STUDIES ON THE MECHANISM OF SHOCK

THE ACTIVITY OF THE RETICULO-ENDOTHELIAL SYSTEM AFTER LIMB ISCHAEMIA IN THE RAT

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INTEREST in the possible role of bacterial products in the response of the body to injury (Fine, 1961) has focused attention on the efficiency of the reticulo-endothelial system (RES) in the injured animal. Because of the importance of this system in the body's defence against both bacteria and their products, schemes in which these products play a significant part in the response to injury include a lowered efficiency of the RES after injury. The latter has been demonstrated after thermal injury in dogs (Warner and Dobson, 1954) and after haemorrhage in rats (Zweifach and Benacerraf, 1958) and dogs (Wiznitzer, Better, Rachlin, Atkins, Frank and Fine, 1960) and has been inferred after these and other injuries in other species. Artificial depression of the efficiency of the RES (" blockade ") increases the sensitivity of animals to such injuries (McKenna and Zweifach, 1956; Zweifach, Benacerraf and Thomas, 1957; Wise, Knecht, Pence and Ondash, 1959; Fine, Rutenburg and Schweinburg, 1959; Fine, Frank, Ravin, Rutenburg and Schweinburg, 1960). Stimulation of the RES, on the other hand, is accompanied by greater resistance to these injuries (Zweifach and Thomas, 1957; Zweifach *et al.*, 1957; Fine *et al.*, 1959; 1960).

The object of the present experiments was to extend this type of investigation to another form of injury (limb ischaemia) in the rat in order to see if the apparent connection between the activity of the RES and the response to injury is a general phenomenon. While maintaining that bacterial products are concerned in the response to haemorrhage in the rat (Friedman, Schweinburg, Yashar and Fine, 1957), Schweinburg and Fine (1960) do not think that they are involved in the rat's response to tourniquet injury which they consider to be a " reversible " form of shock. Their experiments were not a good test since the limb ischaemia used seems to have been a relatively mild injury with the rats surviving more than 18 hr. In the present work two more severe forms of limb ischaemia have been used which led in one case (4 hr. ischaemia) to a " reversible " form of shock and in the other (10 hr. ischaemia) to an " irreversible " form. An irreversible state of tourniquet shock can be defined as one in which death cannot be prevented by giving large volumes of 0·9 per cent NaCl. In both forms of shock the rat's ability to clear its blood of colloidal matter was found to be reduced and the experiments were continued in an attempt to assess the importance of this.

MATERIALS AND METHODS

Albino rats of the Porton strain (body wt. 240 $\pm$ 35 g. S.D.) fed on MRC diet 41b (Bruce and Parkes, 1956) were used. In experiments with a 4 hr. period of limb ischaemia food

was available until the start of the experiment. When a 10 hr. period was used food was removed from the cage on the afternoon before the experiment and coprophagy prevented. Water was freely available. Limb ischaemia was produced as in previous experiments by the method of Rosenthal (1943), a short ether anaesthetic being given for the application of the tourniquets. Control rats were given a similar short period of anaesthesia. Colon temperatures were taken with a mercury thermometer. The environmental temperature was 18–22°.

The following chemical preparations were used. Carbon was given as a colloidal suspension free from shellac (Pelikan Ink C11/1431a; Gunther Wagner, Hanover) as used by Biozzi, Benacerraf and Halpern (1953). For injection this preparation was centrifuged for 20 min. at 1070 *g* to remove any large aggregates and diluted with a 5 per cent (v/v) aqueous solution of polyvinylpyrrolidone (Polyvidone, May and Baker) so that 0·27 ml. of the final suspension contained 8 mg. carbon. Saccharated iron oxide was given as Ferrivenin (Benger Laboratories). This solution contains 20 mg. Fe per ml. and was injected very slowly. Thorium dioxide was given as a stabilized colloidal preparation of 24–26 per cent thorium dioxide in a 25 per cent aqueous dextrin solution (Thorotrast; Testagar, Detroit). Beryllium sulphate was given as an aqueous solution (0·25 mg. Be per ml.) neutralized to about pH 5. Yeast cell membranes were given in the form of zymosan (Zymosan, Type A; Standard Brands, Stamford, Connecticut). Zymosan was prepared for injection by making an 0·5 per cent (w/v) suspension in 0·9 per cent NaCl, heating for 10 min. in a boiling water bath and then exposing the suspension to ultra-sound in a MSE ultrasonic generator at 1·3 amps. The endotoxin preparation used was a lipopolysaccharide prepared from *Shigella dysenteriae* by Dr. D. A. L. Davies of the Microbiological Research Establishment, Porton.

Carbon clearance was determined by a modification of the method of Biozzi *et al.* (1953) on rats lightly anaesthetized with Na pentobarbitone (Veterinary Nembutal; Abbott). Carbon, 8 mg. per 100 g. body wt., was injected into a tail vein and the blood samples were withdrawn from an iliac vein by a needle inserted through the femoral vein. The exponential nature of the fall in the concentration of carbon in the blood was confirmed and routinely 2 samples (0·05 ml.) were taken from each rat. The samples were added to 4·0 ml. 0·1 per cent Na_2CO_3 and compared with suitable carbon standards in an Unicam spectrophotometer S.P. 500 at 675 mμ. Heparin was used as an anticoagulant. The "phagocytic index" (K) was calculated from the formula

$$K = \frac{\log C_{t_1} - \log C_{t_2}}{t_2 - t_1}$$

where C is the concentration of carbon in the blood (mg. per 100 ml.) at time t (min.) The "corrected phagocytic index" (α) was calculated from the formula

$$\alpha = \frac{W}{WLS} \cdot \sqrt[3]{K}$$

where W = body wt. (g.) and WLS = combined weight (g.) of liver and spleen (Biozzi *et al.*, 1953). The theoretical initial concentration of the carbon in the blood (C_{t_0}) was obtained by extrapolation.

Paraffin sections were prepared from tissues fixed in Helly's fluid and stained by routine methods. Blood for smears was collected without anticoagulant in polythene test-tubes after the rat had been guillotined and was transferred to the microscope slide with a polythene tube. The smears were stained with Leishman's stain.

Where possible results are expressed as the mean $\pm$ standard deviation (S.D.) and the means compared statistically by Student's t test as modified by Fisher (1934) for small samples. LD_{50}s were calculated by Weil's method (1952) using groups of 4 animals.

RESULTS

Many of the relevant physiological and metabolic changes following a 4 hr. period of bilateral hind-limb ischaemia in the rat have been described previously (Stoner, 1958*a*, *b*; Stoner and Threlfall, 1960). A 10 hr. period of ischaemia

leads to a more severe state of shock (Haist, 1960), the average survival time being reduced from about 13 hr. to about 4 hr. Whereas the intraperitoneal administration of 12 ml. 0·9 per cent NaCl per 100 g. body weight at the time of removing the tourniquets prevented the death of rats after 4 hr. limb ischaemia it only saved 30 per cent of those given a 10 hr. period.

Clearance of carbon from the blood after limb ischaemia

Shortly after a 4 hr. period of bilateral hind-limb ischaemia the phagocytic index (K) was strikingly reduced. This was accompanied by increases in the " corrected " phagocytic index (α) and in the ratio W/WLS (Table I). The

TABLE I.—*The Effect of Bilateral Hind-limb Ischaemia on the Clearance of Carbon from the Blood of the Rat*

Duration of ischaemia (hr.)	Interval after ischaemia (hr.)	Number of rats	K	α Mean ± S.D.	W/WLS
			Fed rats		
0	—	21	0·0421±0·0107	7·12±0·77	20·6±5·7
4	2·25–3·5	10	0·0263±0·0111‡	7·75±0·85*	26·8±2·0‡
4	5–7	5	0·0260±0·0046†	7·48±0·70	25·5±1·2‡
			Fasted rats		
0	—	5	0·0320±0·0079	7·30±0·39	23·2±2·2
10	0·67–1·83	3	0·0143±0·0017†	6·86±0·0017	28·3±1·1*

Dose of carbon = 8 mg. per 100 g. body wt. K = phagocytic index, α = " corrected " phagocytic index (see text), W = body wt., WLS = combined wt. of liver and spleen. Environmental temperature 18–22°.

* Significantly different from the control at $P < 0·05$.
† " " " " " " $P < 0·01$.
‡ " " " " " " $P < 0·001$.

change in the latter was due to a very obvious contraction of and expulsion of blood from the spleen, there being no change in the weight of the liver at this stage (Threlfall and Stoner, 1961). These changes were maintained for at least 7 hr. after removal of the tourniquets. Fasting overnight did not affect the phagocytic indices in the controls. A 10 hr. period of ischaemia in these rats led to a greater depression of K without significant change in α (Table I).

Although the intravenous injection of 8 mg. carbon per 100 g. body weight was never fatal in normal rats this dose of carbon sometimes caused death after limb ischaemia. Death was more likely to occur the longer the interval between removal of the tourniquets and the injection of the carbon. In these cases apnoea quickly occurred after the injection and death ensued from respiratory failure.

Distribution of injected carbon after limb ischaemia

In smears of blood taken between 5 and 60 min. after the injection of 8 mg. carbon per 100 g. body weight carbon was found within the platelets and in association with clumps of platelets as described by Stehbens and Florey (1960). In these respects there did not seem to be any significant difference between the injured and normal rats. Most of the carbon, however, was free in the plasma unassociated with cells.

The stability of the carbon suspension in the blood of normal animals has been shown by Stehbens and Florey (1960) and sections of tissues from animals given large doses of carbon (48 mg. per 100 g. body weight) showed that the aggregates formed by fixation were well-dispersed in the blood vessels (Fig. 1). Rats given 8 mg. carbon per 100 g. body weight were usually examined histologically 1 hr. after the injection when there were very few intravascular carbon granules. A different picture was seen after limb ischaemia. While there were very few circulating granules, carbon was found adhering to the endothelial lining of the vessels of the hind-limb (Fig. 2) and also as solid masses plugging some of the smaller ones. Both phenomena were most commonly seen in tissue which had been directly compressed by the tourniquet. Their incidence was reduced, but not eliminated, by giving 200 I.U. heparin per 100 g. body weight intravenously 15 min. before injecting the carbon. Felted masses of carbon were also found obstructing the smaller vessels of the lungs (Fig. 3*a*). In another part of this section carbon was seen incorporated into an antemortem thrombus in a pulmonary artery (Fig. 3*b*). Intravascular agglomerations of carbon were very rarely seen in the lungs of control rats after this dose although they were visible in injured rats killed 5 min. after the injection of carbon.

The histological appearance of the liver in rats killed 1 hr. after the intravenous injection of 8 mg. carbon per 100 g. body weight, given 1 hr. after 4 hr. bilateral hind-limb ischaemia, was compared with that of the liver of controls killed at 1 hr. and at 35 min. after this dose of carbon. The average concentration of carbon in the blood of the controls 35 min. after the injection was the same as that at 1 hr. in the injured rats. Although the amount of carbon in the livers of the control and injured rats appeared similar at low magnification (Fig. 4) its distribution was very different (Figs. 5 and 6). In the controls (Fig. 5) the carbon was almost entirely situated within the large Kupffer cells (type G, Lison and Smulders, 1948) both at 35 min. and at 1 hr. after its injection although at the earlier time a few granules appeared to be adherent to the walls of the sinusoids. In the livers from the experimental rats carbon was also found in these large Kupffer cells but they were not as well filled as in the controls. In addition

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EXPLANATION OF PLATES

FIG. 1—.Carbon particles in a large hepatic blood vessel of a rat 2 hr. after the intravenous injection of 48 mg. carbon per 100 g. per body wt. H. and E. ×260.

FIG. 2.—Carbon adherent to the endothelium of an artery and vein in the rectus femoris muscle of a rat given 8 mg. carbon per 100 g. body wt. intravenously 1 hr. after 4 hr. bilateral hind-limb ischaemia and killed 1 hr. later. H. and E. ×165.

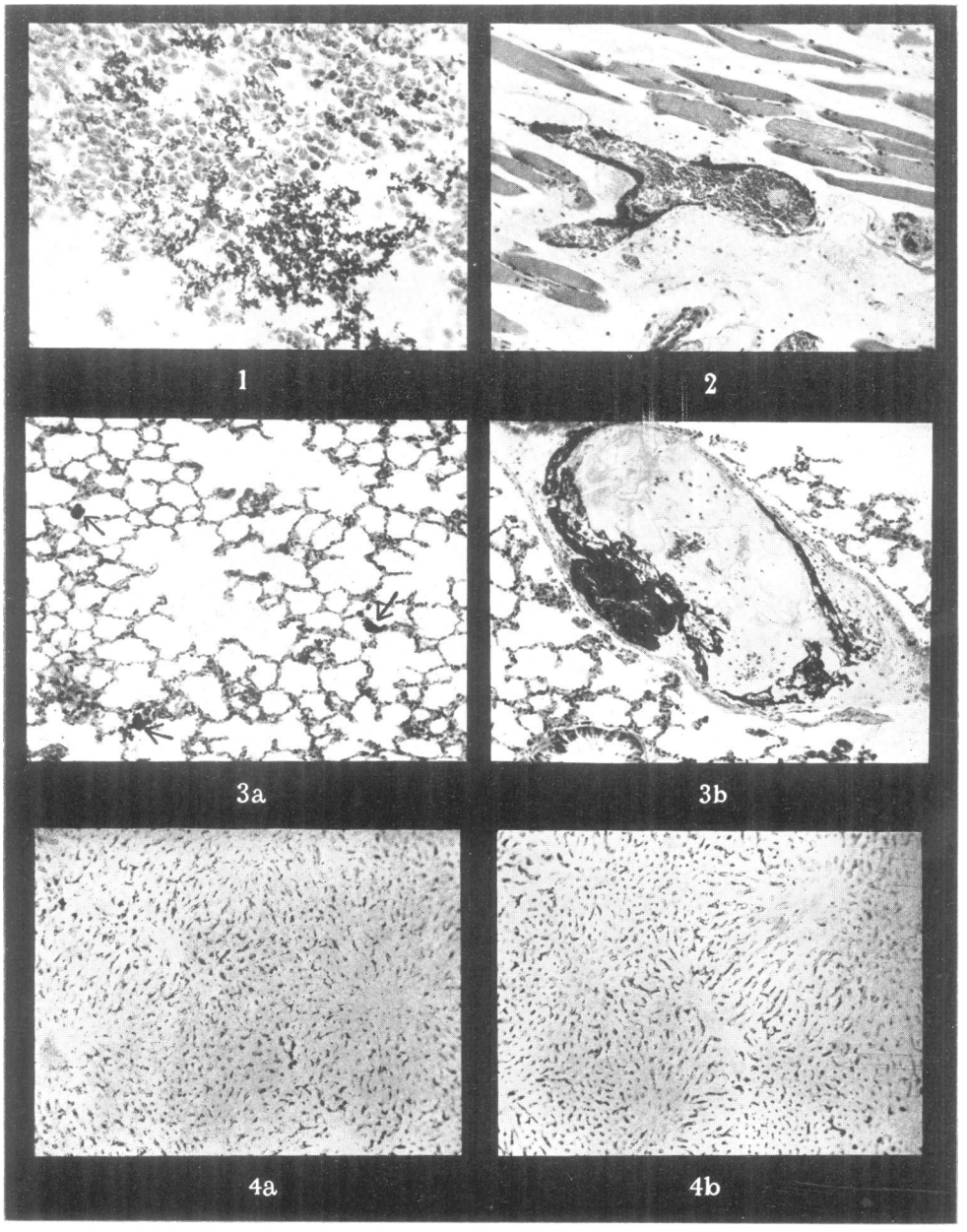
FIG. 3.—(*a*) Carbon masses, shown by arrows, occluding small vessels in the lung. (*b*) Carbon-containing thrombus in a pulmonary artery of the same lung. 8 mg. carbon per 100 g. body wt. injected intravenously 5 hr. after 4 hr. bilateral hind-limb ischaemia and the rat killed 1 hr. later. H. and E. ×165.

FIG. 4.—The distribution of carbon in the liver 1 hr. after the intravenous injection of 8 mg. carbon per 100 g. body wt. into (*a*) control rat; (*b*) rat 1 hr. after 4 hr. bilateral hind-limb ischaemia. Unstained. ×66.

FIG. 5.—Carbon in the liver of a control rat (*a*) 35 min. after and (*b*) 1 hr. after the intravenous injection of 8 mg. carbon per 100 g. body wt. H. and E. ×530.

FIG. 6.—(*a* and *b*) Carbon in the liver of rats injected intravenously with 8 mg. carbon per 100 g. body wt. 1 hr. after 4 hr. bilateral hind-limb ischaemia and killed 1 hr. later. H. and E. ×530.

FIG. 7.—The distribution of carbon in the spleen 1 hr. after the intravenous injection of 8 mg. carbon per 100 g. body wt. into (*a*) control rat; (*b*) rat 1 hr. after 4 hr. bilateral hind-limb ischaemia. Unstained. ×66.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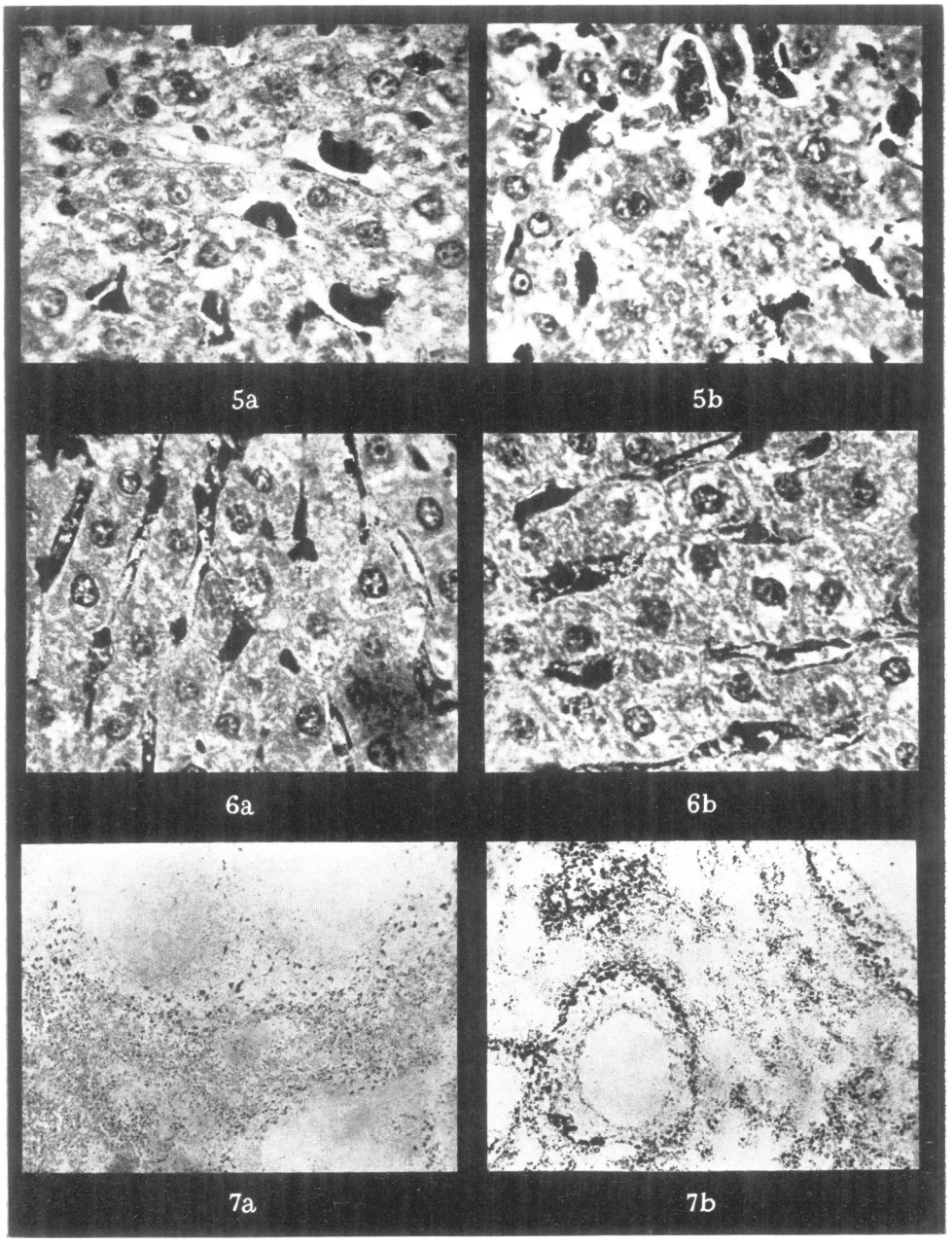

5a 5b

6a 6b

7a 7b

carbon was also found in the small littoral RE cells (type F, Lison and Smulders, 1948) and considerable amounts of it appeared to be extracellular, adherent to the walls of the sinusoids (Fig. 6). This was reduced but not eliminated by giving heparin as above before injecting the carbon.

Limb ischaemia had a similar effect on the intra-hepatic distribution of saccharated iron oxide (2 mg. Fe per 100 g. body weight) injected intravenously at the same time after removal of the tourniquets.

Despite these changes in the behaviour of the RE cells of the liver towards injected carbon after limb ischaemia it was not possible to detect any certain abnormality in their appearance in haematoxylin and eosin stained sections from injured rats not given carbon and killed at intervals up to the time of death.

The distribution of carbon in the spleens of control rats 1 hr. after the injection of 8 mg. carbon per 100 g. body weight is shown in Fig. 7*a*. The carbon was mainly massed in RE cells in the outer part of the shell of cells surrounding the Malpighian bodies and in cells scattered through the splenic pulp. Under a higher magnification the endothelium of capillaries in the outer part of the Malpighian systems was frequently seen to be studded with carbon granules. After limb ischaemia the spleen contracted and appeared to contain more carbon (Fig. 7*b*). The masses of carbon in the RE cells were larger but the carbon along the endothelial lining of the capillaries was not so obvious as the vessels were contracted.

For comparison the distribution of injected carbon was also observed in rats with the RES damaged by " blockading agents ". Outlining of the liver sinusoids was not a feature after single doses of 48 mg. carbon per 100 g. body weight or when 8 mg. carbon per 100 g. body weight was injected intravenously after " blockade " of the RES with Be. In the spleen after Thorotrast the carbon was displaced from its position around the Malpighian bodies by the " blockading " agent. After beryllium sulphate the appearance of the injected carbon in the spleen was similar to that after limb ischaemia probably because Be causes more damage to the RES of the liver (Cheng, 1956).

Sensitivity to endotoxin after limb ischaemia

The sensitivity of the normal rats to the endotoxin used was rather variable and an exact LD_{50} could not be determined but was about 300 μg. per 100 g. body weight by intravenous injection. An injection of 160 μg. per 100 g. body weight was never fatal and only caused a very slight transient illness.

The administration of Thorotrast (0·6 ml. per 100 g. body weight i.v.) to give RE " blockade " increased the sensitivity so that 2·5 μg. endotoxin per 100 g. body weight intravenously 4 hr. later was a certainly fatal dose. The 1000-fold increase in sensitivity after Thorotrast reported for rabbits by Good and Thomas (1952) was not consistently found in the rat. After carbon (48 mg. per 100 body-weight i.v.) or beryllium (0·025 mg. per 100 g. body weight i.v.) which also " blockade " the RES, the certainly lethal dose was 20 μg. per 100 g. body weight intravenously.

The effects of endotoxin were not altered by large parenteral doses of saline. In 2 groups of 6 rats given 400 μg. endotoxin per 100 g. body weight intravenously one group was also given 12 ml. 0·9 per cent NaCl per 100 g. body weight intraperitoneally 1 hr. later and 4 rats in each group died.

The survival of rats after 4 hr. bilateral hind-limb ischaemia varies from experiment to experiment despite attention to such factors as nutrition, time of day and environmental temperature (Stoner, 1961*b*). Nevertheless, when 2 groups of 8–12 rats are compared at the same time under the same conditions

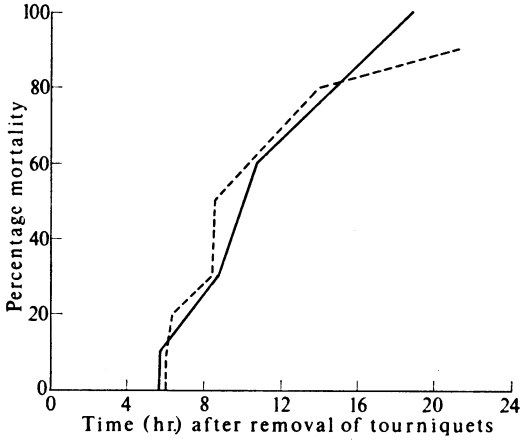

FIG. 8.—Mortality rate and survival time after 4 hr. bilateral hind-limb ischaemia in 2 groups of 10 rats. Environmental temperature 18°.

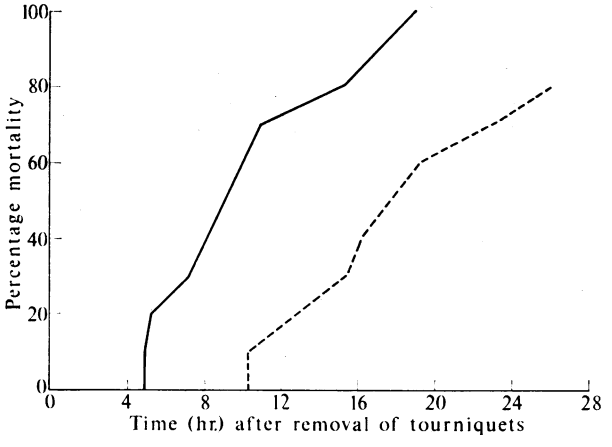

FIG. 9.—Mortality rate and survival time after 4 hr. bilateral hind-limb ischaemia in 2 groups of 10 rats, one group (————) being given 100 μg endotoxin per 100 g. body wt. intravenously 1 hr. after removal of the tourniquets. Environmental temperature 20°.

the 2 groups agree in their overall mortality rates, times to 50 per cent mortality and survival times (Fig. 8). Such groups can be used to test the effect of various treatments. Fig. 9 shows the shortening of the survival time after a 4 hr. period of bilateral hind-limb ischaemia by 100 μg. endotoxin per 100 g. body weight given intravenously 1 hr. after removal of the tourniquets. The threshold dose of endotoxin to produce a clinical effect after 4 hr. limb ischaemia was about 20

μg. per 100 g. body weight. Although rats after 4 hr. limb ischaemia were more sensitive to endotoxin, 100 μg. did not shorten the survival time to that commonly seen after 10 hr. bilateral hind-limb ischaemia nor did the rate of the fall in colon temperature during the first 3 hr. after its administration to them differ from that in a group of untreated rats after limb ischaemia.

Effect of " blockade " of the RES on the response to limb ischaemia

The compounds used to " blockade " the RES and their effects on its phagocytic activity are shown in Table II. The doses of Thorotrast, Ferrivenin and

TABLE II.—*The Effect of Compounds Causing " Blockade " of the RE System on the Clearance of Injected Carbon (8 mg. per 100 g. body weight) from the Blood*

Compound	Dose per 100 g. body wt.	Number of rats	K	a Mean ± S.D.	W/WLS
Thorotrast	0·6 ml.	3	0·0216±0·0020†	5·15±0·26‡	18·8±2·7
,, ,,	0·3 ml.	5	0·0210±0·0088‡	6·06±0·53†	22·8±1·5*
Ferrivenin	0·4 ml.	3	0·0197±0·0135†	5·48±1·20†	21·0±0·3
Carbon	48 mg.	3	0·0286±0·0052*	5·09±0·15‡	18·7±1·3
Beryllium	0·025 mg.	11	0·0199±0·0052‡	5·32±0·57‡	19·7±0·9
Controls	—	21	0·0421±0·0107	7·12±0·77	20·6±5·7

For the significance of K, a and W/WLS see text. The control rats are the same as in Table I.

* Significantly different from controls at $P < 0{\cdot}05$.
† ,, ,, ,, ,, ,, $P < 0{\cdot}01$.
‡ ,, ,, ,, ,, ,, $P < 0{\cdot}001$.

carbon used were of the order commonly advised for this purpose. They were given intravenously shortly before the tourniquets were applied so that the phagocytic activity of the RES would be depressed when they were removed after 4 hr. and would remain low for the next 16 hr. *i.e.* during the usual survival period.

After " blockade " of the RES by doses of Thorotrast (0·6 ml.), Ferrivenin and carbon which caused similar depressions of K and α, the mortality rate after 4 hr. bilateral hind-limb ischaemia was increased and the survival time shortened, the 3 compounds producing a similar effect (Fig. 10). This effect of Thorotrast was not reversed completely by giving 12 ml. 0·9 per cent NaCl per 100 g. body weight intraperitoneally when the tourniquets were removed although in the injured controls this therapy was completely effective, reducing the mortality rate from 80 per cent to zero (Fig. 11). A " reversible " form of shock was thus converted to an " irreversible " one by the blocking agent.

A 2 hr. period of bilateral hind-limb ischaemia at an environmental temperature of 18–22° is never fatal in normal rats. When 0·6 ml. Thorotrast per 100 g. body weight was given 4 hr. before the tourniquets were removed after a 2 hr. period of limb ischaemia 3 out of 6 rats died. In a similar experiment with Ferrivenin (0·4 ml. per 100 g. body weight) 7 out of 12 rats died and when the experiment was repeated with 48 mg. carbon per 100 g. body weight 3 out of 9 rats died.

Substances which damage the RES are naturally toxic. The LD_{50}s of Thorotrast and Ferrivenin were approximately 0·8 ml. per 100 g. body weight.

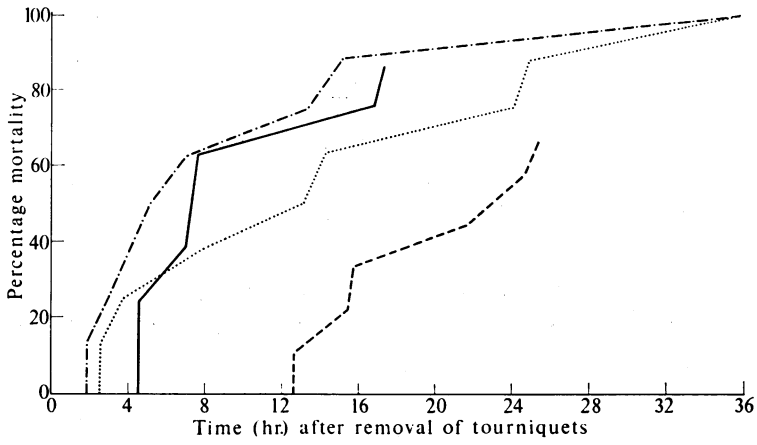

FIG. 10.—Mortality rate and survival time after 4 hr. bilateral hind-limb ischaemia in 4 groups of rats.

– – – – – – – – Controls (9 rats).
—.—.—. Pretreated with 0·6 ml. Thorotrast per 100 g. body wt. i.v. (8 rats).
———— Pretreated with 48 mg. carbon per 100 g. body wt. i.v. (8 rats).
. . . . . Pretreated with 0·4 ml. Ferrivenin per 100 g. body wt. i.v. (8 rats).
Environmental temperature 21°.

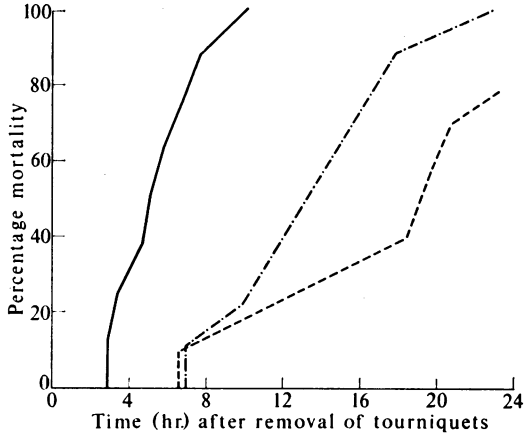

FIG. 11.—Mortality rate and survival time after 4 hr. bilateral hind-limb ischaemia in 4 groups of rats.

– – – – – – – – Controls (10 rats).
———— Pretreated with 0·6 ml. Thorotrast per 100 g. body wt. i.v. (9 rats).
—.—.—. Pretreated with 0·6 ml. Thorotrast per 100 g. body wt. i.v. and treated with 12 ml. 0·9 per cent NaCl per 100 g. body wt. i.p. at the time of removing the tourniquets (9 rats).

None of 4th group of 10 rats treated with 12 ml. 0·9 per cent NaCl per 100 g. body wt. i.p. at the time of removing the tourniquets died. Environmental temperature 19°.

The LD_{50} of the carbon suspension was not determined but according to Halpern, Benacerraf and Biozzi (1953) 48 mg. carbon per 100 g. body weight is the maximum single dose which can be tolerated by the rat. It is difficult to damage the Kupffer cells alone for after a time the toxic material they have engulfed diffuses out to the detriment of neighbouring parenchymal cells. In beryllium poisoning in the rat this process has been worked out by Cheng (1956). In this species particulate matter is most actively removed by Kupffer cells associated with the afferent terminal vessels (see Rappaport, Borowy, Lougheed and Lotto (1954) for terminology) and the subsequent changes produce the so-called " midzone " lesion. Although Thorotrast and Ferrivenin in the above doses caused only a transient

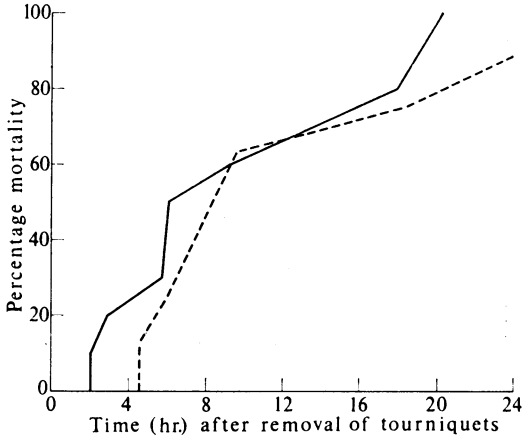

FIG. 12.—Mortality rate and survival time after 4 hr. bilateral hind-limb ischaemia in 2 groups of rats.
- - - - - - - - Controls (8 rats).
———— Pretreated with 0·3 ml. Thorotrast per 100 g. body wt. i.v. (10 rats).
Environmental temperature 20°.

outward disturbance in the rat small islets of hepatic necrosis were often found in this area 24–48 hr. later. The appearance and distribution of these lesions were very similar to those produced by endotoxin. Their size was very variable and they appeared reparable. After 48 mg. carbon per 100 g. body weight only occasional necrotic cells were seen near groups of Kupffer cells packed with carbon but carbon granules were also seen within most of the parenchymal cells of the liver.

When the dose of Thorotrast was reduced to 0·3 ml. per 100 g. body weight no parenchymal cell damage was seen during the subsequent 48 hr. This dose reduced K to the same level as 0·6 ml. but the fall in α was less (Table II). In this case the effect on the response to limb ischaemia was relatively slight (Fig. 12). A similar effect was observed in a number of experiments with this dose of Thorotrast.

To obtain a situation where the effect on the parenchymal cells was better defined, RE " blockade " was produced with beryllium sulphate. This has been used for this purpose in mice by Dr. G. E. Paget (personal communication). The dose used (0·025 mg. Be per 100 g. body weight) did not produce any

parenchymal cell damage as judged by the absence of necrotic cells and of regenerative mitoses 48 hr. after the injection. These changes were seen after 0·5 mg. Be per 100 g. body weight. Gross biochemical changes are, of course, required to produce structural damage to the liver cells and even then the histological changes lag behind the chemical ones (Majno, La Gattuta and Thompson, 1960). Nevertheless, experiments in this laboratory with much larger does of Be (Aldridge, Barnes and Denz, 1950 ; Cheng, 1956 ; Stoner, 1956) suggest that the parenchymal cells would be little affected by this dose during the 24 hr. following its injection. Although this dose did not visibly disturb the rats its

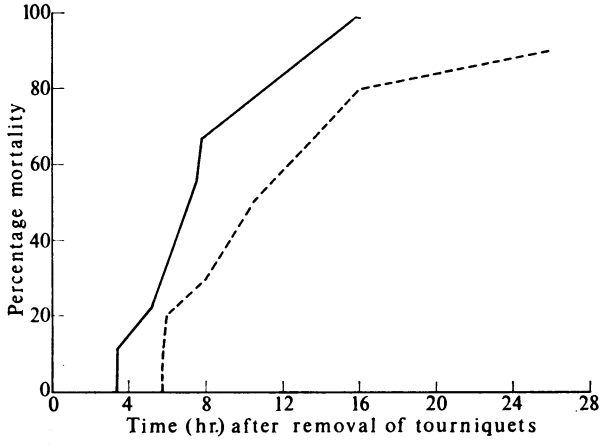

FIG. 13.—Mortality rate and survival time after 4 hr. bilateral hind-limb ischaemia in 2 groups of rats.

– – – – – – – Controls (10 rats).
——————— Pretreated with 0·025 mg. Be per 100 g. body wt. i.v. (9 rats).

effect on the RES in respect to carbon clearance is shown in Table II and its effect on the sensitivity to endotoxin has been mentioned above. Blockade of the RES by this dose of Be only moderately increased the effects of a 4 hr. period of bilateral hind-limb ischaemia (Fig. 13).

Effect of stimulation of the RES on the response to limb ischaemia

To increase the activity of the RES with minimal disturbance of other tissues, rats were given 3 daily doses of 1·0 mg. zymosan per 100 g. body weight and used 24 hr. later. Three rats treated in this way had values for K, α and W/WLS of 0·0706 $\pm$ 0·0044, 7·2 $\pm$ 2·5 and 17·5 $\pm$ 0·9 respectively when tested with intravenous doses of 8·0 mg. carbon per 100 g. body weight. The greater activity of the RES after zymosan can lead to anaemia through the increased phagocytosis of erythrocytes (Gorstein and Benacerraf, 1960). Twenty-four hours after 3 daily doses of zymosan the haematocrit was 36 $\pm$ 2 (3 rats) without change in the total blood volume calculated from the C_{t_0} obtained in the carbon clearance experiments. The haematocrit in 13 normal rats was 42 $\pm$ 3 so that there was a significant ($p<0·01$) loss of erythrocytes but it is doubtful if this reduction would seriously impair the oxygen carrying capacity of the blood. Mice repeatedly

injected with zymosan have an increased sensitivity to endotoxin (Benacerraf, Thorbecke and Jacoby, 1959) but this change had not occurred in the rats used for they still tolerated the intravenous injection of 160 μg. endotoxin per 100 g. body weight.

Increasing the activity of the RES in this way had no effect on the response to either 4 or 10 hr. periods of bilateral hind-limb ischaemia.

DISCUSSION

From the results of the carbon clearance experiments it would seem that the phagocytic ability of the RES was depressed after limb ischaemia as after other forms of injury. Comparing these results with those of Zweifach and Benacerraf (1958) in haemorrhagic shock in the rat it is seen that, although the values of K were different, the percentage reductions in " reversible " and " irreversible " haemorrhagic shock were similar to those after 4 and 10 hr. limb ischaemia respectively which could also be said to lead to " reversible " and " irreversible " forms of shock.

However, before a significant depression of the cellular activity of the RES after limb ischaemia can be accepted some other points must be considered.

It is difficult to attach a biological meaning to the expression $W/WLS. \sqrt[3]{K}$ and it will have been observed that the changes in α after limb ischaemia differed from those after the administration of " blocking " agents (compare Tables I and II). This was due to the different behaviour of the spleen in the two conditions. After limb ischaemia it contracted so that, at least after 4 hr. limb ischaemia, the increase in W/WLS more than compensated for the fall in K. This perhaps illustrates some of the artificiality of α.

The phagocytosis of particles by the RES is dependent upon a number of factors a change in which could alter the clearance of carbon without necessarily implying any intrinsic alteration in the RE cells. Among such factors are the blood flow through the RE tissues and the body temperature (Benacerraf, Biozzi, Halpern and Stiffel, 1957 ; Dobson, 1957). The dose of carbon was above the level at which its clearance is determined by the liver blood flow but in the injured rat the failing blood flow to the RE tissues will gradually cease to be compensated by the increased efficiency of extraction from the slower flow and will eventually become a limiting factor. As it has been shown that the liver blood flow after 4 hr. limb ischaemia is maintained within the normal range for some hours (Stoner, 1958*a*) it is doubtful if this would play a significant part at the times studied. It could be more important after the 10 hr. period of ischaemia but data on the blood flow changes under these conditions are not available.

Carbon clearance is affected by changes in body temperature (Halpern, Dick, Biozzi and Mené, 1951) and in experiments on the isolated rat liver with $Cr^{32}PO_4$ Brauer, Holloway and Leong (1957) found that the Q_{10} for the overall reaction of colloid uptake was 1·92. From this it can be calculated that for the results to be explained by the lowered liver temperature after injury it would have to be 30° in the rats studied after 4 hr. hind-limb ischaemia and 21° in those after the 10 hr. period. In the former this temperature would not be reached until 8–10 hr. after removal of the tourniquets (Stoner, 1958*a*) and in the latter the colon temperature at the time of testing had not fallen below 30°.

A further possibility arises from the peculiar kinetics for the clearance of

injected carbon for not only does the carbon concentration in the blood decline exponentially but K × dose = constant, *i.e.* the larger the dose the slower its rate of removal. In the injured rat the effective dose of carbon is increased by the fall in blood volume. The effect of this is shown in Table III and it is seen that this factor alone could not account for the changes observed.

TABLE III.—*The Effect of Alteration in Blood Volume on Carbon Clearance from the Blood*

Controls : Mean initial concentration (C_{t_0} = 114·3 mg. carbon per 100 ml. blood.
Dose (D) = 8 mg. carbon per 100 g. body wt.
Blood volume $\left(\frac{100 \cdot D}{C_{t_0}}\right)$ = 7·0 ml. per 100 g. body wt.
$K \cdot D$ = 0·3368 (fed) ; 0·256 (fasted).

Experimental	Mean C_{t_0} mg. carbon per 100 ml. blood	Equivalent dose (D') of carbon for blood volume of controls	K Calculated $\left(\frac{K \cdot D}{D'}\right)$	K Observed
4 hr. limb ischaemia :				
2·25–3·5 hr. after	124·0	8·68	0·0388	0·0263
5–7 hr. after	126·2	8·83	0·0381	0·0260
10 hr. limb ischaemia :				
0·67–1·83 hr. after	140·3	10·0	0·0256	0·0143

Another point emerged from this exercise. The blood volume of the injured rat can be calculated either from C_{t_0} and corrected for the volume of the injection assuming that it remained in the circulation during the test or from previous data on the haematocrit levels in these rats (Stoner, 1958*b* ; Stoner, Heath and Collins, 1960) assuming that the erythrocyte mass was unchanged. The values obtained by the 2 methods differed, those by the first method being higher (Table IV). This could imply that a fraction of the injected carbon, up to 24 per cent, was rapidly removed from the circulation and did not enter into the determination of carbon clearance. An indication of the fate of this " lost " carbon is given by the histological studies.

TABLE IV.—*Effect of Limb Ischaemia on Blood Volume.*

	Blood volume (ml. per 100 g. body wt.)		
	From C_{t_0} corrected for volume of injection	From haematocrit.	Difference per cent
Control	6·7	—	—
4 hr. limb ischaemia :			
2·25–3·5 hr. after	6·2	5·0	19·4
5–7 hr. after	6·1	4·7	22·9
10 hr. limb ischaemia :			
0·67–1·83 hr. after	5·4	4·3	20

The distribution of the carbon in the tissues of the injured rats differed both from that in the controls and that in rats in haemorrhagic shock (Zweifach and Benacerraf, 1958). Their illustrations show that after severe haemorrhage the uptake of carbon in the liver was restricted to Kupffer cells related to the afferent terminal vessels. Under their conditions the other Kupffer cells seem to have been damaged in the hypotensive episode. After limb ischaemia Kupffer cells throughout the liver contained carbon although in reduced amounts. The distinguishing feature after limb ischaemia was the large amount of carbon associated with the walls of liver sinusoids and of blood vessels in the lungs and elsewhere. This was not observed after haemorrhage by Zweifach and Benacerraf (1958). By light microscopy much of this carbon appeared extracellular but electron microscope studies would be required to decide its precise location. This is thought to be the " lost " carbon mentioned above.

This appearance was not due to precipitation of circulating carbon in fixation since there was very little circulating at the time and it was not seen in controls killed when the concentration of carbon in their blood was the same as in the injured rats (Fig. 5*a*). The effect was not due to the overloading of the rat with carbon. Benacerraf, McCluskey and Patras (1959) have described the attachment of carbon to the vascular endothelium in mice which were either given very large amounts of carbon or injected with carbon after the RES had been extensively damaged with " blocking " agents. Their results suggest that in the present experiments the dose of carbon would be too low and the depression of the RES too slight to give overloading. The effect was not seen in the rats pretreated with Be. Benacerraf *et al.* (1959) have also shown how this appearance can be provoked by giving adrenaline before the carbon. They attributed this to a change in the coagulability of the blood. The appearance of the carbon masses particularly in the lungs of the injured rats certainly suggested a thromboembolic process. Hyperadrenalinaemia was almost certainly present in these rats and it has also been shown that extracts prepared from the ischaemic hind-limb muscles have increased clot-promoting activity (Quastel and Racker, 1941 ; Stoner and Green, 1947, 1951). The deaths which occurred among the injured rats shortly after the injection of carbon are thought to have been due to pulmonary embolism. Their manner of death was the same as that described by Green and Stoner (1947) and rats are known to be very sensitive to the lethal action of pulmonary emboli after limb ischaemia (Green and Stoner, 1950 ; Whiteley, 1954). Limb ischaemia appears to reduce the stability of the colloidal suspension of carbon in the blood as do various clotting factors (Halpern *et al.*, 1953).

From the work of Warner and Dobson (1954) it was reasonable to find apparently more carbon than normal in the spleens of the injured rats. However, this may, in part, be an optical illusion due to the contraction of the spleen. Attempts to determine the percentage of the injected dose in the spleen gravimetrically by the method of Halpern, Biozzi, Mené and Benacerraf (1951) were unsuccessful. The apparent recovery of carbon from the liver, spleen and lungs always exceeded the amount injected and no way of further purifying the extracted carbon was found. This is hardly surprising in view of the adsorptive properties of finely divided carbon. Consequently the carbon in the spleen could not be determined. However, a more important point was that after limb ischaemia the carbon was not displaced from the cells around the Malpighian bodies.

Since carbon attached to vascular endothelium is effectively cleared from the circulation the observed values of K may overestimate the phagocytic ability of the RES after limb ischaemia. The results support the recent criticism of this method by Stehbens and Florey (1960). In a pathological situation of this type it only gives qualitative information and might even be misleading.

A precise explanation of the slower removal of colloid from the blood after limb ischaemia cannot be given. A number of factors have been shown to be incapable of producing the effect single-handed but it is difficult to assess their combined action and so determine the influence of limb ischaemia on the functional ability of the RE cells themselves. Interference with the latter could arise through their metabolic derangement or through their saturation by phagocytosed material and no conclusion can be reached on this.

However, from the practical standpoint the fact remains that after limb ischaemia the rat is not able to remove foreign colloidal material from its blood stream at the normal rate and such material could then be expected to exert an enhanced effect. From the point of view of the endotoxin theory of shock there seems little reason for isolating tourniquet shock in the rat from haemorrhagic shock in that species or tourniquet shock in other species (Schweinburg and Fine, 1960). However, the importance of such material in the response seems small. Further depression of the RE activity increased the response but the effect was relatively slight when parenchymal liver damage was avoided. The hepatotoxic action of the agents used to " blockade " the RES has attracted little attention from their users but doses of Thorotrast which do not cause visible damage produce biochemical changes (Fisher and Fisher, 1961). Even if the effect observed in the experiment of Fig. 13 was not caused by undetected liver damage it need not be due to the action of bacterial products but could represent failure to remove embolic blood clot or fat to which these rats are sensitive. Whatever the nature of the material concerned the normal role of the RES under these conditions would seem relatively unimportant since increasing its phagocytic capacity had no effect. Further experiments are planned to see if the depression of RE function after limb ischaemia assumes greater importance under other experimental conditions as, for instance, when the intestinal flora is altered by dietary changes. While the experiments described in this paper do not investigate the endotoxin theory directly their results do not offer much support for it.

SUMMARY

The effect of hind-limb ischaemia on the functional activity of the reticulo-endothelial system has been studied and an attempt made to assess its importance in the response to this injury in the rat.

As judged by the ability of the rat to clear its blood stream of injected colloidal carbon the phagocytic activity of the reticulo-endothelial system was depressed by hind-limb ischaemia. The degree of depression could not be assessed accurately. In the normal rat the injected colloid remained stable in the blood stream but after limb ischaemia a significant fraction of the dose rapidly became attached to the walls of liver sinusoids and blood vessels in the hind-limbs and obstructed blood vessels in the lungs.

In confirmation of this reduced activity of the reticulo-endothelial system

doses of bacterial endotoxin without effect in normal rats shortened the survival time after limb ischaemia.

" Blockade " of the reticulo-endothelial system by a number of agents also shortened the survival time after limb ischaemia but the effect was not gross if damage to the parenchymal cells of the liver was avoided.

Increasing the phagocytic ability of the reticulo-endothelial system had no effect on the survival time after limb ischaemia.

It is concluded that under the experimental conditions used the reticulo-endothelial system did not play a very important part in the rat's response to limb ischaemia. The value of the carbon clearance method for assessing reticulo-endothelial activity in pathological conditions is also discussed.

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