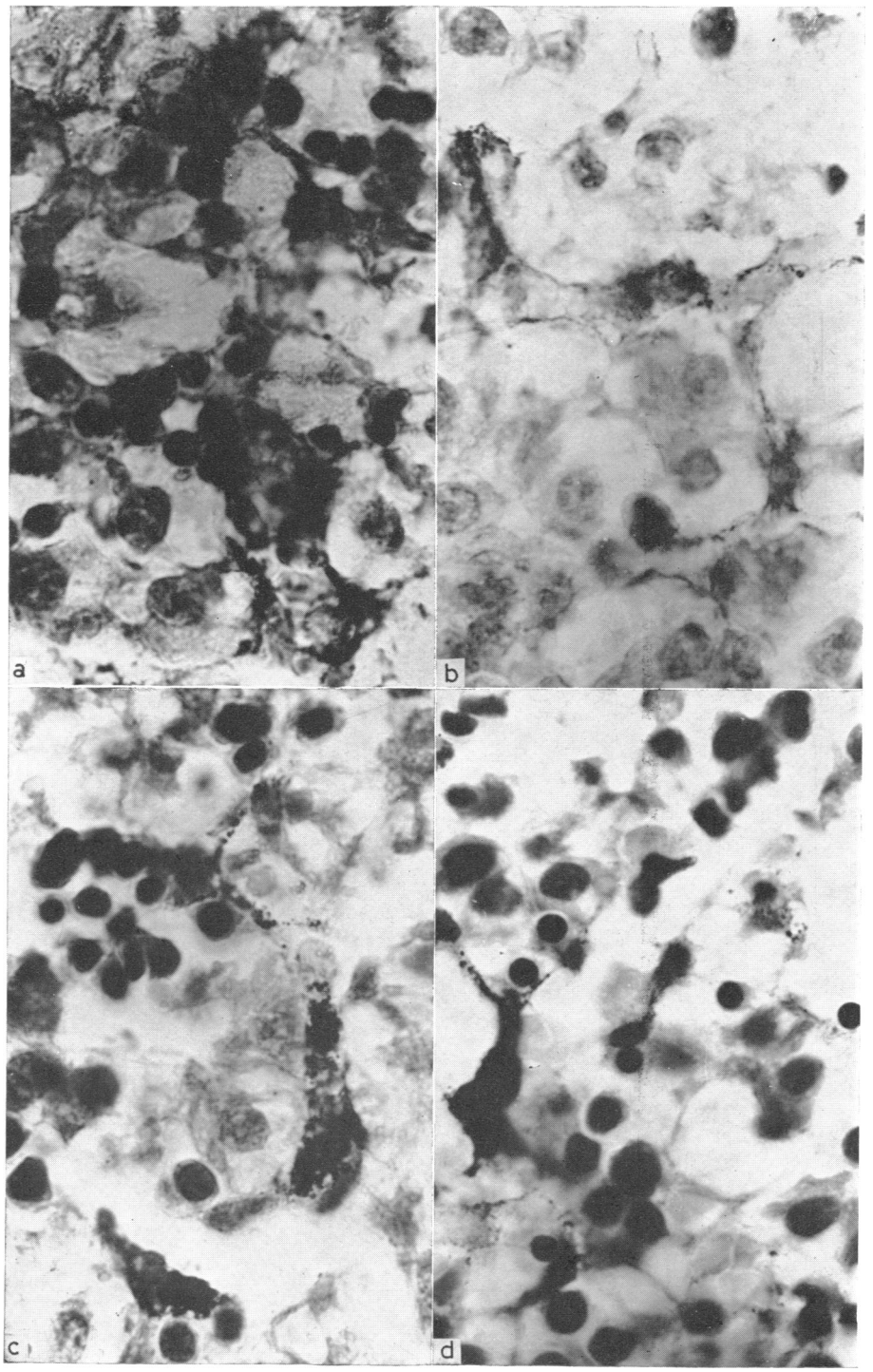

FIG. 2. Photomicrographs of livers from 20-day foetal rats at time intervals after the intravenous injection of 0·4 mg carbon. (a) 6 minutes; (b) 8 minutes; (c) 15 minutes; (d) 30 minutes. Haematoxylin and eosin, ×650.

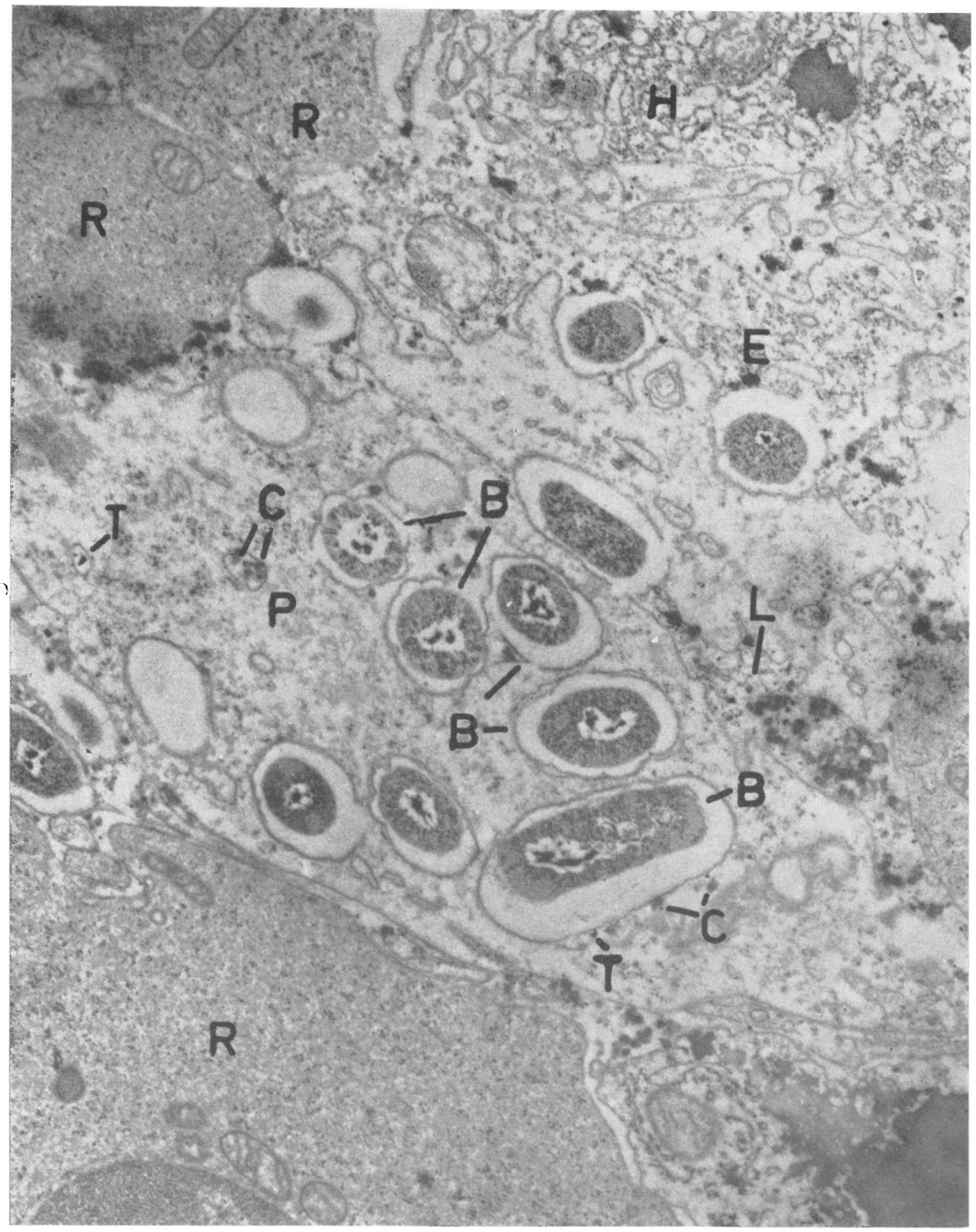

FIG. 3. Electron micrograph of a 20-day foetal rat liver showing bacteria (B), carbon (C) and thorotrast (T) inside vacuoles in a hepatic phagocyte (P). E = endothelial cell; R = early red blood cell; L = lumen; H = hepatic parenchymal cell. × 20,000.

(*Facing p.* 65)

The results of this investigation demonstrated quite clearly the phagocytic ability of the foetal hepatic macrophages. In addition to this it demonstrated the anatomical relationship which these cells have to parenchymal cells and haemopoietic cells of foetal livers. Because of the considerable proportion of haemopoietic tissue it was not possible to demonstrate the well defined relationships which have been shown by Jezequel (1962) to exist between the parenchymal cells and sinusoidal macrophages of adult livers. Nevertheless foetal hepatic macrophages were observed to have similar characteristics to natal macrophages. They were large prominent cells with micro-villi, few small mitochondria and sparse endoplasmic reticulum, interspersed amongst sessile endothelial cells which lined the blood sinusoids.

These cells were observed to contain injected particles in cytoplasmic vacuoles. On most occasions representatives of all the particles injected were to be seen in the one cell. Sometimes they were seen to be segregated in separate vacuoles but most often a mixture of particles was to be seen in any one phagocytic vacuole.

Fig. 3 demonstrates this point by showing in an electron micrograph portion of a foetal hepatic macrophage containing bacteria (B) and carbon (C). It can be seen that several bacteria, together with carbon particles, are collected in the one large vacuole. In addition to the presence of particles within the hepatic macrophages it was noted that hepatic parenchymal cells had also taken up and enclosed both bacteria and other particles in membrane enclosed vacuoles. These observations will be discussed more fully elsewhere.

DISCUSSION

The histological investigations described in this paper have shown that phagocytic cells are responsible for the clearance of foreign particles from the blood of foetal rats and that these cells have similar morphological characteristics to equivalent cells in natal animals. It has been demonstrated in this study that the sinusoidal macrophages of the liver, like those of adult animals play the major role in the uptake of intravenously introduced particles. These findings indicate that foetal rats, above the age of 14 days at least, have a functional reticulo-endothelial system and this evidence supports the findings reported by Reade and Jenkin (1965) who demonstrated an increasing phagocytic capacity as the foetal animals mature to parturition.

The property of particle uptake exhibited by some of the hepatic parenchymal cells of the foetal rats studied has also a parallel in natal animals. Intravenously injected carbon and saccharated iron oxide were found by Foot (1921) and Cappell (1930) respectively in liver parenchymal cells. It was considered that this was part of a redistribution of these materials following the initial rapid clearance from the blood by hepatic macrophages. These cells have also been credited with a role in the removal of chylomicrons from the blood as a step in normal lipid metabolism (Waddell, Geyer, Clarke and Stare, 1954). In a subsequent communication we shall describe experiments similar to those presented here where it was found that bacteria and various particles are taken up by hepatic parenchymal cells of adult rats.

While adult livers are well known to have a highly regular structure it was seen that such was not the case in the foetal rat livers. The influence of a regular architecture was reflected in the difference in distribution of injected carbon particles. In the adult animal carbon was seen to be collected in a regular manner with the greatest concentration in the vicinity of the periphery of each lobule. This distribution can be explained by the direc-

tion of blood flow. In the foetal livers, however, the hepatic phagocytes which had taken up carbon were distributed more or less evenly throughout the substance of this organ. It is suggested that the way in which the foetal liver is organized allowed most of the cells an opportunity to phagocytose carbon. The circumstances existing in adult livers, however, allowed the phagocytes at the periphery of each lobule to have the greatest chance of taking up the carbon as the blood percolates from the portal to the intralobular veins.

The presence of a functional reticulo-endothelial system in foetal rats may be attributed to the necessity for disposal of effete material produced during embryogenesis. For example, endogenous ferritin, presumably arising from erythrocyte destruction, was observed in hepatic macrophages. It was also shown in this study that macrophages in the foetal adrenal cortex were capable of phagocytosing foreign material in the form of intravenously injected carbon particles. The macrophages involved in this process are concentrated towards the periphery of the gland and it is from this site that the 'true' cortex infiltrates the 'foetal' cortex and eventually takes its place during neonatal life. If the macrophages are present in this location for the purpose of disposing of effete self-components and they can also take up introduced foreign material, then it seems that a tangible link has been provided between physiological waste disposal and host defence mechanisms by the same system.

ACKNOWLEDGMENTS

This work was supported in part by a Nuffield Grant, by Grant No. AI 03226-05 from the United States Public Health Service and by a Grant from the National Heart Foundation of Australia.

We wish to thank Professor A. A. Abbie for the use of facilities in his Department, Dr C. R. Jenkin for his helpful advice and criticism and Dr B. G. Radden for his assistance in preparing the histological material.

REFERENCES

Biozzi, G., Benacerraf, B. and Halpern, B. N. (1953). 'Quantitative study of the granulopectic activity of the reticulo-endothelial system. II. A study of the kinetics of the granulopectic activity of the RES in relation to the dose of carbon injected. Relationship between the weight of the organs and their activity.' *Brit. J. exp. Path.*, **34**, 441.

Cappell, D. F. (1930). 'The late results of intravenous injection of colloidal iron.' *J. Path. Bact.*, **33**, 176.

Florey, H. W. (1961). *General Pathology*, 3rd edn. Lloyd-Luke, London.

Foot, N. C. (1921). 'Studies on endothelial reactions. V. The endothelium in the healing of aseptic wounds.' *J. exp. Med.*, **34**, 625.

Jezequel, A. M. (1962). 'Microscopie electronique du foie normal.' *Path. et Biol.*, **10**, 501.

Kupffer, C. (1878). 'Uber Sternzellen in der Leber.' *Arch. mikroskop. Anat.*, **12**, 352; cited by W. Mollendorff (1923) in *Handbuch der mikroskopischen Anatomie des Menschen*, p. 418. Springer, Berlin.

Kupffer, C. (1899). 'Uber die sog. Sternzellen der Saugetier-Leber.' *Arch. mikroskop. Anat.*, **54**, 254; (cited by W. Mollendorff, (1923) in *Handbuch der mikroskopischen Anatomie des Menschen*, p. 364. Springer, Berlin).

Parks, H. F. (1956). 'The hepatic sinusoidal endothelial cell and its histological relationships in electron microscopy.' *Proceedings of the Stockholm Conference* (Ed. by F. S. Sjostrand and J. Rhodin), p. 151. Almqvist & Wiksell, Stockholm.

Reade, P. C. and Jenkin, C. R. (1965). 'The functional development of the reticulo-endothelial system. I. The uptake of intravenously injected particles by foetal rats.' *Immunology*, **9**, 53.

Waddell, W. R., Geyer, R. D., Clarke, W. and Stare, F. J. (1954). 'Function of the reticulo-endothelial system in removal of emulsified fat from the blood.' *Amer. J. Physiol.*, **177**, 90.

Wardlaw, A. C. (1963). 'The complement bacteriolytic activity of normal human serum. II. Cell wall composition of sensitive and resistant strains.' *Canad. J. Microbiol.*, **9**, 41.