A QUANTITATIVE STUDY OF THE KINETICS OF BLOOD CLEARANCE OF P³²-LABELLED ESCHERICHIA COLI AND STAPHYLOCOCCI BY THE RETICULO-ENDOTHELIAL SYSTEM*

By B. BENACERRAF, M.D., MARTHA M. SEBESTYEN, M.D., AND STUART SCHLOSSMAN, M.D.

(*From the Pathology Department, New York University–Bellevue Medical Center, New York*)

The fate of bacteria injected intravenously and the mechanism of their disappearance from the circulation have been studied by numerous investigators in various animal species. Many observations have established that:

(*a*) Bacteria can be cleared from the blood very rapidly in very large numbers (1–4, 6–8),

(*b*) The reticulo-endothelial system (RES), of the liver and spleen principally, is largely responsible for removing the bacteria from the blood (1, 3, 4, 6, 8, 9),

(*c*) The efficiency of clearance of some bacteria by the portal circulation can be high in specific instances, in the order of 40 per cent for *Klebsiella* in the dog (4) or 60 per cent for *Micrococcus aureus* in the rabbit (10); attempts to block the RES with colloidal particles, such as thorotrast, have resulted in significant decreases in the efficiency of removal of circulating bacteria (8, 10).

(*d*) The rapid clearance of injected bacteria is followed, in the case of virulent organism, by a second phase when the blood concentration of the microorganisms increases and the animal eventually dies (2, 9).

(*e*) Previous immunization of the animals increases greatly the ability of the RES to extract both avirulent and virulent bacteria from the blood and improves its ability to kill the phagocytized organisms (1–4, 6, 9),

(*f*) The circulating leucocytes do not seem to play an important protective role in the clearance of bacteria from the blood (11, 7) except in cases in which bacteria are enmeshed in fibrin clots or adhere to the endothelium of capillaries (12, 13) and hence can be removed by surface phagocytosis.

The role played by the RES in the control of bacteriemia is therefore of primary importance.

In the past few years, much has been learned about the phagocytic activity of the RES from experiments dealing with the kinetics of blood clearance of standardized suspensions of carbon from the blood by various organs of the RES (14). When carbon particles of homogeneous size, about 250 A are injected intravenously, nearly

90 per cent of the injected material is removed by phagocytes of the liver and spleen and the rate of clearance follows an exponential function of the time $C = C_0 \, 10^{-Kt}$ (15). When relatively small amounts of such avidly phagocytized colloids are injected, the clearance is very rapid and the maximal efficiency of removal by the Kupffer cells is of the order of 80 to 90 per cent (16). As larger amounts are injected, one reaches a critical dose of particles above which the rate of clearance K becomes "dose-dependent" ($K \times D =$ constant), reflecting first the decreasing efficiency of the phagocytes to clear the blood of larger concentrations of colloids and then the saturating effect of phagocytized particles on the RES (15, 16). In this range of dosage above the critical concentration, the colloid clearance test may be used to measure the phagocytic capacity of the RES. This test was the basis for many studies of the phagocytic activity of the RES in experimental infections (17–19). It was shown that several infectious processes are associated with a considerable increase in the phagocytic capacity of the RES, at least in an early phase; later, in the case of virulent organisms or high infecting doses, the phagocytic function appears depressed. Other experiments have also shown that the rate of clearance of carbon by the RES is greatly increased in animals treated with agents which improve natural resistance to infections, such as endotoxins from Gram-negative bacteria (20–23), zymosan (21), or B.C.G. infection (18, 24).

Since the behavior of the RES towards colloidal carbon may not necessarily be extended to bacterial suspensions, it was felt that some useful data could be obtained from a quantitative study of bacterial clearance from the blood (*Staphylococcus aureus, Escherichia coli*), with the same techniques and approaches which had been used for the study of phagocytosis of colloidal particles.

Materials and Methods

Animals.—The experiments were carried out on white male Swiss Webster mice weighing 20 to 30 gm., guinea pigs weighing 400 to 600 gm., and rabbits weighing about 2 kg.

Bacteria.—*E. coli* 0111 B_4[1] and *Staphylococcus aureus*, Giorgio strain[2]. The bacteria were grown on a medium prepared as follows:

    Na₃ citrate 3H₂O   0.1 gm.
    MgSO₄ 7H₂O   0.02 gm.
    Glucose   0.4 gm.
    Casein amino acids[3] 2.0 gm.
    P³² phosphate   1 mc.
    Distilled water to make a total of 200 ml.

E. coli cultures were grown for 24 or 48 hours. The slower growing staphylococci were allowed to grow for 3 to 4 days. In preliminary experiments in mice with live *E. coli*, it was established that the clearances were similar for live and heat-killed (60°C.) bacteria. In all subsequent experiments, killed bacteria were used. *E. coli* or staphylococci were killed by

---

[1] Provided kindly by the Difco Laboratories, Detroit.

[2] From Dr. Dubos' Laboratory, The Rockefeller Institute, kindly provided by Dr. Boehme.

[3] Mixed amino acids from enzymatic hydrolysis of casein N-Z amine (type B), Sheffield Farms Co., Inc., New York.

heating at 60°C. for 20 minutes. They were washed repeatedly with 0.9 per cent NaCl until the radioactivity of the supernatant was negligible and finally suspended in the saline solution. The number of *E. coli* per ml. was calculated from optic density measurement at 490 mμ, after calibration with a known suspension on which the bacterial count was carried out. In the case of staphylococci, the number of bacteria was not counted, but suspensions were prepared for injection which showed similar degree of optical density as the *E. coli* suspensions in order to inject comparable numbers of *E. coli* or staphylococci. The bacterial preparation kept at refrigerator temperature (4°C.) were stable and could be used reliably to measure blood clearance of bacteria over a period of 4 to 6 weeks until decay of the isotope rendered measurements inaccurate. Successive bacterial preparations gave identical results.

Measurement of Blood Clearance of P^{32}-Labelled Bacteria.—The animals were injected with heparin intravenously 10 minutes before the bacterial suspension to avoid any possibility of intravascular clotting or fibrin deposition on the bacteria modifying the kinetics of clearance by the RES. This precaution was observed although, in early experiments, no significant differences were observed in the blood clearances of *E. coli* or staphylococci in normal or heparinized mice. Mice received 0.1 ml.; guinea pigs 0.25 ml., and rabbits 0.5 ml. of a solution of heparin containing 1000 U.S.P. units per ml. The blood clearance of *E. coli* was investigated in mice, guinea pigs, and rabbits in the following dose range: mice 2×10^8 to 5×10^9 bacteria, guinea pigs 2.5×10^8 to 1.5×10^9 bacteria, rabbits 1.5×10^8 to 6×10^9 bacteria, per 100 gm. of body weight. The blood clearance of staphylococci was only investigated in mice, using doses of bacteria which were equivalent with respect to turbidity to 4×10^8 and 2×10^9 *E. coli* per 100 gm. of body weight, in the range in which most of the experiments were carried out with this microorganism.

The rate of clearance of P^{32}-labelled bacteria from the blood was measured by drawing, at various times, 0.05 ml. of blood with a calibrated glass pipette from the retroorbital venous plexus (25) in mice and guinea pigs and from the cut artery of the ear in rabbits. The blood was then spread on circles of filter paper about 2.5 cm. in diameter, cemented on glass slides, and the radioactivity measured with a Geiger-Müller counter keeping the geometry constant. The radioactivity of the injected suspension of bacteria was measured in the same way. The logarithms of the counts per minute in the blood samples were calculated and plotted with respect to time. The radioactivity of the blood decreases according to an exponential function of the time down to about 10 per cent of the amount injected or sometimes less, and then the rate of clearance generally begins to slow down irrespective of the dose injected. A straight line can be drawn through the points down to such a concentration (Figs. 1 and 2) and, as in the case of the clearance of colloidal particles by the RES, its slope K which measures the rate of clearance can be calculated from the equation: $\dfrac{\text{Log } C_1 - \text{Log } C_2}{T_2 - T_1} = K$ (phagocytic index), in which C_1 and C_2 are the radioactivity of the blood at the times T_1 and T_2.

Measurement of Radioactivity in the Organs.—The radioactivity in the organs of mice and guinea pigs injected with P^{32}-labelled bacteria was measured. The animals were sacrificed by decapitation when the radioactivity in the blood had fallen to 10 to 15 per cent of the injected amount. The liver, spleen, lungs, and kidneys were digested in 10 per cent NaOH in a water bath and the radioactivity measured by taking 0.1 ml. aliquot samples and spreading them on filter paper as above. The amount recovered in each organ was expressed as per cent of radioactivity injected. In a few mice which received 10^9 *E. coli* per 100 gm. of body weight, histoautoradiography of the liver were done which showed the radioactivity to be in the Kupffer cells.

Measurement of Agglutination Titer of Sera.—Measurements were made of agglutination titers against *E. coli*, of sera from normal mice, guinea pigs, and rabbits, and of sera from

immunized animals and from animals treated with bacterial endotoxins. Agglutination titers against staphylococci in normal mice sera were also measured. Serial double dilutions were prepared of the sera using a volume of 0.4 ml. per tube. To each tube was added an equal volume of a suspension containing 2×10^9 E. coli/ml. of the same heat-killed bacteria used in the clearance studies. The staphylococcal suspensions used were standardized to the same optical density as the E. coli suspension. The tubes were incubated at 55°C. for 2 hours and then left overnight at 4°C. before reading the agglutination titer.

Preparation of Immune Sera.—Rabbits and mice were immunized with the lipopolysaccharide from E. coli 0111 B₄ prepared by the Difco Laboratories. Rabbits were injected intravenously every day with increasing amounts of lipopolysaccharide for 10 successive days
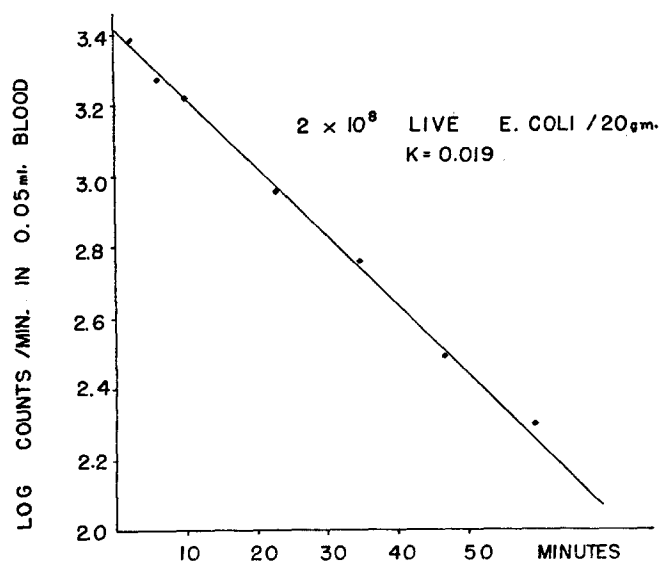
FIG. 1. Blood clearance of 10^9 P³²-labelled live E. coli per 100 gm. of body weight in a normal mouse.

starting with 5 μg. up to 100 μg., then once a week for 4 weeks with 100 μg. The animals were bled a week after the last injection. Two groups of 10 mice were immunized with the same lipopolysaccharide; each animal received 10 μg., 48 hours later 100 μg., and 100 μg. twice a week for 3 weeks, by intraperitoneal injections. The mice were then bled and the sera pooled. A strong rabbit antiserum and the pooled mouse antiserum were analyzed for anti E. coli agglutinins; the agglutination titer of the mouse antiserum was 1/512 and that of the rabbit serum was 1/4096. These two sera were used in a quantitative study of the opsonizing effect of antibody on the phagocytosis of E. coli by the RES in mice. The rabbit antisera was further analyzed for precipitating antibodies against the purified polysaccharide by the Heidelberger precipitin technique (26), using a purified polysaccharide from E. coli prepared by alkaline hydrolysis, kindly given to us for this purpose by Dr. Anne-Marie Staub of the Pasteur Institute. This antiserum contained 90 μg. AbN/ml. Owing to the limited amount of mouse antiserum and its low titer, quantitative precipitin determination could not be made, but the antibody content of this serum against the same purified polysaccharide was assayed by passive cutaneous anaphylaxis (P.C.A.) in the guinea pig (27). 0.1 ml. of this serum induced P.C.A. reactions down to a dilution of 1/50.

Measurement of Opsonizing Effect of Specific Antibody on Phagocytosis of E. coli by the RES.
—These experiments were carried out in mice, who showed a normally slow blood clearance
rate of these bacteria, using a dose of 10^9 *E. coli* per 100 gm. After the usual intravenous in-
jection of heparin, 0.5 ml. of a suitable dilution of the rabbit or mouse antisera described
above were injected intravenously. 10 minutes later, the P^{32}-labelled *E. coli* suspension was
injected intravenously. The blood clearance was studied and the phagocytic index K cal-
culated as described above in each case. The following dilution of the sera were used: in the
case of the mouse antiserum pool 1/50, 1/100, 1/200, 1/300, 1/500, 1/1000, 1/3000, in the
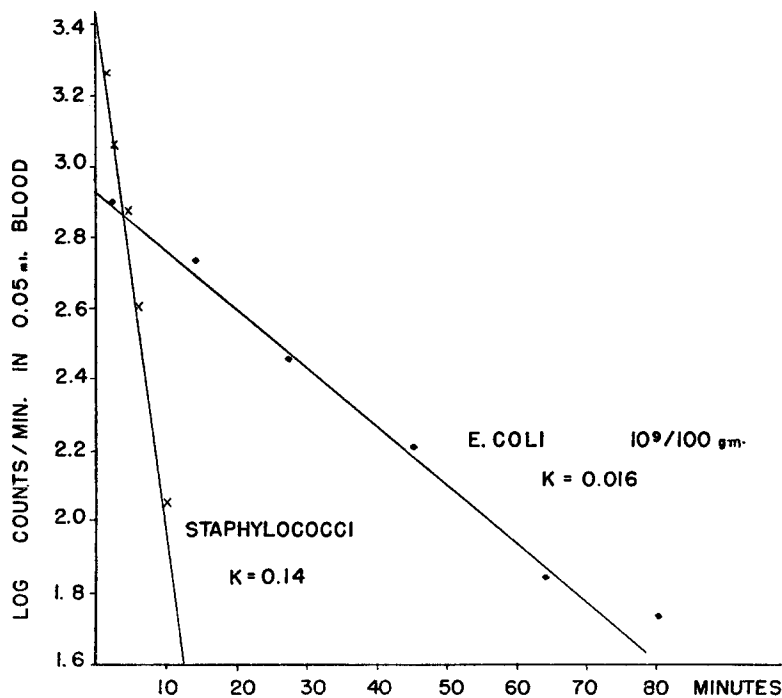
Fig. 2. Blood clearances in two normal mice of 10^9 heat-killed *E. coli* per 100 gm. and of
a dose of heat-killed staphylococci equivalent to 4×10^8 *E. coli* per 100 gm. with respect
to optic density measurement.

case of the rabbit antiserum 1/500, 1/5000, 1/7500, 1/10000, 1/20000; each dilution was
injected into 3 to 12 mice.

Treatment with Bacterial Endotoxins.—The purified lipopolysaccharides used in these
experiments were prepared and kindly given to us for these studies by Dr. Maurice Landy.
The lipopolysaccharides used were prepared from *Salmonella typhosa* (lot B_{12}), *Serratia mar-
cescens*, *Salmonella typhimurium*, mouse red cell, and mouse lung. The clearance of P^{32}-
labelled 10^9 *E. coli* per 100 gm. body weight was investigated in mice treated with various
doses schedules of these lipopolysaccharides intraperitoneally. 12 mice received 25 μg. of
S. typhosa endotoxin and the blood clearance of *E. coli* was investigated in groups of these
mice after 3 hours, 10 hours, 24 hours, and 6 days. Other groups of mice received repeated
injections of the various endotoxins used and the clearance of *E. coli* was tested after 10 days.
The following dosage schedule was usually employed: 10 μg. followed by 100 μg. every 48

hours for 3 doses. Some of the mice which had received this treatment were bled on the 10th day and the power of the serum to transfer opsonizing capacity for *E. coli* was investigated; 0.5 ml. of these sera was injected intravenously into new mice 10 minutes before the usual dose of P^{32}-labelled bacteria, $10^9/100$ gm. and the clearance investigated.

Competition Effect of Injected Carbon Particles on the Phagocytosis of E. coli by the RES.— To verify that the bacteria investigated were phagocytized by the RES by the same process as inert carbon particles, the competitive effect of the phagocytosis of carbon particles on the clearance of *E. coli* was investigated. Studies on the phagocytic function of the RES have shown that colloidal particles present in suitable amounts can interfere with the phagocytosis by the RES of other particles by a phenomenon of competition (15, 28). This is manifested by a sharp change in the rate of clearance of the suspension investigated as soon as the competing colloid in injected. These experiments were carried out in mice with P^{32}-labelled *E. coli*, $10^9/100$ gm.; after enough samples had been obtained to draw the clearance curve, 16 mg. of carbon per 100 gm. (14) were injected intravenously and the rate of clearance of *E. coli* measured again. The competitive effect exerted by the carbon particles is measured by the ratio of the rates of clearance K_1 over K_2 before and after the injection of carbon.

RESULTS

Blood Clearance of E. coli and Staphylococci.—When P^{32}-labelled *E. coli* or staphylococci are injected intravenously, the blood radioactivity measuring the number of bacteria decreases according to an exponential function of the time down to a concentration of 10 per cent or less; then, the rate of clearance becomes progressively smaller. This has been observed for *E. coli* in mice, guinea pigs, and rabbits and for staphylococci in mice. The curves in Figs. 1 and 2 represent the results of typical experiments in mice. These bacteria are therefore cleared from the blood as a homogeneous suspension, but there seems to be a small number of microorganisms which are removed with a lesser degree of efficiency than the general population of *E. coli*. The kinetics of blood clearance of bacteria follow the same laws as the clearances of colloidal suspensions of carbon (14), iron oxide (15), or denatured proteins (16) by the RES. The same equation:— $\dfrac{\text{Log } C_1 - \text{Log } C_2}{T_2 - T_1} = K$, describing these clearances can be used in the case of bacterial clearances, in which K referred to as the "phagocytic index," measures the rate of clearance of the substrate used.

Several other important points have to be considered: (*a*) what are the elements of the RES which phagocytize the bacteria; (*b*) what determines the efficiency with which they are phagocytized; (*c*) what is the effect of the number of bacteria upon the rate of clearance; does the blood clearance of *E. coli* become dose-dependent above a certain concentration as in the case of other RES clearances. If one compares the rate of clearances of similar doses of *E. coli* in mice, guinea pigs, and rabbits (Table I, Fig. 3) and relate these results with the antibody titers in the sera of these animals, the following conclusions can be drawn. The rate of clearance of *E. coli* in mice is slow, without considerable variations in a range of dosage from $2 \times 10^8/100$ gm. to $5 \times 10^9/100$ gm., $K =$

TABLE I

Rate of Clearance from the Blood of P^{32}-Labelled E. coli in Mice, Guinea Pigs, and Rabbits, and Agglutination Titer of the Sera

Mice

	E. coli heat-killed				E. coli Live	Agglutination titers (pooled serum from 5 mice)
	2×10^8/ 100 gm.	10^9/100 gm.	5×10^9/ 100 gm.	10^9/100 gm.		
Phagocytic index K	0.015 0.011 0.010 0.012	58 mice 0.015	0.013 0.013 0.013 0.011 0.016	0.016 0.010 0.018 0.023		1/16±, 1/8±, 1/8±, 1/8±, 1/4+
Mean values.............	0.012	0.015±0.005	0.013	0.016		

Guinea pigs

	2.5×10^8/100 gm.	1.5×10^9/100 gm.	Agglutination titers
Phagocytic index K	0.040 0.029 0.057 0.055 0.024	0.028 0.025 0.038 0.037	1/16+, 1/16+, 1/16±, 1/32±, 1/32±, 1/32±
Mean values.............	0.041	0.032	

Rabbits

	1.5×10^8/100 gm.	6×10^8/100 gm.	3 to 6 $\times$ 10^9/ 100 gm.	Agglutination titers
Phagocytic index K	0.370 0.460 0.230 0.420	0.380 0.240 0.300	0.066 0.056 0.055 0.070	1/128±, 1/128±, 1/64+, 1/64±, 1/32+, 1/32±
Mean values.............	0.370	0.307	0.061	

0.013. Contrary to this, the rates of clearance of the same bacteria, 1.5×10^8 to 6×10^8/100 gm. is very rapid in rabbits, K is greater than 0.300. Correspondingly, the agglutination titers against *E. coli* are 1/4 to 1/8 in normal mouse sera and 1/32 to 1/64 in normal rabbit sera. Guinea pigs show a rate of clearance of *E.*

coli which is intermediate between what is observed in mice and rabbits, and the agglutination titers of guinea pigs sera range between 1/16 and 1/32. It seems therefore that the most important factor to consider in the clearance
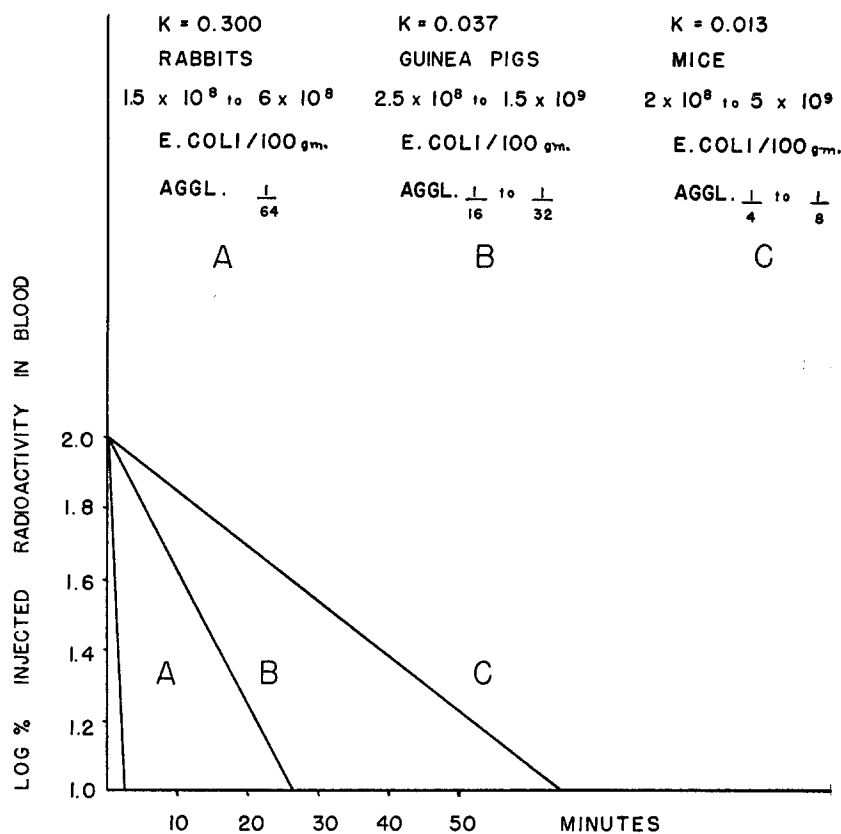

FIG. 3. Average blood clearances of P^{32}-labelled heat-killed *E. coli* in mice, guinea pigs and rabbits.

A, rabbits 1.5 to 6 X 10^8 *E. coli*/100 gm. K = 0.30
B, guinea pigs 2.5 X 10^8 to 1.5 X 10^9/100 gm. K = 0.037
C, mice 2 X 10^8 to 5 X 10^9/100 gm. K = 0.013

of *E. coli* is the level of antibodies which, unless present in adequate amount, is insufficient to opsonize the bacteria properly and becomes rate limiting.

If a relatively small number of *E. coli* are injected intravenously into normal rabbits, when the level of antibodies is sufficient to properly opsonize the bacteria, the rates of clearances vary from K = 0.240 to K = 0.460 and the clearances are predominantly effected by the liver Kupffer cells. These values of K are of the same order of magnitude as those reported for the clearances of avidly phagocytized sus-

pensions (16) by the liver which are removed with an efficiency of about 80 per cent in one passage through this organ. Therefore, well opsonized *E. coli* are phagocytized by the rabbit's liver with an extremely high efficiency, in the order of 80 per cent. If larger doses are injected, 3 to 6 $\times$ 10^9/100 gm., the phagocytes can no longer remove the greater number of bacteria as efficiently, and the value of K drops to an average of 0.061 (Table I). In normal mice, the rate of clearance is slow, K = 0.015 $\pm$ 0.005 in an average of 58 mice injected with 10^9 *E. coli*/100 gm.; the bacteria are phagocytized by the liver and spleen of the mouse with a low degree of efficiency. Since the maximum clearance rate in mice is an average of K = 0.400 for colloids removed, with a maximum degree of efficiency by the liver Kupffer cells (16), the *E. coli* are phagocytized by the RES of the liver and spleen of the normal mouse
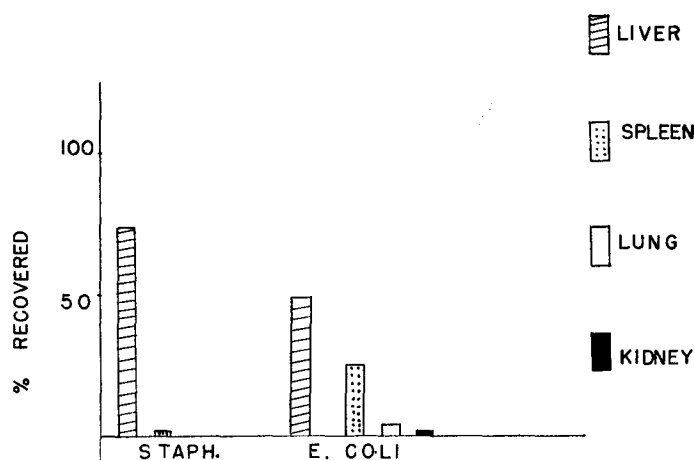
FIG. 4. Percentage of the injected radioactivity recovered in the organs of normal mice injected with 10^9 P^{32}-labelled *E. coli*/100 gm. or with an amount of P^{32}-labelled staphylococci equivalent to 4 $\times$ 10^8 *E. coli*/100 gm. with respect to optic density measurement.

with a degree of efficiency no greater than 3 per cent in one passage through these organs. This low efficiency remains constant in the dose range of 2 $\times$ 10^8 to 5 $\times$ 10^9 *E. coli*/100 gm., as the antibody level is still the rate limiting factor. Under these conditions, the rate-decreasing effect of large numbers of bacteria cannot be demonstrated in mice as it was in the rabbit for the dose of 5 $\times$ 10^9 *E. coli*/100 gm. However, if a sufficient amount of antibody is injected so that it is no longer the rate-limiting factor, the effect of the number of bacteria decreasing the clearance rate can be observed (Table IV).

In this experiment, the mice were injected with 0.5 ml. of 1/50 dilution of the mouse anti *E. coli* polysaccharide serum, an amount sufficient, as will be demonstrated, to opsonize the bacteria adequately. When 1.5 $\times$ 10^8 to 1 $\times$ 10^9 *E. coli* were injected, the rate of clearance was maximum and not limited by the amount of antibody; when 5 $\times$ 10^9 bacteria per 100 gm. are injected, the

clearance becomes dose-dependent as in the case of a sufficient amount of avidly phagocytized colloidal particles (15). (Table V).

The experiments affords a comparison of the maximum clearance rates of well opsonized *E. coli* in rabbits and mice. The maximum clearance rate in mice (Tables IV and V) is smaller than 0.200, while in rabbits it is about 0.340, indicating that all other factors being equal, antibody levels, number of bacteria, the mouse's liver is not as efficient a filter for bacteria, than the rabbit's liver.

$$\frac{K_1}{K_2} = \frac{0.011}{0.004} = 2.7$$

E.COLI 10^9/100 gm.

↓ CARBON 16 mg/100 gm.

$$\frac{K_1}{K_2} = \frac{0.014}{0.007} = 2$$
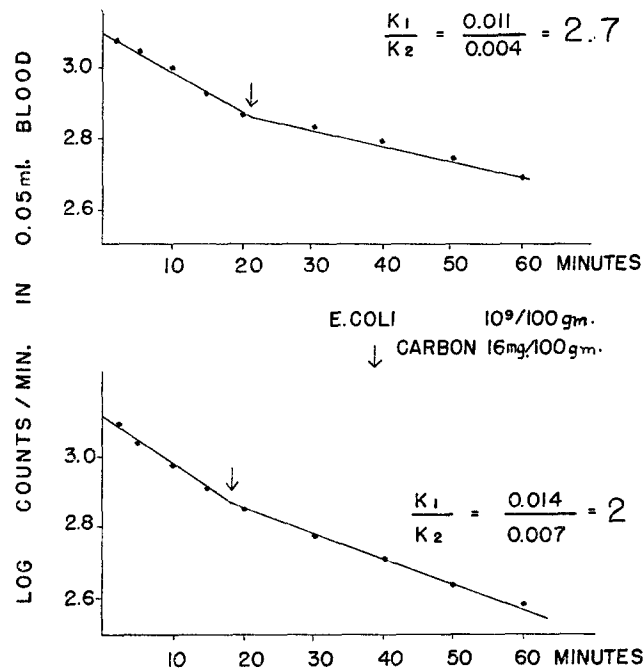
Fig. 5. Competition exerted by the injection of carbon particles, 16 mg./100 gm. of body weight on the phagocytosis of P^{32}-labelled *E. coli* by the RES in mice.

This relatively poorer efficiency of the mouse liver had already been observed in the case of avidly phagocytized particles by Dobson (29, 30) and is attributed to the greater speed of blood flow in the liver of this small animal, which makes contacts between the macrophages and the material to be phagocytized more difficult.

In Table II and Figs. 2 and 4, the blood clearances of *staphylococci* and of *E. coli* are compared in mice. Staphylococci injected in equivalent amounts are cleared from the blood much faster than *E. coli*. The rates observed are of the same order of magnitude as the maximum rates observed in the mouse for *E. coli* (Table V, Fig. 2). Mice sera show an agglutinin titer against staphylo-

cocci of 1/32; this level of antibody may be responsible for good opsonization. However, it is not possible to establish whether antibody is really required for efficient phagocytosis of staphylococci, since the rate of phagocytosis in normal mice is already maximum and cannot be improved upon by more antibody, as in the case of E. coli.

To verify whether bacteria and colloidal particles, such as carbon, are phagocytized by the same mechanism, by the same cells of the RES, the competitive effect of carbon particles on the phagocytosis of E. coli was investigated. This phenomenon of competition had been observed when two colloids, such as carbon or iron oxide particles, that are phagocytized by the RES with com-

TABLE II

Rate of Clearance from the Blood and Distribution in the Organs of P^{32}-Labelled Staphylococci in Mice

Phagocytic index K	Mean agglutination titer	Recovery of injected amount	
		Liver	Spleen
		per cent	*per cent*
0.176			
0.072			
0.220			
0.138	1/32	70*	2*
0.162			
0.110‡			
0.160‡			

* Results of analysis made on combined organs of 4 mice.

‡ These two mice were injected with 5 times the dose used in the preceding animals without significant change in rates.

parable avidity, are both present in the circulation in suitable amounts (28). The results presented in Fig. 5 illustrate that as soon as 16 mg. of carbon are injected, the rate of clearance of E. coli (10^9 bacteria/100 gm.) decreases, the ratio of the values of K before and after the injection of the carbon suspension $\dfrac{K_1}{K_2}$ measures the degree of interference exerted by the carbon particles on the phagocytosis of E. coli.

The P^{32}-labelled E. coli or staphylococci injected intravenously in mice are phagocytized for the largest part by the reticulo-endothelial cells of the liver and spleen (Tables II and III, Fig. 3), a small amount, about 5 per cent, is found in the lungs and traces in the kidneys. However, the respective amounts phagocytized by the liver or the spleen for a given dose vary with the rate of clearance of the bacteria, that is, with the efficiency with which they can be extracted in one passage by the principal filtering organs, the liver and the spleen. Well opsonized, or otherwise easily phagocytized

bacteria can be removed with great efficiency by the liver; therefore, because of its predominantly large circulation, this organ becomes the main filter for such bacteria. In the case of bacteria which are not avidly phagocytized, such as poorly opsonized $E.$ $coli$ in normal mice, the share removed by the spleen phagocytes increases considerably at the expense of the liver. The spleen, because of its slower circulation and better chances of contact between bacteria and macrophages, is a more efficient filter for bacteria which are less avidly phagocytized. It is interesting to note that this differ-

TABLE III

Distribution of the Radioactivity in the Organs of Mice and Guinea Pigs Injected with P^{32}-Labelled E. coli

	Mice						Guinea pigs*			
	E. coli heat-killed				E. coli live		E. coli heat-killed			
	10^9/100 gm.				10^9/100 gm.		2.5×10^8/100 gm.			
	Liver	Spleen	Lung	Kidneys	Liver	Spleen	Liver	Spleen	Lung	Kidneys
	55	27.5	3.7	1.3	38.5	21	72.5	16	5	2
	44	20.5	9	1.5	42	33				
	43	18	5.5	2.5	45	15				
	28.5	21.6	3.2	1						
	37	20	7.2	1.9						
	50	31	3	4.1						
	33	29	2.4	1						
	45	37	3.8	1						
	62	16	3	—						
	58	20	4	—						
	54	29	7	3						
Mean values...	46	24.5	4.7	1.5	42	23				

Values refer to percentage of radioactivity injected.

Mice were sacrificed when level of radioactivity in the blood reached about 10 per cent of the injected amount.

* Results of analysis made on combined organs of 2 guinea pigs.

ence in behavior between the liver and spleen has been observed already in the study of phagocytosis of colloidal particles (15, 30). It is generally found that unstable, avidly phagocytized colloids have almost exclusively a liver clearance, if injected in small amounts; while well stabilized colloids are removed in greater amounts by the spleen and bone marrow.

The Effect of Antibody on the Clearance of E. coli from the Blood.—When suitable dilutions of anti-lipopolysaccharide rabbit or mouse antibody are injected into mice, the rates of clearance of P^{32}-labelled $E.$ $coli$ from the blood is accelerated. A quantitative study was carried out with both antisera to investigate the relationship between the level of antibody and the efficiency with

which bacteria are phagocytized by the RES and to establish what is the amount of antibody required to opsonize the bacteria adequately. The results of these experiments are presented in Table IV and Figs. 6 and 7. If the rates

TABLE IV

Opsonizing Effect of Mouse and Rabbit Antibodies against E. coli Lipopolysaccharide

Phagocytosis of P^{32}-labelled *E. coli* by the RES in mice, measured by the phagocytic index K for the dose of *E. coli* $10^9/100$ gm.

Rabbit antiserum

Antiserum dilution injected..........	1/500	1/5000	1/7500	1/10000	1/20000	Controls 58 mice
AbN injected μg....	0.09	0.009	0.006	0.0045	0.0022	
Phagocytic index K	0.170	0.060	0.122	0.063	0.042	
	0.170	0.130	0.100	0.042	0.047	
	0.204	0.098	0.088	0.033	0.025	
	0.230	0.116	0.092	0.073	0.025	
		0.170	0.087	0.086	0.027	0.015 ± 0.005
		0.175	0.104	0.072	0.044	
		0.154	0.110	0.080	0.028	
		0.136	0.072	0.055	0.046	
			0.096	0.094		
				0.072		
				0.059		
				0.068		
Mean values....	0.193	0.130	0.096	0.066	0.036	0.015

Mouse antiserum

Antiserum dilution injected	1/50	1/100	1/200	1/300	1/500	1/1000	1/3000
Phagocytic index K	0.118	0.114	0.156	0.102	0.085	0.027	0.015
	0.164	0.158	0.174	0.110	0.057	0.061	0.022
	0.160	0.164	0.144	0.115	0.077	0.041	0.021
	0.158	0.230		0.106	0.068	0.030	
		0.107				0.031	
Mean values...	0.150	0.155	0.158	0.108	0.072	0.038	0.019

of clearance, K, of *E. coli* are plotted as ordinate and the amount of antibody injected as abscissa, the experimental points fall on a straight line showing that there is a direct relationship between the amount of antibody present and the efficiency of phagocytosis of the bacteria, until an excess of antibody is present and the rate of clearance reaches a maximum which cannot be improved upon.

When the curves are extrapolated down to zero antibody injected, they cross the ordinate axis at precisely the value of K observed in control mice injected with the same number of *E. coli*.

TABLE V

Effect of the No. of Bacteria on the Clearance of Well Opsonized E. coli from the Blood of Mice When Antibody Level Is Not the Rate-Limiting Factor

	Dose *E. coli*/100 gm.			
	1.5×10^8	3×10^8	10^9	5×10^9
Phagocytic index K	0.168	0.142	0.118	0.070
	0.164	0.150	0.164	0.070
	0.180		0.160	0.074
			0.158	0.090
Mean values.............	0.170	0.146	0.150	0.076

FIG. 6. Effect of rabbit antibody against *E. coli* lipopolysaccharide injected intravenously in mice (0.5 ml.) on the rate of clearance of *E. coli* from the blood by the RES measured by the phagocytic index K for the dose of 10^9 bacteria per 100 gm.

In the case of rabbit antibody, the amount of antibody was recorded in micrograms of antibody nitrogen (AbN) precipitable by the specific polysaccharide, and also in terms of $\dfrac{\text{Injected dilution}}{\text{Agglutination titer}}$, to compare the opsonizing effect of

antibody with its agglutinating properties. With this antiserum, an amount of antibody 0.005 μg. AbN in the whole mouse is sufficient to opsonize the bacteria to ensure a rapid clearance, and 0.01 μg. AbN only is sufficient to cause almost maximal opsonization. Opsonization by antibody is a more sensitive process than agglutination, since lower dilution than the agglutination titer causes good opsonization of *E. coli* for phagocytosis by the RES.

Identical results were obtained with mouse antiserum against *E. coli* lipo-polysaccharide. A direct relationship is again observed between the antibody level and the rate of phagocytosis until an excess of antibody is reached.
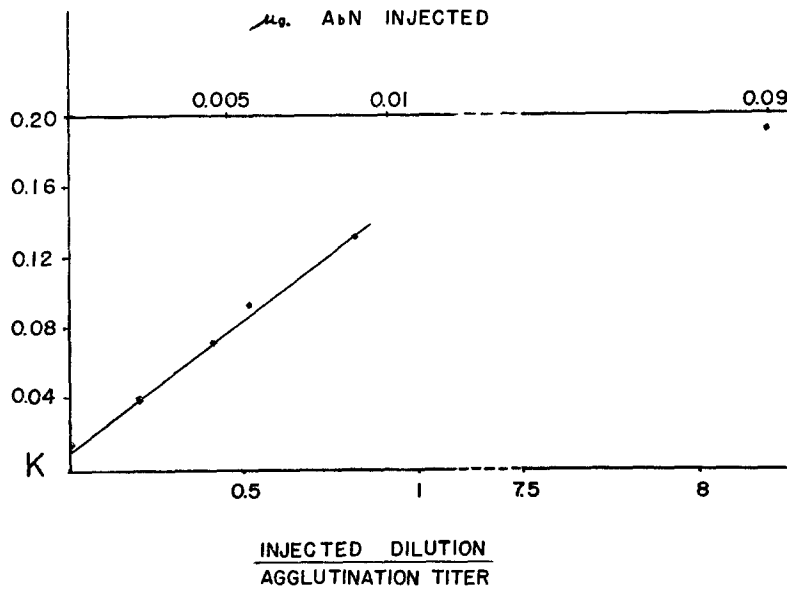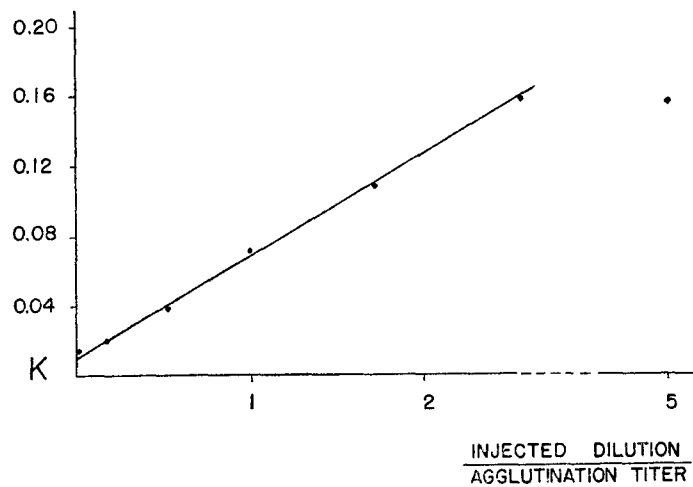

Fig. 7. Effect of mouse antibody against *E. coli* lipopolysaccharide injected intravenously in mice (0.5 ml.) on the rate of clearance of *E. coli* from the blood by the RES, measured by the phagocytic index K for the dose of 10^9 bacteria per 100 gm.

A comparison of the opsonizing efficiency of rabbit and mouse antibodies shows that mouse antibodies seem to be somewhat less efficient than rabbit antibodies, as compared with their respective agglutination titers. However, not much emphasis can be placed upon this difference, as it is based upon a difference in agglutination titer of one tube dilution.

Because of the small amount of mouse antiserum available and of its low antibody content, measurement of antibody by the quantitative precipitin technique was not done. However, the antiserum was assayed for antibody against the purified polysaccharide by passive cutaneous anaphylaxis in the guinea pig P.C.A. (31). The average lowest dilution which induced this reaction was 1/50. The amount of antibody nitrogen of the serum can be estimated with the help of data obtained by Dr. Ovary on the sensitivity of this reaction for mouse antibody. Using mouse antiovalbumin of known antibody content, Dr. Ovary (31) reported the lowest amount of mouse antibody which

induces P.C.A. to be between 0.14 and 0.23 μg. AbN/ml. On the basis of this estimation, the anti-polysaccharide serum would contain about 9 μg. AbN/ml. The amount of mouse antibody which causes maximal opsonization of *E. coli* would be $\dfrac{9 \times 0.5}{200} = 0.02\ \mu$g. AbN which is somewhat more than, but of the same order of magnitude, as the amount of rabbit antibody required for the same effect.

In other experiments, it was verified that *E. coli* could be also well opsonized for phagocytosis by the RES by incubation with antibody *in vitro*. The treated

TABLE VI

Effect of Endotoxin on the Rate of Clearance of E. coli from the Blood as Measured by the Phagocytic Index K for 10^9 Bacteria/100 gm.

Endotoxin treatment	Time after 1st endotoxin injection	No. of animals	Phagocytic index K	Agglutination titer
μg./animal			*mean values*	
58 control mice			0.015±0.005	1/4 to 1/8
S. typhosa				
25	3 hrs.	3 mice	0.02	
	10 "	2 "	0.016	
	24 "	3 "	0.02	
	6 days	4 "	0.037	
10 and 100 × 3	10 "	12 "	0.110	1/64
0.5 ml. serum transfer		4 "	0.057	
Serratia marcescens				
10 and 100 × 3	10 days	4 mice	0.130	
0.5 ml. serum transfer		2 "	0.093	
S. typhi murium				
10 and 100 × 2	1 wk.	4 mice	0.058	
Mouse polysaccharide				
10 and 100 × 3	10 days	5 mice	0.015	

bacteria were injected after washing with saline. This technique allowed the opsonizing effect to be demonstrated with considerably smaller amounts of antibody than required when injected intravenously in the whole animal.

The Effect of Endotoxin on the Phagocytosis of E. coli in Mice.—Because of the remarkable increase in natural resistance to infection caused by treatment with endotoxins from Gram-negative bacteria (22–24) and of the stimulating effect of these endotoxins on the RES (20, 21), it seemed of interest to investigate the effect of these lipopolysaccharides on the phagocytosis of *E. coli* in mice using several different endotoxins. The results of these experiments are presented in Table VI and Fig. 8. Following treatment with 25 μg. of *S. typhi* endotoxin, no significant change in the blood clearance of *E. coli* is observed at

3 hours, 10 hours, or 24 hours; on the 6th day there is a moderate increase in the rate of clearance. If a prolonged treatment is used, 10 μg. followed by 100
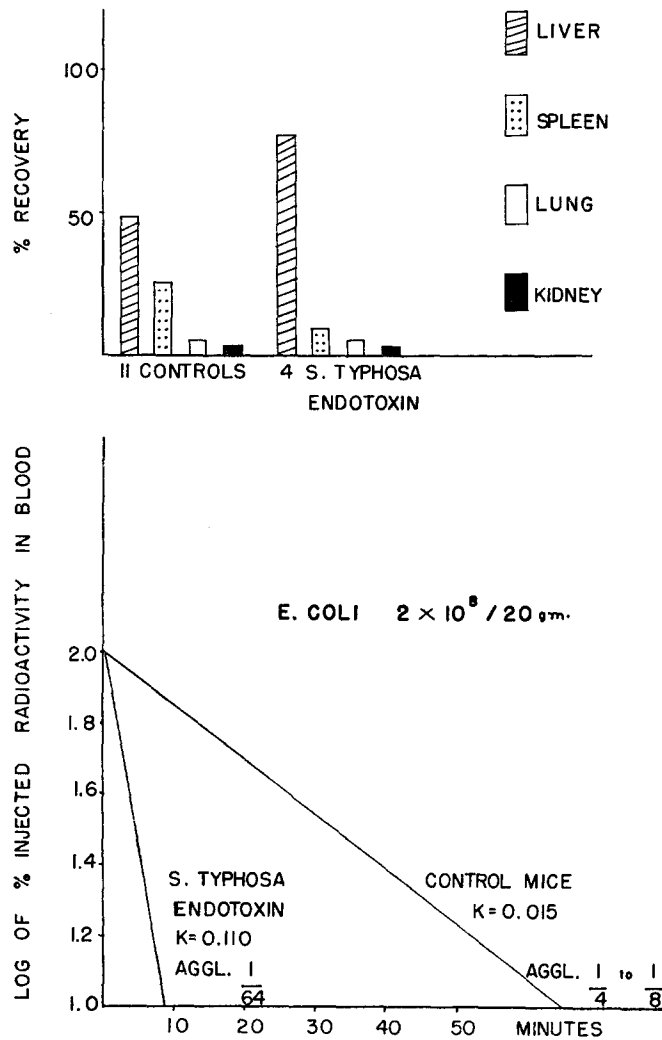
FIG. 8. Effect of prolonged treatment with S. *typhi* endotoxin on the rate of clearance of P^{32}-labelled E. *coli* from the blood of mice by the phagocytes of the RES E. *coli* 10^9/100 gm. Endotoxin 10 μg. then, starting 48 hours later, 100 μg. every 2 days for 3 doses.

μg. every 48 hours for 3 doses, a considerable increase in the rate of clearance is observed. As expected, with the increase in the rates of clearance of E. *coli*, there was also a change in the distribution of the phagocytized bacteria; the

liver in the fast clearing animals became the principal organ of clearance at the expense of the spleen (Fig. 8).

Similar results were obtained with endotoxins from *Serratia marcescens* and *S. typhimurium*, but lipopolysaccharides from mouse lungs and red cells were totally inactive. The increase in the rate of phagocytosis of *E. coli* caused by treatment with these bacterial endotoxins cannot be explained by the greater phagocytic activity of the RES which results also from such a treatment (20, 21); it is determined by an increase of the level of anti *E. coli* antibodies which is the rate-limiting factor in this system. Intravenous injections of serum from endotoxin-treated mice into normal mice transfer to these animals the ability to clear *E. coli* from the blood at a faster rate.

DISCUSSION

A study of the blood clearance of bacteria in mice, guinea pigs and rabbits, using heat-killed P^{32}-labelled *E. coli* as a model, has shown that these bacteria are phagocytized by the RES as are colloidal suspensions, such as carbon (14) or denatured proteins (16). The blood concentration decreases according to an exponential function of the time and the rates of clearance are determined by the avidity with which the bacteria are phagocytized and by the number of bacteria injected, provided a sufficiently large number of bacteria are injected to saturate the clearing mechanism. As in the case of colloidal particles, the respective amounts phagocytized by the liver and spleen (15) depend upon the rates of clearance; the liver, because of its large blood supply, is the principal filter for readily phagocytized bacteria, while the splenic phagocytes are more efficient in the removal of less easily phagocytized bacteria. The avidity with which *E. coli* can be extracted from the blood by the RE cells of the liver and spleen has been shown to be dependent upon the antibody level which in normal mice is the rate-limiting factor. This is in agreement with the recent observations of Howard and Wardlow (32) on the ability of the perfused rat liver to phagocytize *E. coli*. These investigators found that the rat removed *E. coli* very inefficiently unless normal serum was added to the perfusate and concluded that there are three factors in serum which facilitate the phagocytosis of bacteria: specific antibody, complement, and another heat-labile factor devoid of any great specificity. In the present study, the influence of complement could not be investigated, but the opsonizing effect of specific antibody on phagocytosis of *E. coli* was measured. The efficiency with which *E. coli* can be phagocytized by the RES is a linear function of the amount of antibody present. The amount of antibody required to opsonize bacteria optimally to insure clearance from the blood at the maximum rate in the mouse is very small, about 0.01 to 0.02 μg. AbN.

The rate of phagocytosis of *E. coli* in normal mice is slow; under these con-

ditions, is the small amount of antibodies detected in the serum by agglutinin titration necessary for phagocytosis to take place? This cannot be answered with certainty from the data available. However, some insight may be obtained from a study with germ-free mice in which the rate of clearance of P^{32}-labelled *E. coli* from the blood was found to be significantly smaller than in control mice, mean value of $K = 0.0067$ (35), while the ability of the RES to clear carbon was normal. It would appear that as far as *E. coli* is concerned, phagocytosis by the RES is very inefficient in the absence of antibodies. The results obtained with *E. coli* do not necessarily apply to all bacteria, and it is conceivable that some bacterial species do not require antibodies for phagocytosis, if the nature of their surface allows them to be attracted by and to stick to the phagocytes' cell membrane.

The enhanced ability manifested by endotoxin-treated mice to phagocytize *E. coli* is shown as well by normal mice receiving serum from endotoxin-treated mice and therefore it is believed to be related to the increase in antibody titers which the sera of these animals possess, although one cannot exclude the possibility that one may be dealing with non-specific opsonins in such sera. The effect of endotoxin treatment on the rate of clearance of *E. coli* was obtained with lipopolysaccharides from various bacterial species, *Salmonella typhi*, *Salmonella typhimurium*, *Serratia marcescens*. In the case of *S. typhi*, this effect and the increased antibody titer against *E. coli* might be explained on the basis of known immunological cross-reactivity between some strains of *E. coli* and *S. typhi* (33); in the case of *Serratia marcescens*, it is more difficult to explain unless some further cross-reactivities with *E. coli* are postulated, which is the same as saying that antibodies with low specificity exist in the serum.

What is the role of the serological response observed in the increased resistance of mice treated with bacterial endotoxins? It must be stressed that a prolonged administration of big doses is required to obtain a significant increase in antibody titers and in rates of clearance, whereas increased resistance to infections can be demonstrable after one small dose of endotoxin (22, 23). It seems unlikely therefore that the increased antibody titers could alone explain this phenomenon. The enhancement of resistance by endotoxins may indeed depend upon the ability of the macrophages to kill the phagocytized bacteria, which is a definitive requirement for a favorable outcome of any infection. The evidence indicates that serological and cellular factors contribute to an integrated antibacterial defense. Rowley observed that macrophages are unable to kill phagocytized *E. coli* in the absence of a heat-stable serum factor, presumed to be antibody (34).

The unfavorable outcome of an infectious process depends upon the events which follow the initial clearance of bacteria from the blood, when the number of bacteria increases in the circulation in spite of the various defense mechanisms. The present study has not been concerned with that phase of the problem

which must be investigated with other techniques than those used in these experiments.

SUMMARY

1. The clearance of P^{32}-labelled heat-killed *E. coli* and staphylococci from the blood follows an exponential function of the time, and the bacteria are phagocytized principally by the RES of the liver and spleen.

2. The rates of clearance of equivalent number of *E. coli* from the blood is rapid in rabbits and slow in mice and appears to be related to the level of antibodies in the serum of these animals.

3. Unlike *E. coli*, staphylococci are cleared rapidly and efficiently by the RES from the blood of mice which have a sufficient level of serum antibody against these bacteria.

4. The numbers of bacteria, phagocytized by the liver or the spleen respectively, depend upon the rate of clearance and the extent of opsonization of the bacteria. Rapidly cleared, well opsonized *E. coli* are removed almost exclusively by the liver, while less efficiently phagocytized bacteria are also cleared by the spleen in large numbers.

5. The rate of clearance of *E. coli* and the efficiency with which they are phagocyted by the RES in mice have been shown to be directly related to the level of antibody in the serum.

6. Treatment of mice with *S. typhi* or *Serratia marcescens* endotoxins increases the rate of clearance of *E. coli* from the blood and the level of antibody against *E. coli* in the serum. The enhanced clearance of *E. coli* can be transferred to normal mice by the serum of endotoxin-treated mice.

BIBLIOGRAPHY

1. Bull, C. G., The fate of typhoid bacilli when injected intravenously into normal rabbits, *J. Exp. Med.*, 1915, **22**, 475.
2. Wright, H. D., Experimental pneumococcal septicemia and anti pneumococcal immunity, *J. Path and Bact.*, 1927, **30**, 185.
3. Sullivan, F. L., Nekerman, E. F., and Cannon, P. R., The localization and fate of bacteria in the tissues, *J. Immunol.*, 1934, **26**, 49.
4. Kerby, G. P., Holland, B. C., and Martin, S. P., The quantitative estimation of the removal of bacteria from the blood by the various organs of the immunized animals, *J. Immunol.*, 1950, **64**, 123.
5. Hollingworth, J. W., and Beeson, B. P., Experimental bacteremia in normal and irradiated rats, *Yale J. Biol. and Med.*, 1955, **28**, 56.
6. Bennett, I. L., and Beeson, P. B., Bacteremia: a consideration of some experimental and clinical aspects, *Yale J. Biol. and Med.*, 1954, **26**, 241.
7. Rogers, D. E., and Melly, M. A., Studies on bacteriemia, *J. Exp. Med.*, 1957, **105**, 113.
8. Rogers, E., The cellular management of bacterial parasites, The Pasteur Fermentation Centennial (1857-1957), Brooklyn, Chas. Pfizer & Co., 1958.

9. Teale, F. H., Some observations on the relative importance of the reticulo endo-thelial tissues and the circulating antibody in immunity, *J. Immunol.*, 1935, **28**, 133.

10. Martin, S. P., Kerby, G. P. and Holland, B. C., The effect of thorotrast on the removal of bacteria in the splanchnic area of the intact animal, *J. Immunol.*, 1952, **68**, 293.

11. Kerby, G. P., and Martin, S. P., The splanchnic removal of bacteria from the blood stream of leukopenic rabbits, *J. Exp. Med.*, 1952, **68**, 293.

12. Wood, W. B., Jr., Smith, M. R., Perry, W. D., and Berry, J. W., Studies on the cellular immunology of acute bacteriemia, I. Intravascular leucocytic reactions and surface phagocytosis, *J. Exp. Med.*, 1951, **94**, 521.

13. Smith, M. R., and Wood, W. B., Jr., Relations of surface phagocytosis to the fibrinous character of acute bacterial exudates, *Science*, 1949, **110**, 187.

14. Biozzi, G., Benacerraf, B. and Halpern, B. N., 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.*, 1953, **34**, 441.

15. Benacerraf, B., Biozzi, G., Halpern, B. N. and Stiffel, C., Physiopathology of the reticulo-endothelial system: a symposium, Springfield, Illinois, Charles C Thomas, 1957, 52.

16. Benacerraf, B., Halpern, B. N., Biozzi, G., Stiffel, C., and Mouton, D., Phago-cytosis of heat-denatured human serum albumin labelled with I^{131} and its use as a means of investigating liver blood flow, *Brit. J. Exp. Path.*, 1957, **38**, 35.

17. Biozzi, G., Halpern, B. N., Benacerraf, B., and Stiffel, C., Phagocytic activity of the reticulo endothelial system in experimental infections. Physiopathology of the reticulo endothelial system: a symposium, Springfield, Illinois, Charles C Thomas, 1957, 204.

18. Biozzi, G., Benacerraf, B., Grumbach, F., Halpern, B. N., Levaditi, J., and Rist, N., Etude de l'activité granulopexique du système réticulo-endothelial au cours de l'infection tuberculeuse experimentale de la souris, *Ann. Inst. Pasteur*, 1954, **87**, 291.

19. Boehme, D., and Dubos, R. J., The effect of bacterial constituents on the resist-ance of mice to heterologous infection and on the activity of their reticulo endo-thelial system, *J. Exp. Med.*, 1958, **107**, 523.

20. Biozzi, G., Benacerraf, B., and Halpern, B. N., The effect of *Salm. typhi* and its endotoxin on the phagocytic activity of the reticulo endothelial system in mice, *Brit. J. Exp. Path.*, 1955, **36**, 226.

21. Benacerraf, B., and Sebestyen, M. M., Effect of bacterial endotoxins on the reticulo endothelial system, *Fed. Proc.*, 1957, **16**, 860.

22. Rowley, D., Stimulation of natural immunity to *E. coli* infection, *Lancet*, 1955, **1**, 232.

23. Landy, M., Increased resistance to infection developed rapidly after administra-tion of bacterial lipopolysaccharides, *Fed. Proc.*, 1956, **15**, 598.

24. Dubos, R. J., and Schoedler, R. W., Effects of cellular constituents of mycobac-teria on the resistance of mice to heterologous infections. I. Protective effects, *J. Exp. Med.*, 1957, **106**, 703.

25. Halpern, B. N., and Pacaud, A., Technique de prélèvement d'échantillons de

sang chez les petits animaux de laboratoire par ponction du plexus ophtalmique, *Compt. rend. Soc. Biol.*, 1951, **145**, 1465.

26. Kabat, E. A., and Mayer, M., Experimental Immunochemistry, Springfield, Illinois, Charles C Thomas, 1948.

27. Ovary, Z., and Briot, M., Nouvelle méthode de dosage de l'anticorps anaphylactique et son rapport avec l'azote de l'anticorps, *Ann. Inst. Pasteur*, 1951, **31**, 670.

28. Biozzi, G., Benacerraf, B., Halpern, B. N., and Stiffel, C., The competitive effect of certain colloids on the phagocytosis of other colloids by the cells of the reticulo endothelial system and the phenomenon of phagocytic preference, *R.E.S. Bull.*, 1957, **3**, 3.

29. Dobson, E. L., and Jones, H. B., The behaviour of intravenously injected particulate material, *Acta Medica Scandinavica*, 1951, suppl. 144.

30. Dobson, E. L., Factors controlling phagocytosis. Physiopathology of the reticulo endothelial system: a symposium, Springfield, Illinois, Charles C Thomas, 1957, 80.

31. Ovary, Z., Passive cutaneous anaphylaxis in the guinea pig with mouse antibody, private communication.

32. Howard, J. G., and Wardlow, A. C., The opsonic effect of normal serum on the uptake of bacteria by the reticulo endothelial system, *Immunology*, 1958, **1**, 338.

33. Kaufmann, F., Uber antigen hezielingen gzisufen coli. Dysenterie und salmonella bacterien, *Acta Path. et Microbiol.*, 1944, **21**, 72.

34. Rowley, D., Bactericidal activity of macrophages *in vitro* against *Escherichia coli*, *Nature*, 1958, **181**, 1738.

35. Thorbecke, J. J., and Benacerraf, B., Phagocytic activity of the reticulo endothelial system in germ free mice, *Ann. New York Acad. Sc.*, 1959, in press.