THE NORMAL FATE OF ERYTHROCYTES.

I. The Findings in Healthy Animals.

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Plates 55 and 56.

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It has long been recognized that in the healthy body a considerable proportion of the erythrocytes are broken down and replaced every day—exactly what proportion is not known. The bile pigments have been deemed an indicator of the hemoglobin destroyed. Calculations based upon their rate of formation would seem to show that blood destruction is very rapid, in man from one-tenth to one-fifteenth of all the corpuscles being lost and replaced in 24 hours.[1] But the recent work of Whipple,[2] who has proved that the bile pigments may have other sources than the blood, demonstrates a large possible error in such calculations. Perhaps the most certain evidence of blood destruction is to be found in the constant activity of the bone marrow in the production of new cells.

The Phagocytosis of Red Cells.

There exists an extensive but inconclusive literature on the normal method of destruction of the red cells. Pearce and Austin[3] say truly that only two facts regarding this method have been established: first, that certain large endothelial cells, located for the most part in the spleen, take up red corpuscles and destroy them; and, second, that blood pigment is sometimes present in the Kupffer cells of the liver,

[1] Calculated from figures given by Howell, W. H., A Text-Book of Physiology for Medical Students and Physicians, Philadelphia, 1917, 6th edition.

[2] Whipple, G. H., and Hooper, C. W., *Am. J. Physiol.*, 1916, xl, 349.

[3] Pearce, R. M., and Austin, J. H., *J. Exp. Med.*, 1912, xvi, 780.

indicating that they may play a part in the destruction. Some investigators hold that phagocytosis is of itself sufficient to account for blood destruction. In our work, this possibility has been the first considered.

Certain cells of the bone marrow and, exceptionally, of the lymph glands may ingest red corpuscles, but they are in general negligible as compared with the phagocytes in the spleen. Sections of the spleen, as ordinarily prepared, contain so much blood as to conceal the phagocytes in large part and render difficult an estimation of their number. For this reason, we have examined sections of spleens washed out with Locke's solution by way of the vessels, and have searched the centrifuged washings as well. In order to prevent all clotting in the latter, they have been mixed with an equal bulk of an isotonic, watery solution of sodium citrate (3.8 per cent). The spleens of the guinea pig, dog, cat, rabbit, and monkey have been thus examined. That of the guinea pig contains many phagocytes. Numbers of them come away in the washings, a fact easily understood when sections of the half washed organ are studied, for it is then found to contain many wide venules or sinuses in which the majority of the phagocytes lie free mingled with the red cells. The spleen of the rabbit contains similar venules. Phagocytes may be few or plentiful in this animal, depending on individual variation; and not infrequently a few appear in the washings. The numerous phagocytes of the dog spleen are all retained during perfusion in the densely reticulated organ. The bone marrow of this animal may show many cells containing red corpuscles and pigment. The *rhesus* monkey, like man, has few phagocytes normally. The cat shows a practically complete lack of such cells. Indeed, in several adult cats, of which we examined the spleen, bone marrow, lymph glands, and other organs, they were exceedingly rare. It is evident that, in this species, blood destruction must take place by some method other than phagocytosis.

There are wide differences in the size of the spleens of the common laboratory animals, which denote as clearly as the findings just described the dangers of generalization from the results in a single species. In the rabbit, the spleen is so small as to be, relatively speaking, almost vestigial. We have found that it weighs on the

average about 0.6 gm. in an 1,800 gm. animal, thus constituting about 0.033 per cent of the body weight. In the rat, it is proportionally some eight times as large, about 0.27 per cent of the body weight,[4] and in the dog and cat, it represents 0.18 to 0.28 per cent[5] of this weight. These differences would be noteworthy were they dependent merely on differing functional demands of one sort. But the findings as regards phagocytes in the spleens of different species show that at least one function of the organ is subject to wide variation.

Some authors have suggested that the phagocytosis of red cells is not a vital phenomenon, but is agonal or occurs after death. That such a view is erroneous can be shown by opening the splenic vein of an anesthetized guinea pig, which has been washed almost free of blood by means of Locke's solution, introduced through the jugular vein and allowed to escape from the carotid artery. Under these circumstances, if the spleen is gently massaged, many phagocytes filled with intact and fragmented red cells come away in the splenic washings. As in the case of the other animal species examined, the phagocytes are much larger than the mononuclear cells of the circulating blood which take up red cells under pathological conditions, being from 45 to 60 μ or more in diameter. We have attempted experiments on the conditions governing the normal ingestion of red cells, using for this purpose phagocytes washed from the rabbit spleen, but they failed to survive more than a few minutes *in vitro*, as proven by the staining of their nuclei with trypan blue.[6]

The number of corpuscles ingested in the large spleens of the rat and dog is so considerable that, *a priori*, in the absence of definite knowledge of the rate of blood destruction, one might suppose phagocytosis to be the means whereby, in these creatures, the destruction of erythrocytes is accomplished. So, too, in the guinea pig. But in the cat, phagocytosis does not come in for consideration; and in normal man, the monkey, and the rabbit, it cannot be held responsible for the disappearance of any important amount of blood.

[4] Jackson, C. M., *Am. J. Anat.*, 1915, xviii, 75.

[5] This statement is based on isolated instances from several authors to whose papers Professor Jackson has kindly referred us.

[6] Evans, H. M., and Schulemann, W., *Science*, 1914, xxxix, 443. Rous, P. and Jones, F. S., *J. Exp. Med.*, 1916, xxiii, 601.

Extracellular Destruction.

The search for extracellular blood destruction was now begun. Cats were employed for the first experiments. After positive findings had been obtained with them, the observations were extended to other species.

From what is known of the resistance of the red cells, it would seem unlikely that their destruction takes place while they are still in circulation. Tests with hemolytic and hypotonic solutions according to the current methods fail to disclose signs that any of them are in process of breaking down. Some are more frail than others, but the resistance of all is considerable. The washed corpuscles of man and the cat may be shaken in bulk for a considerable time without the destruction of any. All this is difficult to reconcile with the idea that the cells circulate while disintegrating. It would seem either that they are removed by some organ, or set of organs, as soon as they begin to break down, or else that they leave the circulation even before this happens. In either case, one might expect to find somewhere in the body of animals in which phagocytosis is negligible, as, for example, in cats, a locus of disintegrating corpuscles. We have thought to find it by a search of the washings from organs perfused until entirely blood-free, combined with an examination of the washed organs themselves. Such a search presupposes a perfusion fluid in which the corpuscles will not be injured, and a method whereby those that are undergoing the normal disintegration may be collected from out the great mass of intact cells. The first of these requirements is fulfilled by the use of Locke's solution[7] containing a small amount of gelatin, and the second by differential centrifugation of the washings. When freed of serum and placed in normal salt solution, or Locke's or Ringer's solution, the erythrocytes of many species, especially those of the dog and of some rabbits, begin within a few hours to hemolyze. This, as one of us has shown, is the result of mechanical injury, entailed in even the most careful handling. It can be avoided by adding to the solution 0.125 to 0.25 per cent of gelatin.[8] In such a

[7] Locke's modification of Ringer's solution, but without sugar.

[8] Rous, P., and Turner, J. R., *J. Exp. Med.*, 1916, xxiii, 219.

fluid, delicate corpuscles remain intact, although pipetted or even shaken. The present work has constituted itself a severe test of the efficacy of the protection afforded by the gelatin. For the cell shadows that are the product of mechanical injury are easily recognizable, and by the differential centrifugation we have employed, they are brought into the preparations examined. Rarely has one been observed. Indeed, their consistent absence from the specimens obtained on perfusion and from teased preparations of the organs perfused renders it certain that normal blood destruction does not entail the formation of shadows.

We reasoned that disintegrating red cells would probably be lighter than sound ones because of some loss in substance or content. If this were true, they should come down last when the blood is slowly centrifuged in dilute suspension. It proved to be the case in preliminary tests with suspensions of the damaged cells of animals injected intravenously with a specific hemolysin.

Method.

A specimen of the animal's blood was taken by direct cardiac aspiration into a mixture of perfusion fluid and citrate solution, and then, under ether, the viscera were excised one at a time, after the vessels had been temporarily tied or clamped off, and immediately flushed out through the artery with Locke's solution containing 0.25 per cent of gelatin. A large gravity bottle was employed for the perfusion. Before it was begun, each organ was rinsed of surface blood, and during the process it lay in a porcelain dish into which the washings escaped by way of the natural venous channels. The perfusion pressure was ordinarily that of a column of the fluid some 120 cm. high. The washings which came last, and which were thought to represent, not so much the circulating as the residual blood, were caught separately and examined with special care. The kidneys, intestines, liver, and lungs were easily washed out. In order to wash the red bone marrow, a femur was excised, its surface flushed with the perfusion fluid, the bone was chipped away, and the marrow washed directly by means of hollow needles thrust repeatedly into it. In some cases, a limb of the animal was perfused *in situ*. Examination showed that the marrow then failed to wash out. The spleen and bone marrow were the only organs from which the blood failed to come away completely. In them a considerable trace always remained.

To prevent clotting the washings were caught in an equal bulk of isotonic, watery, sodium citrate solution (3.8 per cent). In the later work a mixture of equal parts of the citrate solution and gelatin-Locke's solution was used for the perfusion. 0.25 per cent of gelatin in the wash fluid was found to ensure protection in this ultimate mixture.

Portions of the washing were now slowly centrifuged in tall test-tubes and when the mass of red cells had come down, the shimmering, faintly pink, supernatant fluid was removed to another tube and sedimented at high speed. The slight sediment was suspended in a trace of fluid and examined microscopically. It consisted for the most part of platelets, red cells, and fragments of red cells.

The specific gravity of the wash fluid was such that no distinct leukocytic pellicle was formed.

Bodies Like Degenerated Red Cells.

Examinations of the organs of cats, rabbits, monkeys, and dogs, carried out according to this method, disclosed the presence of peculiar bodies in the washings from the spleen, bone marrow, and especially the liver, which yielded them in great number. They had the appearance of red cells and cell fragments which had lost their hemoglobin, but, unlike shadows, had retained something of their other substance, so that they were still refractile, though never markedly so (Fig. 1). They were not found in the washings from lung, limbs, or intestines. The kidney yielded a few of them. On centrifugation, they came down with the platelets, and when in great numbers, as in the liver washings, they tended on standing to collect into clumps (Fig. 1), which, however, were easily broken up with the pipette. They were spherical, oblong, or sausage-shaped, of all sizes up to that of the red cell or slightly larger, and of many degrees of refractility, some, especially the smaller, being difficult to perceive. The substance of the best preserved had often a greenish yellow cast, and what appeared to be transition forms from red corpuscles were easily found. When placed in blood serum, the appearance of the bodies did not change. Methylene blue and cresyl blue brought out in them a reticulum somewhat more delicate, as a rule, than that of reticulated red cells, but often indistinguishable from it (Fig. 2). Scharlach R and Lugol's solution failed to stain them.

Like red cells, the bodies were laked by water and by bile, acetic acid, saponin in high dilutions (1: 10,000), and by serum hemolysins when complement was present. In all these instances, shadows were left behind similar, except for the variation in size, to those of red cells. The bodies laked somewhat more easily than red cells, as was seen in preparations containing both. On standing in the ice box in salt solution, they became shadows after some hours or days. They

were crenated by hypertonic salt solutions, fixed by formalin and Cupp's fluid (Fig. 3), and agglutinated by specific agglutinins for the red cells. Elements morphologically indistinguishable from them were separated out by differential centrifugation from the blood of rabbits that had become anemic as the result of repeated injections of a serum hemolysin. We repeatedly recovered elements resembling them from the blood of patients with severe pernicious anemia, and from one case of congenital hemolytic jaundice. Other observers[9] have also found them in these conditions. They were regularly found in the circulation of rabbits engaged in breaking down a plethora of blood transfused to them from other rabbits. Furthermore, elements resembling them were observed within the endothelial phagocytes of rabbits injected intravenously with an hemolysin.

Despite all these characters which would seem to identify the bodies with disintegrating red corpuscles, they have another origin, being the products of injured cells of the fixed tissues. This can be shown by washing out at 5 minute intervals the liver of a rabbit, dog, or cat while it is still *in situ*. If ligation of the hepatic artery and the introduction of cannulas into the portal vein and the hepatic end of the inferior vena cava are carried out so rapidly that perfusion supervenes on normal circulation after the lapse of only 1 or 2 minutes, no bodies, or at most only a few will come away in the first washings which free the liver of blood. If there has been delay, the bodies will be found. Always they occur in great number in the washings obtained by perfusion after 5, 10, and 15 minutes. The result is the same whether the fluid used is a salt solution or defibrinated blood. In teased specimens of the liver and spleen, the cells of the parenchyma not infrequently may be seen to give off translucent globules, some of which when free constitute the bodies. Splenic phagocytes containing red corpuscles have been observed to give off "bodies" that are brightly tinted with hemoglobin.

If this is the origin of the bodies, how do they come to circulate in the blood of animals and human beings, the subjects of hemolytic processes? Those seen under such circumstances appear to have

[9] Lee, R. I., Minot, G. R., and Vincent, B., *J. Am. Med. Assn.*, 1916, lxvii, 719.

another source. They are probably reticulated red corpuscles, free of hemoglobin. Though morphologically such elements are characteristic "bodies," their reticulum as demonstrated with cresyl blue is moored in place, so to speak, as is true of reticulated cells in general, whereas that of the bodies usually draws together toward one side into a heavily pigmented skein or ball, some minutes after staining (Fig. 2).

The bodies differ in many ways from Albrecht's myelin, which is a relatively late postmortem product. A discussion of them at such length has seemed warranted because elsewhere we[10] have erroneously reported upon them as disintegrating red cells, and because they do indeed possess many characters which have been regarded as peculiar to erythrocytes. Their study should prove profitable to those interested in the constitution of the red cell.

In the subsequent work, each organ was washed out with all possible speed after the circulation had been interrupted. Often this was done with the organ *in situ*. As a result, the "bodies" have seldom been encountered.

Disintegration by Fragmentation.

The method just described has made possible a close search of the organs, one by one, for disintegrating red cells. Nowhere have hemolyzing cells been found; and the consistent absence from the washings of shadows, as already mentioned, is additional evidence that hemolysis in the gross sense does not occur. Instead, another and unsuspected method of blood destruction has been found; namely, disintegration of the cells, while they are still circulating, by fragmentation without loss of hemoglobin. It occurs in the cat, rabbit, and dog, all the animal species thus far examined.

If 2.5 cc. of blood is taken direct from the heart of a normal animal into 15 cc. of gelatin-Locke's-citrate and submitted to differential centrifugation, microcytes and poikilocytes similar to those of anemic bloods are regularly found, often in considerable numbers, among the platelets and other elements that are last to be thrown down. All stages in their derivation from red cells of normal size can be observed.

[10] Rous, P., and Robertson, O. H., Association of American Physicians, 1916.

Among the few of these latter that are associated with the small forms in the ultimate sediment, some are fragmenting, as shown by deep constrictions in their protoplasm or by their possession of slender processes from which small, hemoglobin-containing bits are separating off. The cells first thrown down by the centrifuge, that is to say the great bulk of the red cells, fail to show any such changes.

The division of red cells into microcytes and poikilocytes—schizocytes, as Ehrlich called them and as we shall for convenience refer to them—can be brought about *in vitro* by the pressure of a coverglass, or by gently heating the fresh preparation (Schultze, Rollet, Ranvier[11]). Such forms may also appear in the blood during caffeine poisoning and after prolonged anesthesia with chloroform.[11] The ease with which they are produced shows the need for careful controls to our observations. These have been carried out. Repeated manipulation of the diluted blood according to the method used in our work does not increase the number of fragmenting forms. They are found when the blood has been taken into oxalated salt solution instead of gelatin-Locke's-citrate. They occur in the blood from anesthetized animals. And, finally, they are present in undifferentiated fresh blood, though they are in general so few that it is easy to see why they have not been recognized as normally present there. They may easily be found in fresh rabbit blood. In looking for them care should be taken to use thick films of the blood, since in thin ones the cells tend to catch on the glass surfaces by which they are compressed and to be pulled in two as the drop spreads, thus producing an artificial fragmentation. This is avoided when the blood is examined in a thick layer or caught directly in a little gelatin-Locke's-citrate and then observed. Under both these circumstances, the number of fragmentation forms is usually small, and indeed some rabbit bloods fail to show them except on long search. In most bloods, though, they are regularly found, one to twenty being noted as a rule in a 5 minutes' search. In the individual the number is remarkably constant from day to day. It may be considerably increased when there is the least anemia.

In the washings from the organs, as obtained by our method,

[11] Cited in Krehl, L., and Marchand, F., Handbuch der allgemeinen Pathologie, Leipsic, 1908, i.

much normal blood is always present, and in consequence schizocytes are always found. It has been necessary to control this factor in the search for accumulations of disintegrating cells. Our practice has been to make in perfusion fluid suspensions of the animal's heart's blood of approximately the same hemoglobin content as each of the organ washings, and to differentiate them in the centrifuge with the latter. Such dilute specimens of the circulating blood yield few schizocytes, and any marked preponderance of them in the washings can with good reason be attributed to the organ's special content.

The results of our search of the organs can be stated briefly. The only evidence encountered of extracellular blood destruction lay in the presence, in the spleen especially, of accumulations of microcytes and poikilocytes such as had been met with in the circulating blood. The spleens of the dog, rabbit, and cat regularly contained numbers of these small forms. They often failed in large part to wash out, being then discoverable in the tissue teased after perfusion. Systematic observations in rabbits have shown that there may be small collections of them elsewhere, most frequently in the kidneys, but these are inconstant and always insignificant as compared with the splenic content, even when the difference in size of the organs is taken into account. The red bone marrow (femur and rib) is notably free of the forms. It is plain that they are not put forth as such by the marrow. Confirmatory evidence on this point is presented in our second paper.

The normal spleen may retain sufficient schizocytes after washing to give the organ a brownish red color, as in the case of many rabbits and cats, or this color may be due in greater part to phagocyted red cells, as in other rabbits and in dogs. In some healthy animals, certainly, the accumulation of schizocytes is not great. Some cat spleens can be washed almost white, and the washings in these cases may contain only moderate numbers of the fragmentation forms. Their number is moderate, too, in the large spleen of the dog. But they are always present and are much more frequent than in the circulating blood. The difficulty with which they are dislodged from the spleen, even on repeated perfusion, is striking. The first

washings in which most of the blood comes away contain but few of them.

A number of control tests have been made which prove that the splenic schizocytes are not artefacts. They are regularly found when the fresh organ is teased in autogenous blood serum. The teasing itself does not cause them, for other organs containing the same amount of blood and similarly teased fail to yield them. They have no relation to the use of ether as an anesthetic. The possibility that they are fragments of corpuscles extruded by phagocytes in animals possessing such cells has been ruled out by means of a differential stain. In fresh preparations in isotonic salt solution, to which a little cresyl blue has been added, the fragments of red corpuscles resulting from intraphagocytic digestion become a deep, opaque blue-black, whereas those produced by extracellular fragmentation fail to stain at all. The specificity of the dye is, in this regard, striking. Red cells when first phagocyted fail to stain. Later they stain copper-green or intensely blue. As breaking down proceeds, the affinity for the stain grows, and the intracellular fragments of red cells stain almost black in solutions of cresyl blue so weak as scarcely to affect other elements. In teased specimens, these blue-black fragments are not infrequently found lying free, a sharp contrast to the non-staining fragments (schizocytes) that result from extracellular destruction.

The Schizocytes.

The shape of the poikilocytes and microcytes found in the peripheral blood and the spleen is to some extent peculiar to the animal species. In the cat spherical or oblong forms predominate (Fig. 4); in the dog, short, blunt rods exist as well; in man, in addition to these, shapes like small asymmetrical starfish are observed; while in the rabbit, there is the greatest diversity of forms—spheres, capsules, shapes like drumsticks, rods, dumb-bells, pears, balls with strings to them, and even short, thick threads brightly tinted with hemoglobin (Fig. 5). If anything, the schizocytes are of a more intense orange hue than ordinary corpuscles. Many very small forms are seen. From the larger forms, fragments so minute as to be at the limit of microscopic visibility can be seen to break off. The

ultimate fate of all would seem to be division and redivision into a fine dust.

Red cells of approximately normal size, from which fragments are in process of separation, are fairly common in the spleens of all the species we have examined. As already stated, they are present in the ultimate sediment obtained on differential centrifugation of the circulating blood. A form always to be found in the rabbit spleen, and sometimes recovered from the blood by differential centrifugation, consists of two deeply tinted portions of corpuscle held together by a median zone of protoplasm so flattened anteroposteriorly as to appear devoid of hemoglobin (Fig. 6), except when viewed from the side. When so viewed this form has a dumb-bell shape, and the connecting zone is seen to be well provided with pigment. In the end the zone gives way, and the cell portions become separate microcytes. We have never found any indication of the decolorization of microcytes in the spleen. In the cat spleen there may sometimes be encountered a few pale and exceedingly thin corpuscles, still tinted with hemoglobin, which look as though they were dissolving away much as discs of sugar dissolve in water.

SUMMARY.

The phagocytosis of red corpuscles, while frequent in the normal dog, rat, and guinea pig, is slight in man, the *rhesus* monkey, and many rabbits. In cats it is always negligible in amount and frequently absent. Phagocytosis will not suffice as a general explanation of normal blood destruction.

When the liver, spleen, and bone marrow of the cat, dog, rabbit, or monkey are slowly perfused with defibrinated blood or Locke's solution, bodies are given off into the fluid which have the appearance of red corpuscles that have lost their hemoglobin but retained the rest of their cell substance. These bodies possess many of the properties supposedly distinctive of red corpuscles. They are the product of disordered parenchymal cells.

By a special method, it has proved possible to search the body, organ by organ, and the circulating blood also, for disintegrating red corpuscles. Shadows of red cells are not present anywhere, nor are hemolyzing red cells found. A hemolytic process, in the

ordinary sense of the term, can scarcely play an important part in normal blood destruction. Instead, it is certain that some red corpuscles, at least, are destroyed in another way; namely, by fragmentation. Normal blood regularly contains small numbers of fragmentation forms—microcytes and poikilocytes—and accumulations of them are regularly present in the spleen, but are found only inconstantly in the other organs. The fragments are in evident process of further subdivision. They occur not only in species in which phagocytosis as a means of cell destruction is negligible (cats), but also in animals in which it is an important process (dogs, some rabbits).

The method of study that we have employed is well suited to disclose how the blood is destroyed. The importance of cell fragmentation in this connection is indicated by our failure to find any other means of destruction, save only the phagocytosis already known. Further facts indicating the importance of fragmentation are presented in our second paper, where a general discussion will also be found.

EXPLANATION OF PLATES.

PLATE 55.

FIG. 1. "Bodies" from the liver washings of a dog. Five ordinary red cells and a leukocyte are also to be seen. The arrows point to the bodies, which are clumped as the result of centrifugation. Fresh preparation.

FIG. 2. "Bodies" stained with cresyl blue. A, immediately after staining; diffuse reticulum. B, later effect; the reticulum has massed to one side. From the liver washings of a dog.

PLATE 56.

FIG. 3. "Bodies" fixed with Cupp's fluid (a formalin-potassium-bichromate-acetic-acid mixture). A, bodies; B, red cells. From the liver washings of a monkey.

FIG. 4. Microcytes and poikilocytes collected by differential centrifugation from the blood of a normal cat. Wright's stain. The upper arrow points to a round microcyte, and the lower to a poorly focused oblong form.

FIG. 5. Microcytes and poikilocytes collected from the blood of a normal rabbit. The dark dots are platelets. The arrows point to the schizocytes of which the diversity of form is well shown. Wright's stain.

FIG. 6. Fragmenting red corpuscles from the spleen of a rabbit. Wright's stain. There is no real loss of hemoglobin from the pale portions of the cells, but here the layer of protoplasm is very thin.

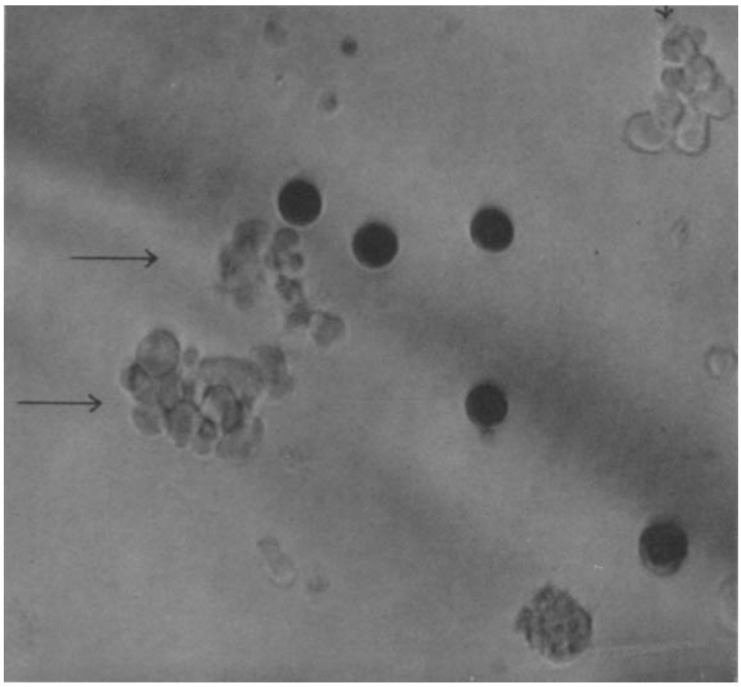

FIG. 1.

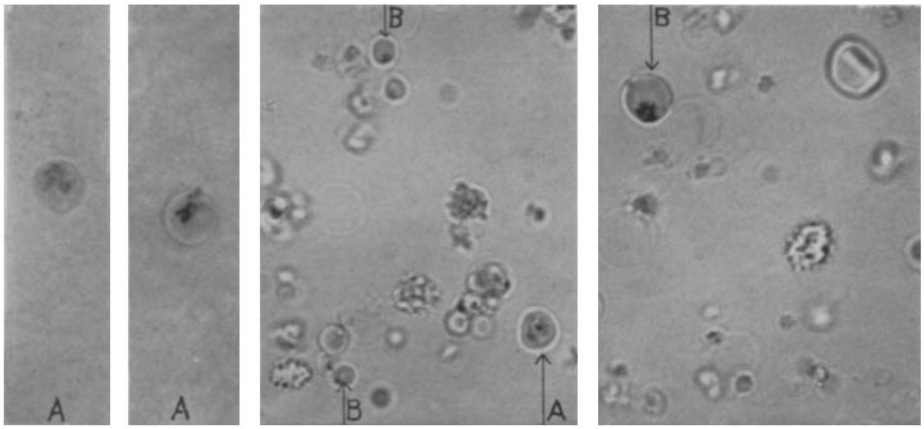

FIG. 2.

(Rous and Robertson: Normal Fate of Erythrocytes. I.)

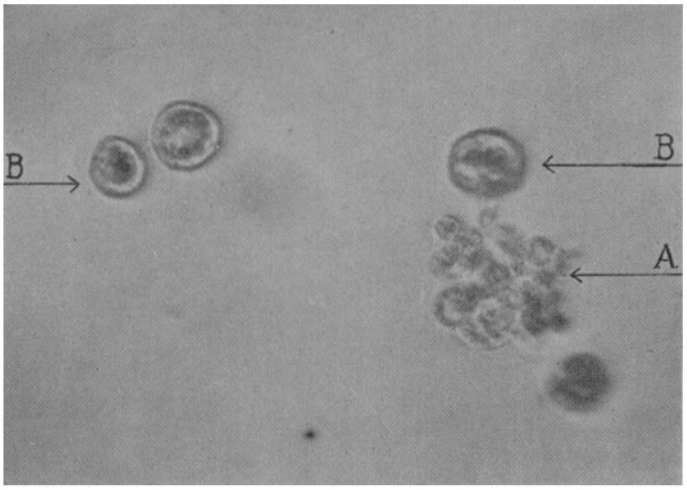

FIG. 3.

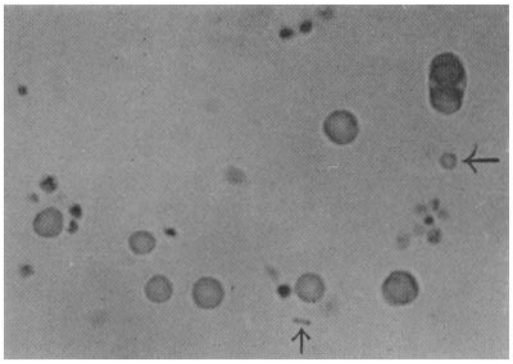

FIG. 4.

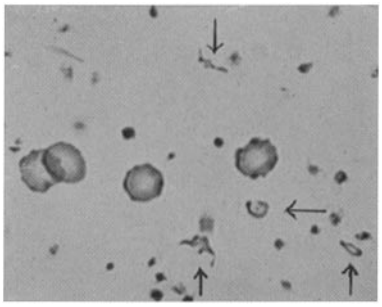

FIG. 5.

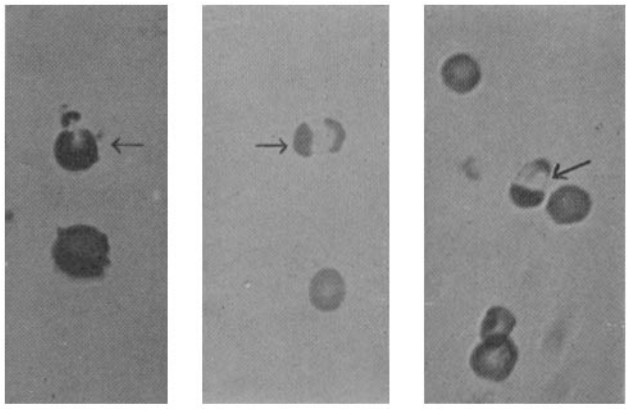

FIG. 6.

(Rous and Robertson: Normal Fate of Erythrocytes. I.)