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The Functional Development of the Reticulo-Endothelial System

I. THE UPTAKE OF INTRAVENOUSLY INJECTED PARTICLES BY FOETAL RATS

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Summary. A study has been made of the uptake of various particles by the reticulo-endothelial system of foetal rats. It was shown that the rate of uptake varied both with the age of the foetus and with the particles used. This indicated that the phagocytic properties of this system develop progressively during foetal life.

INTRODUCTION

The reticulo-endothelial system of mammals has been described as an important host defence mechanism. Recent studies would suggest that the phagocytic cells of this system play an important role not only in discriminating between the host's 'wanted' and 'not wanted' components, but also in the production of antibodies (Fishman, 1961; Fishman and Adler, 1963; Nossal, Ada and Austin, 1964). A more complete understanding of the functional development of this system in the foetus might well provide some clue as to the role of the macrophage in antibody production in the adult animal. In this particular study the clearance and distribution of intravenously introduced particles has been used to investigate the phagocytic properties of the cells constituting the reticulo-endothelial system of foetal and natal rats.

MATERIALS AND METHODS

Bacterial strains

The strains of bacteria used in these studies were as follows: *Salmonella typhimurium* (C5 and M206), *Salmonella gallinarum* (9S), *Salmonella enteritidis* (Se795), a smooth strain of *Escherichia coli* (E2206), and two rough strains of *Escherichia coli* (Lilly and K12).

Maintenance of foetal rats in an external environment

A technique was required which would allow the maintenance of foetal rats in an external environment. For this purpose it was necessary to modify techniques that have been previously used for work on larger animals (Huggett, 1927).

An anaesthetic method was employed that required the supply of a mixture of O_2, N_2O and ether vapours by way of tracheal cannulation to the maternal animal. This mixture

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was delivered by an anaesthetic machine supplied by Commonwealth Industrial Gases, Torrensville, South Australia. The machine was fitted with flow meters and a Goldman Halothane vapourizer.

Following this anaesthetic procedure the pregnant animal was fixed to a rigid support and transferred to a bath of Ringer-Locke solution maintained at 37°. The rat was submerged in this bath to the lower border of its thoracic cage. The gravid bicornuate uterus was then exteriorized via an incision in the abdominal wall and rested on a submerged platform (Fig. 1). The foetal rats were then presented by dividing the uterine wall and the embryonic membranes and were arranged in the order of their attachment on the submerged platform (Fig. 2).

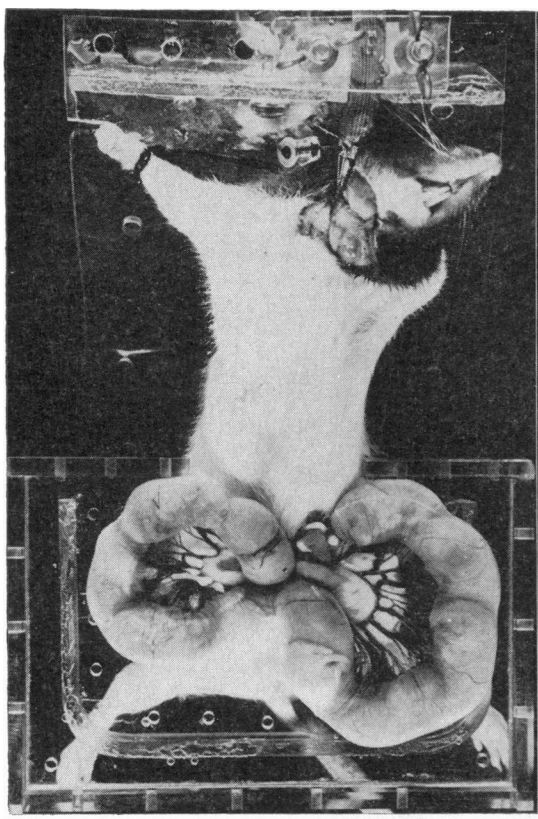

FIG. 1

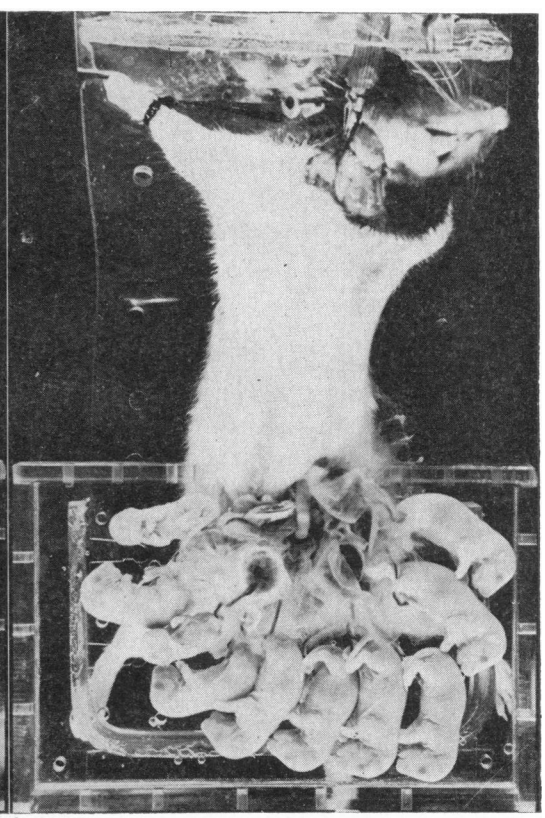

FIG. 2

FIG. 1. A pregnant rat with gravid uterus exteriorized and placed on a support in physiological saline at 37°.

FIG. 2. Foetal rats removed from the uterus and from the foetal membranes.

Intravenous injection was by way of the orbital branch of the anterior facial vein (Fig. 3), and was greatly facilitated by the use of a foot operated syringe to which was attached a length of fine polythene tubing cemented to a 30 gauge hypodermic needle. Blood (0·01 ml) was collected from the foetuses by cardiac aspiration using graduated glass pipettes to the end of which had been cemented 26 gauge needles (Fig. 4).

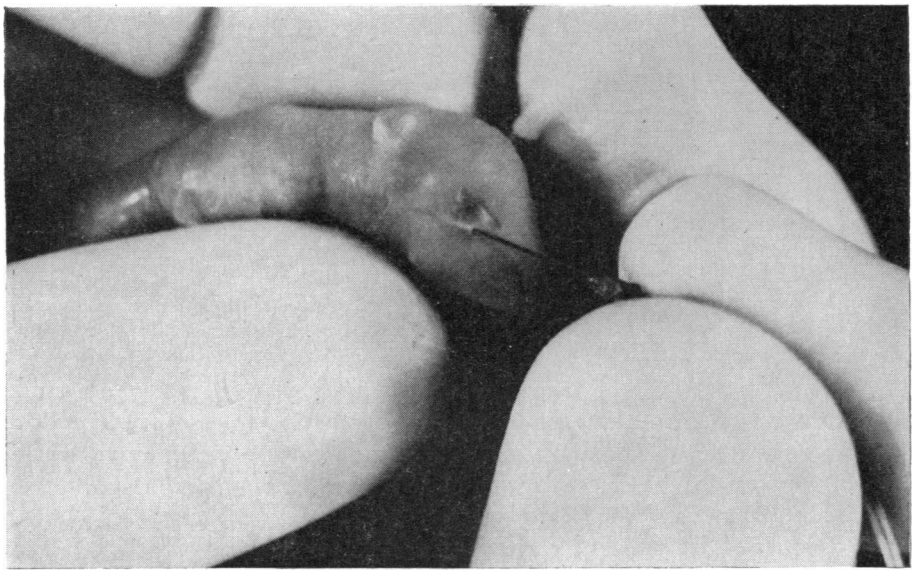

FIG. 3. Injection with a 30 gauge needle into the orbital branch of the anterior facial vein of a foetal rat.

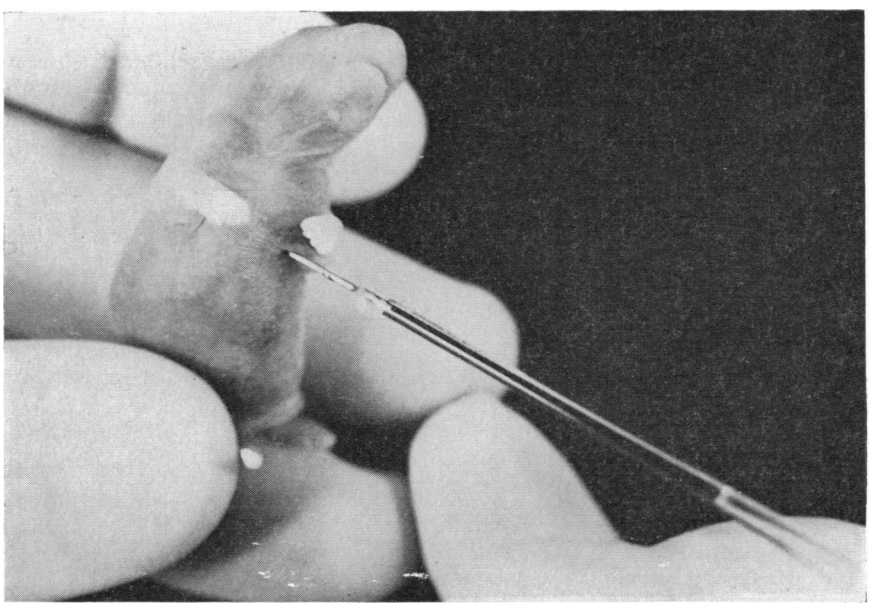

FIG. 4. Aspiration of blood by cardiac puncture from a foetal rat using a 26 gauge needle cemented to a 0·01 ml glass pipette.

In vivo *studies on the phagocytosis of particles*

Bacteria were labelled with ^{32}P and stored as previously described (Jenkin and Rowley, 1961). The foetal rats were prepared for injection according to the method described above. Natal rats were injected intravenously via the femoral vein. Following a dose dependence study in foetal rats it was decided to use a dose of 5×10^7 bacteria for foetuses of all ages

up to birth. After intravenous injection via the orbital branch of the facial vein, a blood sample (0·01 ml) was aspirated by cardiac puncture and pipetted onto stainless steel planchettes. The rate of elimination of the bacteria from the foetal circulation was followed over a period of 10 minutes, one foetus being used for each time period. Repeated bleedings from the same foetus was found to be an unsatisfactory procedure. The rate of elimination of bacteria was expressed as the phagocytic index K where

$$K = \frac{\log C_1 - \log C_2}{T_1 - T_2}$$

C_1 and C_2 being the concentration of bacteria at times T_1 and T_2. The corrected phagocytic index (α) was calculated according to Biozzi, Benacerraf and Halpern (1953). Natal rats were injected with 10^9 bacteria per 100 g weight. Repeated bleedings were taken from natal animals older than 14 days. The final phagocytic indices given in the tables and figures are average values obtained from at least twenty foetuses or natal animals at any one age. The clearance curve of bacteria in the foetus was exponential until approximately 70–80 per cent of the injected bacteria had been removed.

Carbon C11/1431a (Günther Wagner, Hanover, Germany) was injected at a dose of 8 mg/100 g body weight, and the samples treated as previously described (Biozzi *et al.*, 1953).

Estimation of ^{32}P label in various organs

Various organs were dissected from the injected animals and homogenized in a tissue grinder. The homogenates were digested with 10 per cent sodium hydroxide and an aliquot of the digest assayed for radioactivity. The total blood volume of the foetus could be calculated from a knowledge of the number of radioactive counts injected in a certain volume and the dilution factor obtained by extrapolating the clearance curve to zero time.

Opsonization of bacteria

Opsonization was carried out as previously reported (Karthigasu and Jenkin, 1963).

RESULTS

RATE OF ELIMINATION OF BACTERIA FROM THE CIRCULATION OF FOETAL AND NATAL RATS

Studies were carried out in greatest detail using *Salmonella typhimurium* C5 which is not virulent for adult rats. The results of these experiments are given in Fig. 5 where it may be seen that there is an increase in the rate of elimination of this organism up to birth followed by a plateau until the age of weaning and a further increase following this event. This increase in the rate of phagocytosis cannot be correlated with the increase in liver size, a point which will be discussed later and is illustrated in Table 1. The clearance of a number of other strains of bacteria in foetal rats was also investigated and it is apparent from these results (Table 2) that the reticulo-endothelial system shows with time an increasing capacity to phagocytose these particles. It is interesting to note that rough strains of bacteria in general were cleared more rapidly than smooth strains in the age groups studied. This difference in the rate of clearance between, for example, *Salmonella typhimurium* (C5) and *E. coli* (Lilly) cannot be accounted for by lack of opsonins since the rate of clearance of *S. typhimurium* (C5) in foetal rats following treatment with specific antibody gives a K value similar to that which one obtains using unopsonized bacteria.

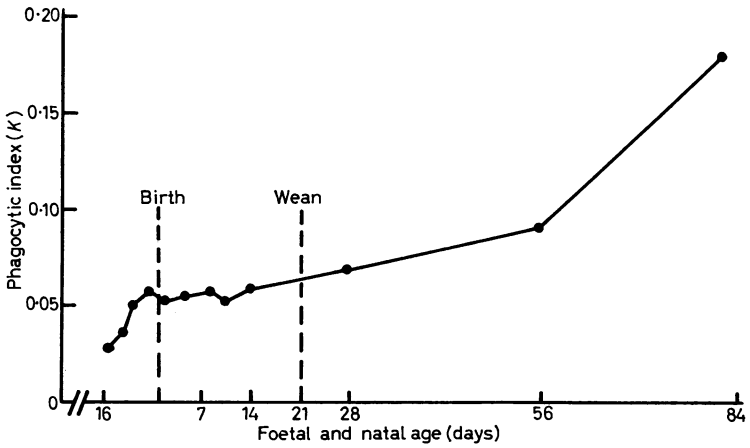

FIG. 5. The rate of clearance K of *Salmonella typhimurium* C5 from the circulation of foetal and natal rats.

	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

TABLE 1

THE PHAGOCYTIC INDICES (K AND α) DETERMINED IN RATS OF VARIOUS AGES USING *Salmonella typhimurium* C5

Age (days)	Dose (orgs.)	Weight (g)			K	α
		Body	Liver	Spleen		
Foetal						
16	5×10^7	1·12	0·11	—	0·026	3·0
18	5×10^7	2·55	0·23	0·001	0·034	3·6
20	5×10^7	3·31	0·33	0·002	0·052	3·7
22	5×10^7	4·85	0·41	0·007	0·055	4·4
Natal						
4	5×10^7	7·2	0·35	0·02	0·045	7·4
7	5×10^7	11·2	0·46	0·04	0·058	9·3
10	10^8	16·5	0·71	0·05	0·052	8·1
14	10^8	21·7	0·99	0·06	0·058	8·2
28	2×10^8	68·7	4·04	0·46	0·066	6·2
56	4×10^8	119·6	6·37	0·57	0·090	7·6
84	6×10^8	180·1	8·68	0·68	0·18	10·9

TABLE 2

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 Se795	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

PHAGOCYTOSIS OF CARBON BY FOETAL AND NATAL RATS

Foetal and natal rats were injected with colloidal carbon and the rate of elimination of this particle followed with time. It can be seen from the results given in Fig. 6 and Table 3, that the capacity of the reticulo-endothelial system of the foetal rat to ingest carbon increases with age of the embryo. Again this increased efficiency cannot be accounted for solely in terms of increased size of the liver.

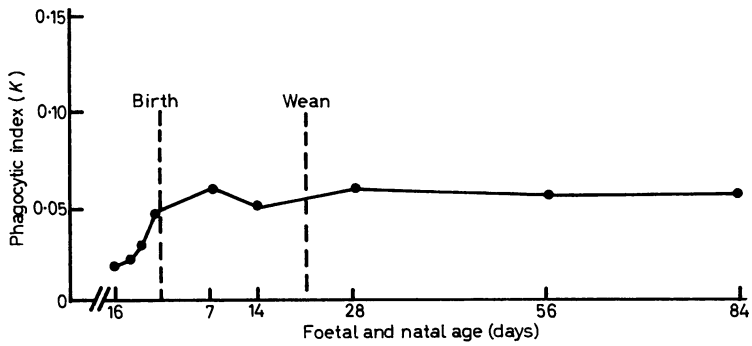

FIG. 6. The rate of clearance K of carbon from the circulation of foetal and natal rats. Foetal rats injected with a standard dose of 0·4 mg and natal rats a dose of 8 mg per 100 g body weight.

	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

TABLE 3

PHAGOCYTIC INDICES (K AND α) FOR FOETAL AND NATAL RATS AT VARIOUS AGES USING A DOSE OF 8 mg OF CARBON PER 100 g BODY WEIGHT IN NATAL ANIMALS AND A CONSTANT DOSE OF 0·4 mg FOR FOETUSES

Age (days)	Dose (mg)	Weight (g)			K	α
		Body	Liver	Spleen		
Foetal						
16	0·4	1·12	0·11	—	0·016	2·6
18	0·4	2·55	0·23	0·001	0·022	3·1
20	0·4	3·31	0·33	0·002	0·028	3·0
22	0·4	4·85	0·41	0·007	0·048	4·2
Natal						
7	0·9	11·2	0·40	0·02	0·058	9·3
14	0·8	21·7	0·99	0·04	0·051	7·8
28	5·4	68·7	4·04	0·46	0·062	6·1
56	9·6	119·6	6·37	0·57	0·056	6·5
84	14·4	180·1	8·64	0·68	0·056	7·4

DISTRIBUTION OF ^{32}P LABEL IN FOETAL RATS

From Table 4 it may be seen that the liver of the foetal rat accounts for the major portion of the ingested material. Unlike the chick (Karthigasu and Jenkin, 1963) no radioactivity was associated with the foetal membranes. The relatively high proportion of radioactivity associated with the placenta of 14-day foetuses seemed to be due to two

TABLE 4

PERCENTAGE DISTRIBUTION OF RADIOACTIVITY IN THE ORGANS OF DEVELOPING RAT FOETUSES

Time (minutes)	Liver			Placenta			Lung			Foetal carcass			Total recovery		
	14*	18	22	14	18	22	14	18	22	14	18	22	14	18	22
10	12	30	62	20	16	11	12	4	4	50	44	15	94	94	92
20	21	55	69	25	1	7	3	2	4	43	27	15	92	85	95
60	20	54	67	28	10	7	2	2	4	53	30	17	103	96	95
120	34	49	64	20	10	7	2	2	4	36	34	17	92	95	92

* Age in days.

possibilities, either mechanical trapping or contained blood, since little evidence for phagocytosis could be obtained by histological examination (Reade and Casley-Smith, 1965).

DISCUSSION

A quantitative study of the rates of phagocytosis of various bacteria and other particles in the foetal rat has shown that the phagocytes of the reticulo-endothelial system of this animal are functional in this regard at a very early age. That the particles were actually ingested by these cells was demonstrated histologically (Reade and Casley-Smith, 1965). It was found in most cases that rough strains of bacteria were phagocytosed more rapidly than smooth strains at all the ages studied, a similar observation having been made with chick embryos (Karthigasu and Jenkin, 1963). Whilst in the chick embryo the difference in the rate of clearance of these two phases, i.e. rough and smooth, could be accounted for by the presence of opsonins for one phase and not for the other, such an explanation in the case of the foetal rat was lacking. The rate of clearance of the smooth strains of bacteria never approached that observed with the rough strains, even if the former had been pre-treated with specific antibody. It is difficult to provide an explanation for these results unless one can postulate that in the presence of equivalent amounts of antibody rough strains of bacteria adhere more readily to the surface of macrophages than do smooth strains. However, as will be shown later (Reade, Turner and Jenkin, 1965), the clearance of bacteria was dependent on the presence of serum opsonins. It was also apparent that the efficiency of the reticulo-endothelial system as a phagocytic organ increased with increasing age for both bacteria and carbon particles. This increase in efficiency in foetal animals could not be accounted for solely by an increase in liver size. It would appear from histological examination of foetal livers that there is an increase in the numbers of phagocytic cells that is not dependent on the increase in mass of the liver. It is also likely that changes in the structure of the liver, leading to changes in the circulation during foetal development, also increase the chances of particle and cell contact, reflecting itself in an increasing efficiency. In agreement with the results described by Benacerraf (1958) the efficiency of the reticulo-endothelial system of the rat with respect to carbon and bacteria seemed to be most active in the first few days following birth. It is possible that at this time, perhaps owing to exposure to various bacterial antigens following birth, there is an accelerated proliferation of macrophage elements. It is interesting to note that in this regard Howard and Michie (1962) have commented on the great increase in the size of

the spleen of the newborn mouse during the first 6 days after birth and suggested by analogy with changes observed in the spleen of adult animals during antigenic stimulation that the rapid increase is due to intense antigenic stimulation. Comparable changes were observed in animals used in this study. Further evidence for a period of intense antigenic stimulation with bacterial antigens following birth is suggested by the work of Miler (1962) on the increasing susceptibility of newborn rats to bacterial endotoxin, since it is believed that this susceptible state may be related to progressive sensitization with bacterial antigens (Stetson, 1959).

This rapid immunological response of newborn animals to stimulation by the external environment appears to be an adaptation of a pre-existing phagocytic system to host defence. The implication of these observations in relation to the evolution of the immune response will be discussed in more detail later (Karthigasu, Reade and Jenkin, 1965).

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REFERENCES

BENACERRAF, B. (1958). 'Quantitative aspects of phagocytosis.' *Symposium on Liver Function.* Publ. 4, American Institute of Biological Sciences, Washington, D.C.

BIOZZI, G., BENACERRAF, B. and HALPERN, B. M. (1953). 'Quantitative study of granulopectic activity of the reticulo-endothelial system. II. A study of the kinetics of the granulopectic activity of the R.E.S. in relation to the dose of carbon injected.' *Brit. J. exp. Path.*, **34**, 441.

FISHMAN, M. (1961). 'Antibody formation *in vitro*.' *J. exp. Med.*, **114**, 837.

FISHMAN, M. and ADLER, F. L. (1963). 'Antibody formation *in vitro*. II. Antibody synthesis in X-irradiated recipients of diffusion chambers containing nucleic acid derived from macrophages incubated with antigen.' *J. exp. Med.*, **117**, 595.

HOWARD, J. G. and MICHIE, M. (1962). 'Induction of transplantation immunity in the newborn mouse.' *Transplant. Bull.*, **29**, 91/1.

HUGGETT, A. S. G. (1927). 'Foetal blood-gas tensions and gas transfusion through the placenta of the goat.' *J. Physiol.*, **82**, 373.

JENKIN, C. R. and ROWLEY, D. (1961). 'The role of opsonins in the clearance of living and inert particles by cells of the reticulo-endothelial system.' *J. exp. Med.*, **114**, 363.

KARTHIGASU, K. and JENKIN, C. R. (1963). 'The functional development of the reticulo-endothelial system of the chick embryo.' *Immunology*, **6**, 255.

KARTHIGASU, K., READE, P. C. and JENKIN, C. R. (1965). 'The functional development of the reticulo-endothelial system. III. The bactericidal capacity of fixed macrophages of foetal and neonatal chicks and rats.' *Immunology*, **9**, 67.

MILER, I. (1962). 'Changes in susceptibility to bacterial endotoxin and infection during the early post natal period in rats.' *Folia microbiol.*, **7**, 223.

NOSSAL, G. J. V., ADA, G. L. and AUSTIN, C. M. (1964). 'Antigens in immunity. IV. Cellular localisation of ^{125}I and ^{131}I labelled flagella in lymph-nodes.' *Aust. J. exp. Biol. med. Sci.*, **42**, 311.

READE, P. C. and CASLEY-SMITH, J. R. (1965). 'The functional development of the reticulo-endothelial system. II. The histology of blood clearance by the fixed macrophages of foetal rats.' *Immunology*, **9**, 61.

READE, P. C., TURNER, K. J. and JENKIN, C. R. (1965). 'The functional development of the reticulo-endothelial system. IV. Studies of serum opsonins to *Salmonella typhimurium* in foetal and natal rats.' *Immunology*, **9**, 75.

STETSON, C. A. (1959). 'Endotoxins and bacterial allergy.' In *Cellular and Humoral Aspects of the Hypersensitive State*, p. 422. Hoeber–Harper, New York.