THE PROTECTION OF PATHOGENIC MICROORGANISMS BY LIVING TISSUE CELLS.

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PLATE 90.

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The discovery by Metchnikoff of the purposeful character of phagocytosis has so stimulated investigation of the defensive activities of tissue cells that phagocytosis and bacterial destruction are at present almost synonymous in the general mind. The old view that leukocytes provide ingested organisms with a culture medium and a means of transport is now mentioned only in historical résumés. It is known that bacteria may be ingested alive—Metchnikoff himself utilizes this fact in his demonstration of the importance of cells for immunity[1]—and it is known also that a cell may take up too many microorganisms and dying of a surfeit, as one might say, may fail to kill them. But such occurrences are regarded as mere incidents in the process of destruction. The possibility that in certain instances cells not only fail to kill the organisms they ingest, but actively protect them from circulating antibodies seems not to have been considered. Yet the question thus raised has more than passing interest. There are a number of important diseases, among them leprosy, tuberculosis, gonorrhea, Leishmania, caused by microbic parasites which live more or less habitually within tissue cells. The part played by the host cells in the life of such microorganisms and also in the distribution within the body of the diseases they induce has obvious importance.

Unfortunately it is not possible to make direct *in vitro* tests with the microorganisms mentioned and the cells in which they live, for the reason that they fail to give rise to circulating antibodies active

[1] Metchnikoff, E., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, 2nd edition, 1913, ii, 679.

enough to be suitable for the tests. But the problem can be approached by means of artificial systems. It is possible, for example, to submit leukocytes that have ingested bacteria to a bactericidal serum and observe its effect on the intracellular organisms.

Protection against a Foreign Antiserum.

In a first experiment we have used *Bacillus typhosus*, the leukocytes of the guinea pig, which, according to Pettersson,[2] contain no substances destructive to typhoid bacilli, and the serum of normal rabbits, which is strongly bactericidal for the organism.

Experiment 1.—Washed leukocytes from two sterile, 18 hour, aleuronat exudates of the guinea pig's peritoneal cavity were made into a single thick suspension with Locke's solution, and incubated with typhoid bacilli in the presence of a much diluted mixture of fresh guinea pig serum and antityphoid rabbit serum. The smallest amount of the mixture that would ensure good phagocytosis had been previously determined. Twice this amount was employed. The suspension of typhoid bacilli consisted of four 24 hour slant agar cultures of different strains[3] made up in 80 cc. of Locke's solution.

After 1 hour's incubation films from the phagocytic mixture and from a control mixture without serum were examined, with Manson's stain. Only in the former was phagocytosis observed. It was profuse, though there were still many free bacteria. Now small portions of the mixtures and of other control mixtures were added to large amounts of fresh, normal rabbit serum, and the incubation was continued 2 hours longer. Plating was then done in equal portions of agar. Duplicate tests were made throughout. For the dilutions Locke's solution was used.

As the experiment shows, leukocytes can protect ingested bacilli from the action of a bactericidal antiserum.

The mixture of leukocytes and bacteria subjected to preliminary incubation without serum (Mixture 2), and consequently free of phagocytosis at the end of the first hour, gave many more colonies in the plates made 2 hours after the addition of rabbit serum than did Mixtures 3 and 4 from which leukocytes were absent. This was probably due to the protection of ingested organisms, despite the fact

[2] Pettersson, A., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1905, xxxix, 423.

[3] These were laboratory strains known respectively as Board of Health, Metchnikoff, Wassermann, and New York Hospital, which had been under cultivation for more than 2 years.

TABLE I.

Mixture.	Locke's solution.	Leukocytes.	Bacteria.	50 % guinea pig serum.	2 % antityphoid rabbit serum.	A.	Colonies per square cm. after 17 hrs.	B.	Colonies per square cm. after 17 hrs.
	cc.	cc.	cc.	cc.	cc.				
1	—	0.2	0.2	0.1	0.1	1 hr.'s incubation 0.3 cc. from each tube was mixed with 2.4 cc. normal rabbit serum; and after 2 hrs.' more incubation 0.5 cc. of this mixture was plated in 6 cc. agar.	211 294	Same as A except that 0.3 cc. was mixed with 2.4 cc. Locke's solution instead of serum.	About 500.
2	0.2	0.2	0.2	—	—		72 49		Innumerable.
3	0.2	—	0.2	0.1	0.1		10 13		Exceedingly numerous.
4	0.4	—	0.2	—	—		25 27		Innumerable.

that the bacteria were all outside the cells at the time the rabbit serum was added. For the rabbit serum itself was able to cause phagocytosis and did actually cause this in the mixture, as the films show. Presumably it brought about the ingestion of some living bacteria which were then protected from its further action by the cells containing them.

The character of the protection was not determined in this experiment. The results of Table I can be interpreted otherwise than wholly in terms of bactericidal action. The serum was agglutinative; and agglutination can of itself produce a reduction in the number of colonies from a bacterial suspension. The leukocytes might have protected the bacilli merely mechanically against clumping and thus have brought about the results seen in the plates. Obviously, for further work a non-agglutinating bactericidal agent was desirable.

An Indicator of Cell Death.

The question came up, furthermore, whether the leukocytes exerting a protective influence were alive. Rabbit serum contains an hemolysin for guinea pig erythrocytes, and might well kill the white cells of this species. To solve the point resort was had to tests with

trypan-blue. Evans and Winternitz[4] state that the dye rapidly colors the nuclei of dead cells but does not stain living ones. The following experiment confirms their observation.

Experiment 2.—A 4 day aleuronat exudate from the pleural cavity of a dog was washed and suspended in Locke's solution. It contained many large mononuclear cells capable of phagocyting rat erythrocytes. A part of the suspension was mixed with rat erythrocytes and dog serum, and incubated for 1 hour, after which an equal bulk of a freshly prepared and filtered solution of trypan-blue (0.02 gm. in 2.0 cc. of Locke's solution) was added and the cells forthwith examined. The nuclei of the majority of them failed to stain. None of the many cells that had phagocyted erythrocytes showed nuclear staining.

Other portions of the original suspension were kept in the ice box for several days and then treated in the same way. Most of the cells now failed to take up the rat corpuscles, and most stained promptly with trypan-blue. Among the few which did not stain were those which had just phagocyted rat cells.

The results of this experiment have been borne out by many subsequent observations involving injury to cells of other types, among them the cells liberated from tissue cultures by digestion of the plasma clot with trypsin.[5] Trypan-blue is a prompt and reliable indicator of whether cells are alive or dead.

Protection against an Inorganic Disinfectant.

The test with trypan-blue showed that rabbit serum is injurious to guinea pig leukocytes subjected to it under the conditions of Experiment 1. At the end of 2 hours' incubation about half the leukocytes were dead, as shown by the nuclear staining, whereas in control specimens incubated in salt solution, they were nearly all alive, very few stained cells being observed. Because of the unfitness of rabbit serum for our work, as thus manifested, it was necessary to find another bactericidal agent, one that would not harm the leukocytes or agglutinate the bacteria. Potassium cyanide proved to have both these qualities.

Clowes[6] was the first to demonstrate the difference in resistance of tissue cells and bacteria to potassium cyanide. As far as we are

[4] Evans, H. M., and Winternitz, M. C., unpublished work, cited by Evans, H. M., and Schulemann, W., *Science*, 1914, xxxix, 443.

[5] Rous, P., and Jones, F. S., *Jour. Exper. Med.*, 1916, xxiii, 549.

[6] Clowes, G. H. A., *Brit. Med. Jour.*, 1906, ii, 1548.

aware, his important observations have not been followed up. He found that tumor cells treated *in vitro* with cyanide in a concentration that killed bacteria remained capable of causing tumors on implantation. Our tests have shown that $\frac{N}{150}$ potassium cyanide in Locke's solution will destroy typhoid bacilli in heavy suspension while failing to kill guinea pig leukocytes, as shown by the trypan-blue test. Furthermore, the bacilli are not agglutinated. In Experiment 3 advantage has been taken of these facts.

Experiment 3.—This experiment closely followed Experiment 1 except that potassium cyanide was substituted for rabbit serum. A watery solution of potassium cyanide, isotonic with 0.95 per cent sodium chloride, was used, diluted with Locke's solution to $\frac{N}{100}$ concentration.

No phagocytosis was observed in Tube 2 after the preliminary incubation; but it was pronounced in Tube 1, though large numbers of bacilli were still free. The leukocytes were tested with trypan-blue at the time of plating and were found to be, in general, still alive, as proved by the fact that their nuclei failed to stain. In more concentrated potassium cyanide solutions they died early as shown by the stain. Some potassium cyanide was carried over into the agar plates but there it was greatly diluted. Control tests with regard to this point showed that the addition to agar of more than ten times the amount of potassium cyanide present in our plates failed to prevent or even to delay the growth of typhoid organisms.

TABLE II.

Mixture.	Locke's solution.	Leukocytes.	Bacteria.	50 % guinea pig serum.	2 % antityphoid rabbit serum.	A.	Colonies per square cm. after 17 hrs.	B.	Colonies per square cm. after 17 hrs.
	cc.	*cc.*	*cc.*	*cc.*	*cc.*				
1	—	0.2	0.2	0.1	0.1	1 hr.'s incubation then 1.2 cc. $\frac{N}{100}$ potassium cyanide added; 2 hrs.' more incubation and 0.2 cc. plated with 6.0 cc. agar.	144 142	Same as A except that 1.2 cc. Locke's solution added instead of the potassium cyanide.	About 1,500
2	0.2	0.2	0.2	—	—		2 2		Innumerable.
3	0.2	—	0.2	0.1	0.1		0 0		About 1,500
4	0.4	—	0.2	—	—		0 0		Innumerable.

Here there was a marked protection of bacteria by the tissue cells containing them (Table II). The potassium cyanide entirely sterilized the suspensions in which the bacilli were free, whereas in the mixtures in which phagocytosis had occurred, many bacilli survived its action and grew in the plates. One or two colonies developed from Mixture 2, in which there was, supposedly, no phagocytosis because of the absence of serum. But, as is well known, phagocytosis takes place to a slight extent in the absence of serum. The result is readily explained on this basis.

Despite the results of this experiment, the evidence for a protection exerted by living cells specifically is not conclusive. For had the cyanide killed but one phagocyte in every several hundred,—and this may well have happened,—the gross number would be sufficient to permit of an interpretation of the results in terms of a protection exerted by dead phagocytes, not by living ones.

Protection against an Homologous Antiserum.

A second objection to experiments such as the foregoing is that they have little in common with the conditions in the animal body. We have sought to meet both these difficulties by employing red cells as the test object, placing phagocytes which contain them in an hemolytic antiserum derived from an animal of the sort furnishing the phagocytes. With such material it is easy to follow the fate of the ingested corpuscles and to determine by means of the trypan-blue stain whether the individual phagocytes exerting protection are alive or dead. And if the time element be disregarded, one can with good reason liken the conditions as regards the ingested rat corpuscles to those affecting pathogenic microorganisms existing within cells bathed with a lymph containing antibodies.

We have employed rat red cells allowing large mononuclear cells from an exudate in the dog's pleural cavity to phagocyte them, after which an anti-rat dog serum has been added to the mixture. In order to follow the fate of the ingested red cells it has been necessary to know what changes they would undergo when injured within the phagocytes by the hemolytic serum. For the corpuscles cannot lake as they would when free. Fortunately these changes proved easily

recognizable when several red cells were present side by side in a single phagocyte. They have been described and figured by Levaditi[7] and others, who noted them, as we have done, within phagocytes that had taken red cells out of an hemolytic mixture after the hemolysin had attached itself. Under such circumstances hemolysis goes on within the phagocyte with the result that the included corpuscles melt together, as it were, forming one or more large, orange-yellow hemoglobin-containing globules, which on pressure escape from the cell to dissolve instantly in the surrounding fluid. The contrast between these intracellular globules and red cells that remain intact after ingestion is pronounced (Fig. 1).

Experiment 4.—The phagocytes used were mononuclear cells of a 4 day aleuronat exudate in the pleural cavity of a dog. The exudate was washed twice and made into a thick suspension. It contained a very few red cells. The opsonizing serum was derived from the same dog, and so too the 25 per cent suspension of washed dog cells needed for the controls. The serum hemolytic for rat cells came from another dog which received three intravenous injections of rat erythrocytes on successive days and was bled 8 days thereafter. Preliminary examination of the two dogs' blood had shown that they did not agglutinate or hemolyze each other.

The form of the experiment was simple. Rat cells, dog exudate, and a little normal dog serum were mixed together, and, when phagocytosis had taken place, anti-rat dog serum was added to some portions of the mixture, and to others an equal quantity of Locke's solution. Incubation was resumed and from time to time the phagocyted red cells were observed for evidence of destruction. Two preliminary tests were necessary.

(*A*) A determination of the least amount of dog serum which would incite to phagocytosis. The dog serum used for its opsonins contained, as is usual, an hemolysin for rat cells. But the test showed that it could be used to incite phagocytosis in an amount far below the one producing visible erythrocytic change.

(*B*) A test to find out how much anti-rat dog serum was required to hemolyze free rat cells so rapidly that they could not be taken up by dog phagocytes mixed with them. For the anti-rat serum was not only hemolytic but an active stimulant to phagocytosis as well. And, had it been added to the phagocytic mixtures in a quantity which permitted the taking up of cells while hemolysis of them was going on, a proper comparison between the corpuscles in the phagocytes submitted to serum and those submitted to Locke's solution would have been impossible. The results of this test were so interesting that they will be given in detail.

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[7] Levaditi, C., *Ann. de l'Inst. Pasteur*, 1902, xvi, 233.

TABLE III.

Mixture.	25 per cent rat red blood corpuscles.	Dog serum.	Exudate.	Anti-rat serum.	Apparent hemolysis.	Real condition as determined microscopically.
	cc.	*cc.*	*cc.*	*cc.*		
1	0.1	0.1	0.25	0.5	Complete within 5 min.	No phagocytosis. Complete hemolysis.
2	0.1	0.25	0.25	0.25	Complete. (?)	Considerable phagocytosis. All free red blood corpuscles hemolyzed.
3	0.1	0.25	0.25	0.25 of 50% solution.	+++	Profuse red sediment, of phagocytes enormously distended with red cells. All free red cells hemolyzed.
4	0.1	0.25	0.25	0.25 of 25% solution.	++	

Incubation was for 1 hour at 37°C.

Only in the first mixture was there complete hemolysis and in this all the red cells had suffered destruction within the first 5 minutes of incubation (Table III). In the other mixtures the degree of color of the supernatant fluid at the end of an hour indicated incomplete hemolysis as did the profuse red sediment. But these findings were not due to the serum's lack of hemolytic power, for, as the microscope showed, all the red cells remaining free had been hemolyzed. Many though, had been ingested by cells of the exudate, and thus were protected from hemolysis. The abundant red sediment consisted of phagocytes distended with red cells. Some of the phagocytes had extended only the thinnest layer of glassy cytoplasm over the red corpuscles which stood out, quite unhemolyzed, as knobs on their surface. Such corpuscles were evidently protected from the serum by their intracellular situation. But most of the ingested red cells had been much injured and had coalesced into orange-yellow globules (Fig. 1).

The test made it evident that in order to avoid phagocytosis in the presence of the hemolytic serum sufficient of this serum must be added to cause hemolysis of all the red cells within 5 minutes.

Now the main experiment was proceeded to. The following mixtures were made in a number of tubes.

Mixture 1.—0.1 cc. 25 per cent suspension of rat red blood corpuscles + 0.5 cc. exudate + 0.5 cc. 25 per cent dog serum.

Mixture 2.—0.1 cc. 25 per cent suspension of rat red blood corpuscles + 0.5 cc. exudate + 0.5 cc. 10 per cent dog serum.

At the end of an hour's incubation the mononuclear cells were found to have ingested numbers of apparently unchanged red cells,—from 6 to 25, as a rule.

To some of the duplicate tubes 0.5 cc. anti-rat dog serum was added, to others the same quantity of Locke's solution, and incubation was resumed. At the end of a further hour the preparations were examined for evidence of destruction of the intracellular red cells. None had occurred. The erythrocytes within the mononuclears submitted to anti-rat serum, like those within the phagocytes treated with Locke's solution, were still intact (Fig. 2). But the anti-rat serum had hemolyzed all extracellular erythrocytes and the phagocytes lay in the midst of masses of shadows.

A still more exacting test was carried out, as follows:

Mixture 3.—0.1 cc. 25 per cent suspension of rat red blood corpuscles + 0.5 cc. exudate + 0.5 cc. concentrated dog serum. After 1 hour's incubation 0.5 cc. anti-rat serum was added to some of the tubes, an equivalent amount of Locke's solution to others, and incubation continued for 2 hours more. In this instance the amount of serum used for opsonization caused injury to the red cells, of which some coalesced into globules immediately after their ingestion, though the majority remained intact. And now when the phagocytes full of these globules and of more or less injured and vulnerable cells were submitted to anti-rat serum and to Locke's solution, respectively, for 2 hours, no microscopic differences in their contents were observed. The anti-rat serum had been powerless to enhance the breaking-down of the red cells.

The dog red cells present in the exudate were far too few to constitute a source of error in the findings. Nevertheless, a number of control tests were made with a 25 per cent suspension of dog cells instead of rat cells. They were not phagocyted. In the experimental tests proper, the free dog cells were easily distinguished by their failure to agglutinate or hemolyze in the anti-rat serum.

The results of this experiment were clear-cut. The phagocytes protected red cells within them from the action of a powerful homologous antiserum (Figs. 2 and 3).

Protection a Function of the Living Cell.

The condition of the leukocytes exerting this protective action remained to be determined. Were they perhaps injured by the antiserum, despite the absence from it of agglutinins and hemolysins? The failure of the serum to penetrate could be explained in this way. Or was the protection a function of the living leukocytes and of living ones only? Tests with the material of Experiment 4 threw light on these points.

Experiment 5.—(*A*) Cells of the ultimate mixtures of Experiment 4 were examined with trypan-blue. The phagocytes which had been incubated with

anti-rat serum and those submitted to Locke's solution alike failed to take the stain. Many of the white cells that had failed to ingest red corpuscles showed nuclear staining.

(*B*) The following mixture was made up with the ingredients of Experiment 4:

0.1 cc. Locke's solution + 0.5 cc. washed exudate + 0.5 cc. concentrated anti-rat serum + 0.5 cc. dog serum.

After 1 hour's incubation the cells were separated out with the centrifuge and made up as follows:

0.5 cc. treated cells + 0.1 cc. 25 per cent suspension of red blood corpuscles + 0.5 cc. dog serum.

At the end of an hour profuse phagocytosis had taken place, proving that the leukocytes could not have been seriously injured by the antiserum.

(*C*) Portions of the ultimate mixtures of Experiment 4, in which phagocytosis had occurred, were kept in the ice box at about 2° C. and examined each day. At the end of the first 24 hours the cellular sediment had largely lost its ruddy color. The microscope showed that this was due to diffusion out of the phagocytes of pigment from the ingested red cells. The majority of the leukocytes had now a ground glass appearance. In Mixtures 1 and 2 there could be seen within the phagocytes the intact stromata of red cells from which the hemoglobin had disappeared. In Mixture 3 the stromata were not so clearly visible. In this instance, one will recall, the majority of the red cells were much damaged previous to ingestion. In all the mixtures there were still some phagocytes containing bright red cells, and in Mixture 3 some with orange-yellow globules. Phagocytes containing one or two intact cells and the shadows of others were not observed; but the protoplasm of many phagocytes was stained light orange, due to the seeping out of the hemoglobin from ingested red elements. With the trypan-blue test it was found that the cells containing bright erythrocytes or globules regularly failed to stain. So too did the cells tinted light orange. Practically all the other leukocytes underwent an immediate nuclear staining.

After 3 days in the ice box the results were identical except that living phagocytes were now rare.

This experiment proved that the protection exerted by the phagocytes in Experiment 4 was not due to injury, but on the contrary was associated with active cell life. When the phagocytes died they became permeable, allowing a rapid diffusion outwards of the hemoglobin from the ingested erythrocytes, as well as diffusion inwards of the trypan-blue stain. It seems highly probable from these facts, as well as from common knowledge of the differences in permeability between dead and living tissues, that when phagocytes die they must lose largely if not entirely their protective power.

DISCUSSION.

There are in the literature a number of detached observations which corroborate our findings. Bordet found that "cholera spirilla injected into the blood stream of cholera immune animals are taken up by the leukocytes even before they can be subjected to lysis by the circulating lytic antibodies."[8] And Metchnikoff, Levaditi,[7] Briscoe,[9] and others have shown that red cells injected into the previously immunized animal may be phagocyted before they can hemolyze. But so far as we are aware no attention has been paid to these indications of protection by cells. Such protection had no practical importance in the instances cited because the phagocytes themselves were capable of destroying the organisms they had ingested. In our experiments as well the phagocytes may have possessed this ability. The demonstration of protection by them is not thereby invalidated. For the ability of cells to protect ingested organisms from the action of outside agencies must be considered as entirely distinct from the disposition they ultimately make of these organisms.

It remains to be determined how far the protection of microorganisms by living tissue cells, especially cells incapable of killing the microorganisms, is important in disease processes. The phenomenon may have much to do with the survival in the animal body of organisms such as the leprosy bacillus which is so often found living within cells of the fixed tissues; and it may serve to explain in part the therapeutic difficulties in such instances. It may throw light, furthermore, on the formation of new disease foci at points of injury in individuals of high general resistance. For if an infective agent can be "walled off" from the action of the body fluids by the protoplasm of a single cell containing it, there is no reason why it should not be carried unharmed wherever this cell goes.

CONCLUSIONS.

1. Living phagocytes are able to protect ingested organisms from the action of destructive substances in the surrounding fluid, and even from a strong homologous antiserum.

[8] See Zinsser, H., Infection and Resistance, New York, 1914.

[9] Briscoe, J. C., *Jour. Path. and Bacteriol.*, 1908, xii, 66.

2. There is evidence that the protection by phagocytes is largely if not entirely conditioned on their being alive.

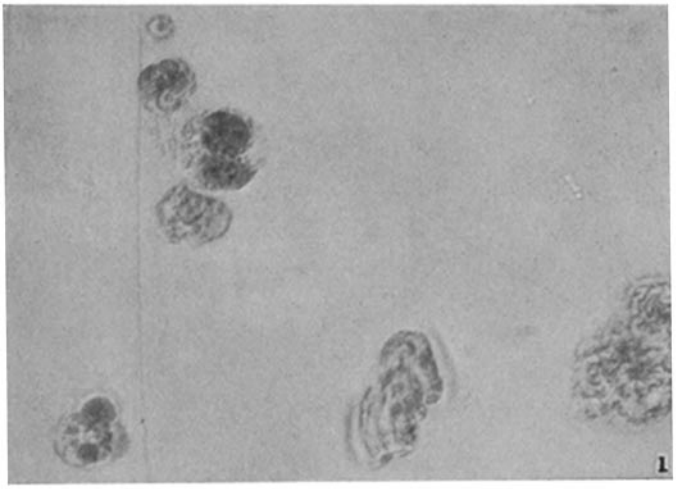
3. These facts should be taken into consideration in the study of diseases caused by infectious agents capable of living within tissue cells.

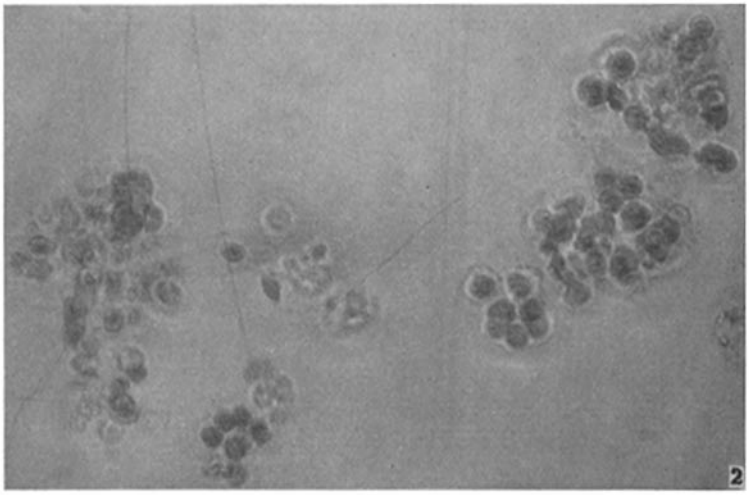
EXPLANATION OF PLATE 90.

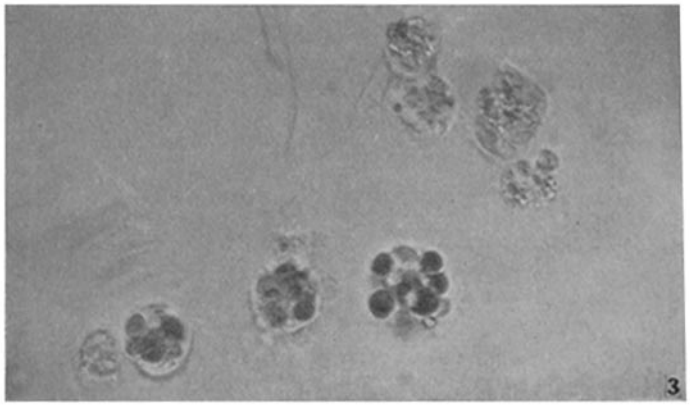
FIG. 1. Red cells hemolyzing within phagocytes. There is a coalescence of the cells into globules. × 625.

FIG. 2. Intact red cells of the rat within dog phagocytes submitted for 1 hour to a powerful anti-rat dog serum. Many of the red cells appear pale because they are out of focus. The only free erythrocytes that have not been laked are a few dog cells. × 625.

FIG. 3. Red cells of the rat still intact within dog phagocytes submitted for 2 hours to a dog serum strong'y hemolytic for rat cells. The shadows of numerous erythrocytes hemolyzed while free are just visible. × 625.







(Rous and Jones: Protection of Pathogenic Microorganisms.)