THE EXAMINATION OF THE TISSUES AND SOME OBSERVATIONS ON THE BLOOD PLATELETS OF RABBITS AT INTERVALS OF FIVE MINUTES, AND LATER, AFTER INTRAVENOUS INOCULATIONS OF *STAPHYLOCOCCUS AUREUS* AND INDIAN INK.

By LEONARD S. DUDGEON, C.M.G., C.B.E., F.R.C.P.,
Professor of Pathology, University of London,
and H. K. GOADBY[1], M.A., M.D. (Cantab.), M.R.C.P. (Lond.),
Louis Jenner Research Scholar.

(*From the Department of Pathology, St Thomas's Hospital.*)

(With Plates I–III).

We have endeavoured in these investigations to obtain a comparison of the immediate reactions in the tissues and blood platelets of rabbits to *Staphylococcus aureus* which is highly pathogenic to these animals, and to suspensions of non-pathogenic particles such as indian ink and colloidal silver, when injected intravenously.

Many workers (Nissen, 1922; Westhues, 1922; Boerner-Patzelt, 1923; Wislocki, 1924; and Lang, 1926) have observed large aggregations of indian ink in the lungs of animals injected intravenously with it.

Cappell (1930) has very carefully studied the fate of colloidal particles injected into the circulation. He found that the injection of indian ink produces at the outset a leucopenia in the peripheral blood, due to an accumulation of polymorph leucocytes in the lung capillaries, some of which take up the particles. The ink, however, is widely circulated, and is found especially in the Kupfer cells in the liver, in the endothelial cells of the splenic sinuses and in the glomerular and intertubular vessels in the kidneys.

Werigo (1894) found in anthracaemia in rabbits that active phagocytosis occurred of *Bacillus anthracis* by the leucocytes in the lungs and the Kupfer cells in the liver. Levaditi (1901) was the first to note that the inoculation of organisms into the circulation results in the accumulation of polymorphs in the lungs. Andrewes (1910) showed that the injection of organisms into the blood stream is followed by an initial leucopenia due to the collection of polymorphs in the lungs, but this occurs only to a slight degree with *S. aureus*.

Bartlett and Ozaki (1917, 1918) found that, in the first 10 minutes after

[1] Working during tenure of a grant given by Mr and Mrs Sannyer Atkin, in memory of their son.

injection of *S. aureus* into the left ventricle, the organism is taken up by polymorphs in the lungs, subsequently by endothelial cells in the liver and spleen, while an increasing number are found free; these eventually lodge in the kidney capillaries, and give rise to abscesses. These authors also state that, as the septicaemia increases, the endothelial cells take up proportionately more and more of the organisms.

Nagao (1920) found that, when he injected formol-killed vaccines of non-haemolytic streptococci intravenously into rabbits, the polymorphs accumulated first and chiefly in the lungs. The extensive researches of Bull (1914–16) led him to the conclusion that non-virulent organisms when injected into the circulation are agglutinated and accumulate in the capillaries of the lungs, liver, spleen and kidneys, where they are taken up by the polymorphs; whereas, if the organisms are virulent for the animal, there is no agglutination and no phagocytosis.

Wright (1927) observed also that it is only in immune rabbits that the agglutination of virulent pneumococci occurs in the lung and liver capillaries with subsequent phagocytosis by the polymorphs and endothelials.

It appears from the literature that the effect of previous immunisation on the rate of clearing of the blood of bacteria varies with each organism. It is stated to be increased in the case of *Vibrio cholorae* (Bail, 1905) and *B. anthracis* (Singer, 1925) while with streptococci (Hopkins and Parker, 1918) and *B. typhosus* (Parker and Franke, 1918) immunisation has no effect.

Our experiments.

Technique.

Live cultures, 24 hours old, grown on agar at 37° C., were suspended in normal saline, and 1 c.c. of the suspension was injected intravenously into rabbits, because these animals are so susceptible to the action of this organism. Formol-killed vaccines of about the same density as the live suspensions of *S. aureus* were used in a similar manner, and also equal parts of Higgin's waterproof indian ink and normal saline filtered through several thicknesses of filter paper so as to obtain a uniform suspension free from large particles. In two instances a 20 per cent. solution of colloidal silver was used. Rabbits were inoculated in the marginal vein of the ear and killed at the required time interval by a blow on the neck. The organs were removed as quickly as possible after death and portions were put into Kaiserling solution and finally embedded in paraffin. Sections were stained with haemalum and eosin and also by Gram Weigert followed by haemalum and eosin.

The distribution of staphylococci (S. aureus) *and indian ink in the tissues, 5 minutes after intravenous inoculation.*

Live agar cultures, 24 hours old, of haemolytic and non-haemolytic *S. aureus*, and their vaccines, were used in all our experiments, but the same results were obtained in each case. When rabbits were inoculated intravenously

the most important microscopic changes were met with in the *lungs*. The alveolar walls were packed with polymorphs actively phagocytosing the cocci which were present in large numbers. The vessels were dilated and full of blood. The liver sinuses contained occasional polymorphs, and a few free cocci were seen. In the spleen, polymorphs were collected in clumps, but the cocci were few in number in the sinuses and mostly free. Cocci were scarce in the kidneys, but were found chiefly in polymorphs in the glomerular tufts. There was a very considerable difference in the experiments made with indian ink. The alveolar walls in the lungs were packed with empty polymorphs, but the ink particles were present in large amount, and phagocytosis of the ink by the large mononuclear cells had commenced. In the liver, however, considerable phagocytosis of the ink particles in the sinuses by the endothelial cells had taken place. The splenic sinuses were full of clumps of empty polymorphs and ink particles which were being engulfed by the endothelial cells. There was also a large amount of free ink in the glomerular and intertubular vessels in the kidneys.

Similar experiments were made at intervals of 20 minutes and 2 hours. The lungs of the *S. aureus* rabbits showed large numbers of polymorphs in the alveolar walls, and a few cocci phagocytosed by polymorphs and endothelial cells. In the liver sinuses large numbers of free cocci were found, and there was also phagocytosis by polymorphs and endothelials. In the spleen free cocci were numerous in the sinuses, and some were phagocytosed by polymorphs. There were very few polymorphs in the kidneys and very few cocci. With indian ink there was very little left in the lungs; mostly phagocytosed by the large mononuclears in the alveolar walls. In the liver, the ink was mostly in the endothelials in the sinuses. There was a massive collection of ink particles in the splenic sinuses, free and engulfed by endothelials. Very few ink particles were found in the kidneys, either free or in the endothelial cells. They were found chiefly in the glomeruli.

The examination of the tissues of a rabbit, killed 7 hours after the intravenous injection of *S. aureus*, showed a few cocci in the lungs and numerous polymorphs. Large numbers of cocci were found in the liver and spleen, where there was accumulation of polymorphs. The kidneys were of great interest. Polymorphs had collected in the glomeruli and in the capillaries of the intertubular tissues, but staphylococci were only found in clumps at the ends of the collecting tubules close to the pelvis.

The only polymorphs in the tissues of a rabbit killed after 22 hours were collected in the glomeruli of the kidneys. There were large numbers of free cocci in the liver sinuses, liver cells and in endothelial giant cells in the liver. In the kidney the staphylococci were present in clumps at the ends of the collecting tubules near the pelvis, and in the subepithelial connective tissue of the pelvis. Dudgeon and Goadby (1930) showed that, in rabbits which had died from *S. aureus* infections, various kidney lesions may occur, but especially (1) a simple acute pyelitis without any obvious lesion in the kidney

tubules; (2) linear abscesses radiating out from the papillae, apparently having started low down in the medulla at the end of the collecting tubules; (3) the whole kidney may be riddled with abscesses localised in the cortex or of the radiating linear type.

Effect of previous immunisation.

Rabbits were "immunised" by weekly injections of an *S. aureus* vaccine followed at a similar interval by two doses of the live organism, and were killed either 6 or 20 minutes after the last dose of the live coccus. In 6 minutes an enormous number of polymorphs had collected in the viscera, especially in the lungs, but not many cocci were found, although phagocytosis by the polymorphs was well shown. After 20 minutes similar polymorph accumulation was present, but many more cocci were visible in the lungs, liver and spleen. It would appear possible from our experiments that the effect of previous immunisation is to increase the immediate collection of polymorphs in the capillaries of the lungs and other organs.

On the injection of "supernatant" saline suspensions of S. aureus.

A haemolytic strain of *S. aureus* was grown on agar for 24 hours. The growth was washed off with sterile normal saline, and the suspension shaken for 30 minutes and finally centrifugalised until the supernatant fluid was clear and *apparently* free of cocci. One c.c. of this clear fluid was injected intravenously into two rabbits, which were killed after 5 and 20 minutes respectively. There was very great cardiac dilatation, engorgement and dilatation of small vessels in the internal organs, and polymorphs had accumulated in the lungs, liver and spleen. In the animals killed 20 minutes after inoculation the venous engorgement had resulted in considerable oedema in the heart muscle and in the liver. Rabbits were injected also with agar cultures of *S. aureus* grown for 24 hours at 37° C. and then suspended in the supernatant saline previously referred to, and killed 5 and 20 minutes later. In these rabbits, although the usual accumulation of polymorphs was present in the lungs, the cocci were not held up there, but were all over the body especially in the liver. Polymorph phagocytosis was active in all the organs, and it is probable that the great vascular dilatation was responsible for the rapid distribution of the cocci. The inoculation of dead staphylococci suspended in this supernatant fluid produced a similar reaction to the saline suspensions. Rabbits inoculated with live *S. aureus*, together with a suspension of washed human red cells, showed the same polymorph accumulation and phagocytosis of the cocci as occurred in the control experiments without red cells, and phagocytosis of the red cells by endothelial cells.

Action of free diphtheria toxin and toxin adsorbed to Indian ink.

(*a*) Within 5 minutes of an intravenous injection of diphtheria toxin into rabbits an accumulation of polymorphs of an unusual type was found in the

lungs, liver and spleen. These cells, instead of containing eosinophil granules characteristic of the polymorphs of rabbits, showed a hyaline non-granular cytoplasm or faintly stained eosin granules.

(*b*) A suspension of indian ink in diphtheria toxin to a concentration of 1 in 5 was left at 0° C. for 24 hours, and part of the mixture was autoclaved at 128 lb. for 30 minutes. Each preparation was shaken at short intervals.

One c.c. of each suspension was injected intravenously into rabbits. Sections of the viscera showed the same aggregations of ink in the capillaries and phagocytosis by endothelial cells, as in the control experiments with indian ink suspended in saline. The toxin had not stimulated the polymorphs to phagocytose the ink particles, and the polymorphs in the spleen and lung near the clumps of ink contained the usual eosin granules.

THE BLOOD PLATELETS.

Our results show that, when a massive dose of foreign particles is injected into the blood stream, they are quickly agglutinated, together with the platelets, which are thus removed from the circulating blood, and the number of platelets lost would appear to depend on the number of foreign particles injected. The platelets are rapidly restored again to their normal numbers, however, after an injection of colloidal silver and indian ink. This may be due to the breaking up of the clumps and not to a fresh output of platelets from the bone marrow. When *S. aureus* is injected in the circulation the platelets seem to be completely removed, for they are not seen in clumps in the shed blood, and take much longer to return to their normal numbers than when ink or colloidal silver is used. Bull (1915) states that when anti-pneumococcal serum is injected intravenously into animals infected with pneumococci, agglutination of the organism occurs in the blood stream, and subsequently phagocytosis by polymorphs in the lungs, liver and spleen. He found experimentally *in vitro* that when the immune serum was added to live pneumococci suspended in defibrinated blood, agglutination occurred at a higher titre than with saline suspensions of the organism. Bull found also that the action of an agglutinating serum is much more rapid and acts at a considerably higher dilution when injected intravenously than when tested *in vitro*, and is equally type specific. Delrez and Govaerts (1918) found that when anti-pneumococcic serum is injected intravenously it causes agglutination of the pneumococci, but they do not clump with the platelets.

We attempted to ascertain whether, in a convalescent case of paratyphoid fever, the patient's platelets had acquired the power of sticking to and causing the agglutination of the specific organism. Blood was taken rapidly from a vein by an ordinary needle (not waxed) direct into sodium citrate solution in a small waxed centrifuge tube, so that the concentration of citrate was 1 per cent. This citrated blood was centrifugalised and the supernatant plasma containing the platelets which was used for our experiments is referred to below as "platelets." Serum was taken at the same time, and was found to

have an agglutinating titre of 1/1000 with our stock *B. paratyphosus* B antigen. A 24 hours' agar culture of *B paratyphosus* B and of a haemolytic *S. aureus* grown at 37° C. were suspended in normal saline. Agglutination tests were then made in waxed tubes at 37° C. as follows:

(1) "Platelets" alone.
(2) "Platelets" and the patient's serum.
(3) "Platelets" and *B. paratyphosus* B.
(4) "Platelets" and *S. aureus*.
(5) "Platelets" and *B. paratyphosus* B and patient's serum.
(6) "Platelets" and *S. aureus* and patient's serum.
(7) *B. paratyphosus* B and patient's serum.
(8) *S. aureus* and patient's serum.

The total volume in each tube was 1 c.c. 0·5 c.c. "platelets" was put into the tubes with a waxed pipette. The serum was diluted 1 in 10. At the end of 2 hours at 37° C. there was good agglutination in the control tube (7) containing *B. paratyphosus* B antigen and serum. Drops were then taken in waxed pipettes from the other tubes and examined as follows:

(1) Hanging drop.
(2) Dried and stained with Leishman.
(3) Smear made and stained with Leishman.
(4) Mixed directly with cresyl blue solution and allowed to dry; counter-stained with Leishman.

Each preparation showed a similar result:

(*a*) The platelets were all quite separate and had not clumped in any of the tubes.

(*b*) Those bacteria, which were not agglutinated, were not adherent to the platelets.

(*c*) Small and large clumps of bacteria were quite separate from the platelets.

This experiment shows that the platelets had not acquired the property of adhering to the specific organisms; they were not clumped and had not agglutinated the bacilli, and that the conglomeration of organisms and platelets which occurs in whole blood in the circulation had not occurred in test-tube experiments such as we have described.

Wright (1927) by *in vitro* experiments showed that a suspension of indian ink, which is stable in saline and blood serum, is precipitated by whole blood in very small particles, some of which are aggregated with platelets.

It is as yet not proven that the platelets are important in the elimination of foreign particles from the circulation.

Delrez and Govaerts (1918) observed a big drop in the circulating platelets following the injection of *S. aureus* or *B. paratyphosus* B as the bacteria became attached to clumps of platelets.

Govaerts (1921) stated that an injection of anti-platelet serum before an animal is inoculated with *B. typhosus* has no effect on the rate of disappearance of the bacillus from the blood stream, and Bull and McKee (1922) confirm these results with *S. aureus* and pneumococci. Taniguchi, Joogetsu and Kasahara (1930) found a rapid disappearance of platelets from the peripheral blood after the intravenous injection of various organisms, but the number of platelets returned to the normal in 1 hour. They observed agglutination of bacteria and platelets in films made from the blood of infected animals.

Using the technique recently described by Goadby (1930) platelets were counted in rabbits before, and at various intervals after, the intravenous injection of live and dead *S. aureus*, indian ink and colloidal silver. The blood examinations were made 1½, 5 and 10 minutes, and 2 hours, after the inoculations. In Table I is shown the effect of the intravenous inoculation of live and dead *S. aureus*. The very rapid fall of blood platelets immediately after the injection of the living organisms or a massive dose of the dead coccus is a very striking phenomenon. Two hours after the injection there is a considerable rise in the number of platelets, although not to the same height as recorded previous to inoculation.

Table I. *The number of platelets per c.mm. of blood, before and after the intravenous inoculation of* S. aureus.

	1 Live organism	2 Vaccine 3000 million	3 Vaccine 30,000 million
Before inoculation	503,400	489,000	1,009,200
Time after inoculation			
1½ minutes	121,000	425,000	187,000
5 minutes	89,400	355,000	184,200
10 minutes	124,000	525,000	356,400
2 hours	364,800	438,000	445,000

After the intravenous inoculation of 20 per cent. colloidal silver, the immediate fall in platelets was even greater than from live *S. aureus*, but amorphous masses of silver and platelets could be seen clumped together, so that the platelets were not countable because the number of free platelets was insignificant. With indian ink, clumps of ink and platelets occurred, but it was possible to count the platelets in the clumps with a fair degree of accuracy. It was noted that the fall in the platelets was very slight and in no sense comparable to the fall following the inoculation of live *S. aureus*.

The results of some experiments done by us suggest that the agglutination of ink and platelets actually occurs in the circulating blood, and not merely in the platelet counting bottle after the blood has been taken for examination, and similarly with colloidal silver. The platelet count in a normal rabbit was done by Goadby's technique, by which 40 c.mm. of blood are taken into a waxed bottle. A small amount of indian ink was then added to the diluted blood in the bottle, so that the concentration of the ink was approximately the same as in the blood of a rabbit injected intravenously with 1 c.c. of

indian ink. The mixture was shaken from time to time for a period of 2 hours, and then the platelets were counted to see whether there was any diminution in their numbers or if they had clumped together with the ink, as occurs after an intravenous injection. It was found that the platelet count was unaltered and that no clumping of the ink and platelets had occurred.

Conclusions.

When any inert suspension such as indian ink or colloidal silver is injected into the circulation, the first effect is the aggregation of the particles with the platelets: these clumps adhere to the walls of the lung capillaries first, some pass on to the liver and spleen sinuses and, in these situations, the foreign particles are taken up by the endothelial cells. At the same time, nearly all the circulating polymorphs collect in the capillaries of the internal organs, *especially in the lungs*, but there is little phagocytosis by these cells. As the particles are removed from the circulation, the platelets are restored to the blood, probably by the breaking up of the clumps, but possibly also by a fresh supply from the bone marrow. There are always in any situation a certain number of particles not ingested, and also a number of empty phagocytes, however much excess of foreign body is present.

When a suspension of *S. aureus* is injected intravenously the same process develops. The cocci are conglomerated with the platelets, but these clumps are filtered off at first almost entirely in the lung capillaries where the available polymorphs collect, and almost instantaneously active phagocytosis of the cocci occurs. This is in great contrast to indian ink which is quickly distributed all over the body and taken up by the endothelial cells. When ink or colloidal silver is injected intravenously, the blood is observed to be very dark in colour within 30 seconds.

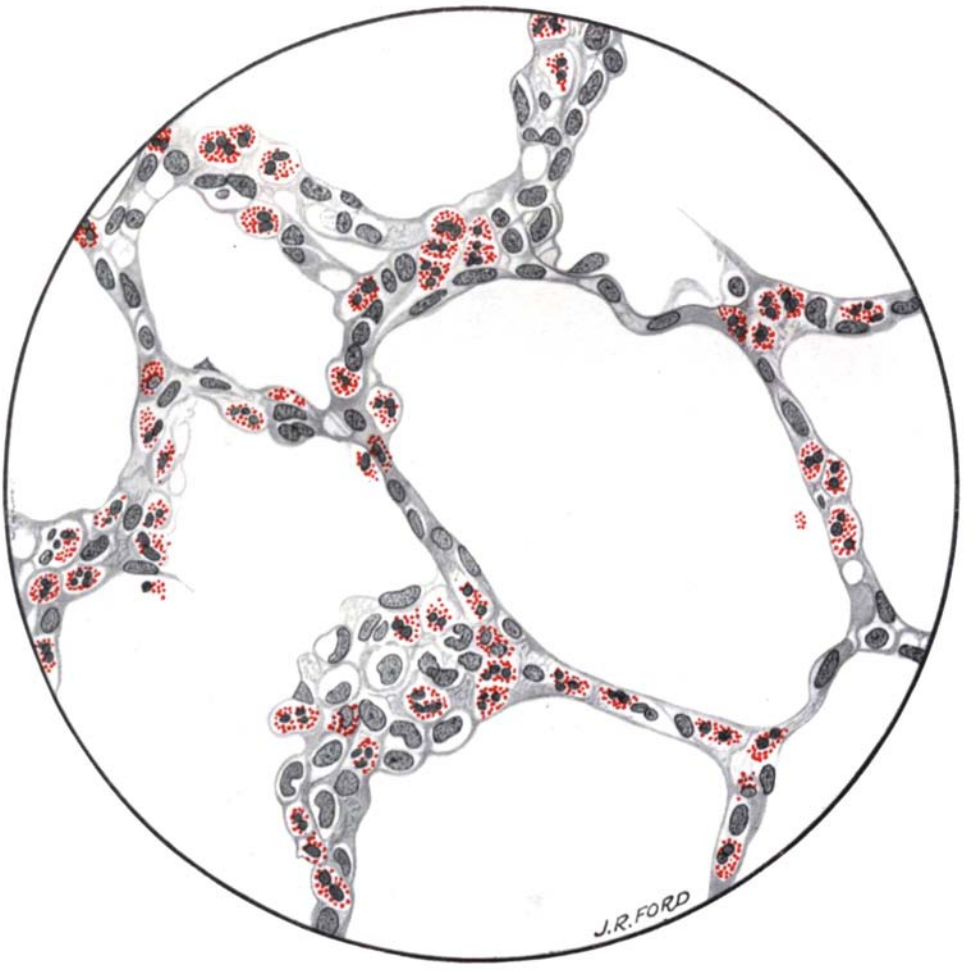
The polymorphs containing the ingested cocci may pass further on into the circulation, and be arrested in the spleen or other organs; some migrate through the alveolar walls and can be seen in the lumen of the alveoli or bronchi; others are phagocytosed by the endothelial cells. As time goes on, more and more cocci are found free in the capillaries in spite of the presence of large numbers of polymorphs which are not phagocytic.

Summary.

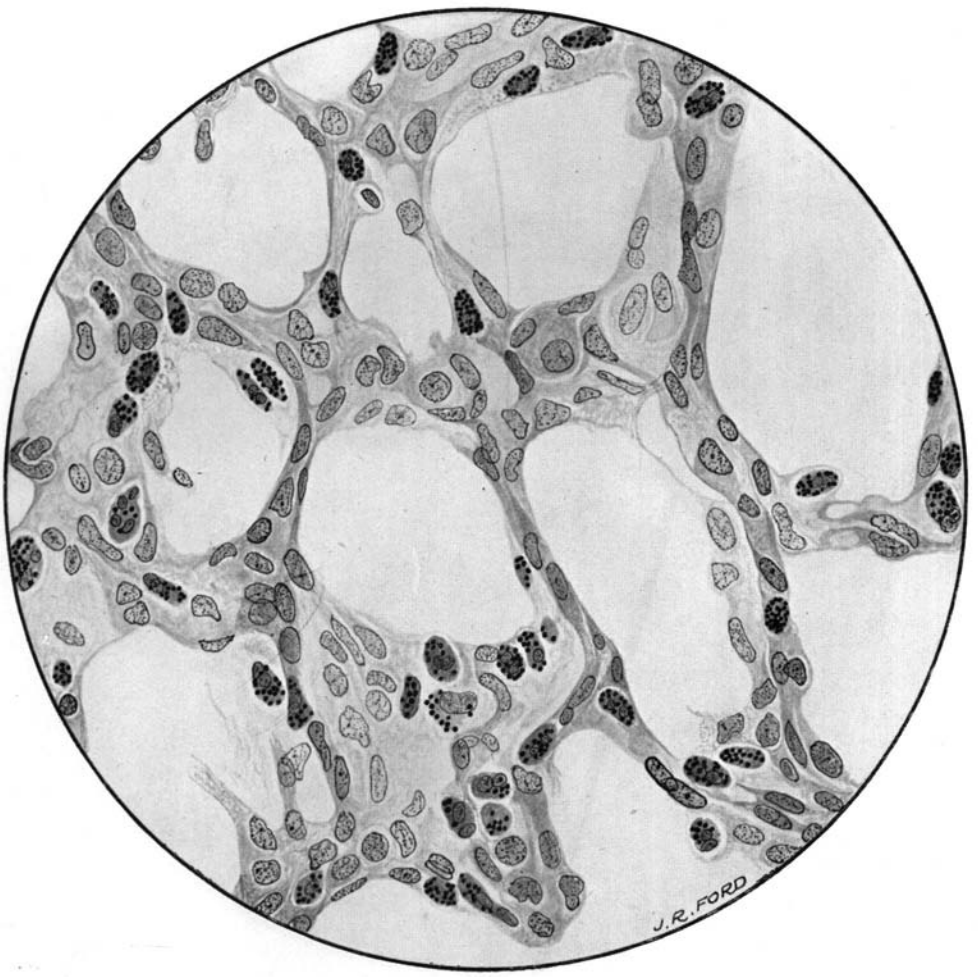
1. The tissue reactions in rabbits from intravenous injections of live and dead *Staphylococcus aureus* and massive doses of indian ink and colloidal silver have been studied.

2. Any particles injected into the circulation cause the accumulation of polymorphs in the lung capillaries.

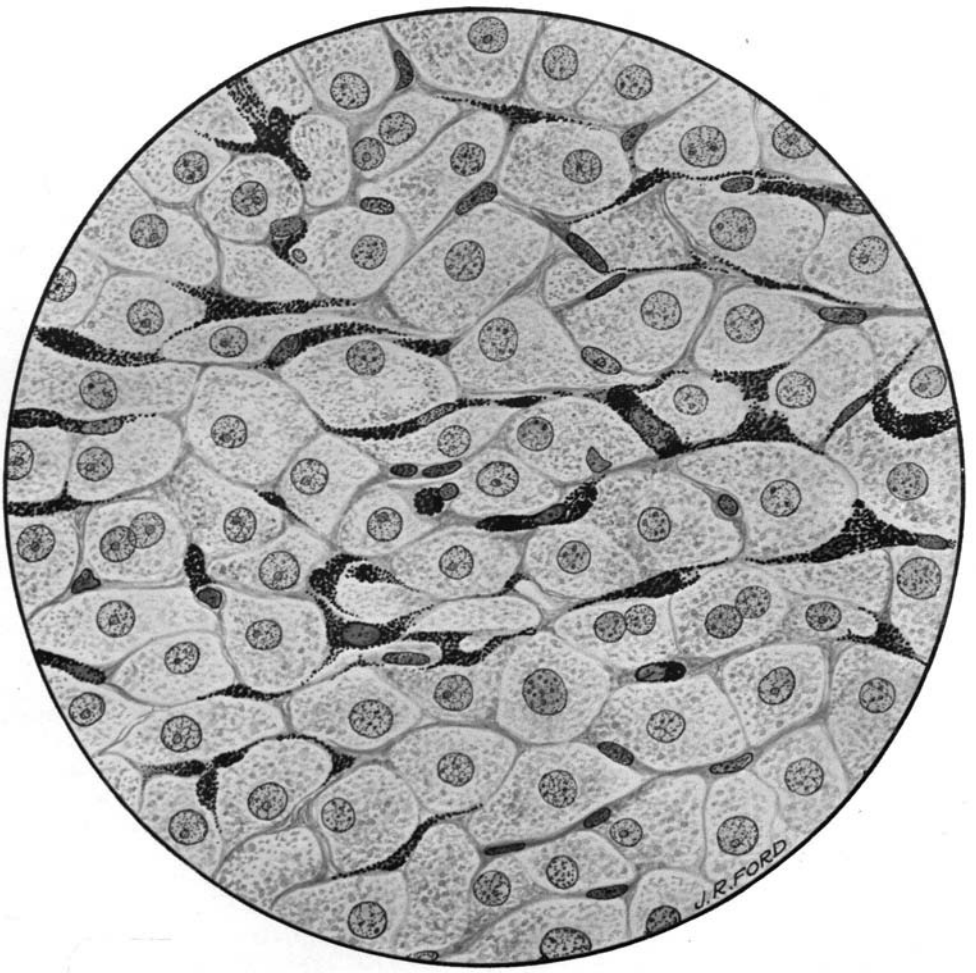
3. Inert colloidal particles such as indian ink are clumped in the capillaries of the lungs, liver, spleen and kidneys, and are phagocytosed by the endothelial cells.



https://doi.org/10.1017/S0022172400010792 Published online by Cambridge University Press



https://doi.org/10.1017/S0022172400010792 Published online by Cambridge University Press



https://doi.org/10.1017/S0022172400010792 Published online by Cambridge University Press

4. Staphylococci (*S. aureus*), live or dead, are nearly all held up in the lungs, where they are actively phagocytosed by the polymorphs within 5 minutes of an intravenous injection.

5. Subsequently the cocci are distributed to the other organs, where phagocytosis continues mainly by polymorphs, but in the liver also by the Kupfer cells.

6. Special attention is drawn to the localisation of the cocci in certain areas in the kidneys.

7. Platelet counting on animals injected with various substances showed that there is an agglomeration of the particles with the platelets, which are consequently removed from the circulation.

8. In the case of the inert particles the platelets are then restored to the circulation. With organisms (*S. aureus*) some of the platelets appear to be completely removed from the blood together with the bacteria.

EXPLANATION OF PLATES I–III.

PLATE I.

Lung of rabbit. Polymorphs in alveolar walls 5 minutes after an intravenous inoculation of *S. aureus*. Section stained with haemalum and eosin. (× 650.)

PLATE II.

Lung of rabbit. Polymorphs in alveolar walls showing very active phagocytosis of *S. aureus* 5 minutes after an intravenous inoculation of this organism. Section stained with haemalum and Gram's stain. (× 650.)

PLATE III.

Liver of rabbit. Large mononuclear cells in liver sinuses showing very active phagocytosis of indian ink particles 5 minutes after an intravenous inoculation of this substance. Sections stained with haemalum and eosin. (× 650.)

REFERENCES.

ANDREWES, F. W. (1910). *Lancet*, i, 1737; ii, 8, 83, 153.
BAIL, O. (1905). *Arch. f. Hyg.* **52**, 272.
BARTLETT, C. J. and OZAKI, Y. (1917). *J. Med. Res.* **35**, 465.
—— —— (1918). *Ibid.* **37**, 139.
BOERNER-PATZELT, D. (1923). *Zeitschr. f. d. ges. exper. Med.* **34**, 336.
BULL, C. (1914). *J. Exper. Med.* **20**, 237.
—— (1915). *Ibid.* **22**, 457, 466, 475, 484.
—— (1916). *Ibid.* **24**, 7, 25.
BULL, C. and MCKEE, H. (1922). *Amer. J. Hyg.* **2**, 208.
CAPPELL, D. F. (1930). *J. Path. and Bact.* **33**, 175–96.
DELREZ, L. and GOVAERTS, P. (1918). *C. R. Soc. Biol.* **81**, 53.
DUDGEON, L. S. and GOADBY, H. K. (1930). *J. Hygiene*, **30**, 180.
GOADBY, H. K. (1930). *J. Path. and Bact.* **33**, 1171.

GOVAERTS, P. (1919). *C. R. Soc. Biol.* **82**, 927.
—— (1920). *Ibid.* **83**, 196.
—— (1921). *Ibid.* **85**, 667.
HOPKINS, J. G. and PARKER, J. T. (1918). *J. Exper. Med.* **27**, 1.
LANG, F. J. (1926). *Arch. Path. and Lab. Med.* **1**, 41.
LEVADITI, C. (1901). *Ann. Inst. Pasteur*, **15**, 894.
NAGAO, K. (1920). *J. Inf. Dis.* **27**, 327.
NISSEN, R. (1922). *Zeitschr. f. d. ges. exper. Med.* **28**, 193.
PARKER, J. T. and FRANKE, E. (1918). *J. Med. Res.* **39**, 301.
SINGER, E. (1925). *Zeitschr. f. Immunitätsforsch.* **43**, 285.
TANIGUCHI, T., JOOGETSU, M. and KASAHARA, T. (1930). *Japan J. Exper. Med.* **8**, 55.
WERIGO (1894). *Ann. Inst. Pasteur*, **8**, 1.
WESTHUES, M. (1922). *Beitr. z. allg. Path. u. Path.-Anat.* **70**, 223.
WISLOCKI, S. B. (1924). *Amer. J. Anat.* **32**, 423.
WRIGHT, H. D. (1927). *J. Path. and Bact.* **30**, 185.

(*MS. received for publication* 30. x. 1930.—Ed.)