DISCRIMINATION OF TWO TYPES OF PHAGOCYTIC CELLS IN THE CONNECTIVE TISSUES BY THE SUPRAVITAL TECHNIQUE.

INTRODUCTION.

By the use of the supravital technique, we think it is now possible to discriminate two distinct types of markedly phagocytic cells in the connective tissues, namely, clasmatocytes, which we shall also designate as *wandering endothelial phagocytes*, and monocytes. The term *monocyte* was used by Naegeli to cover the transitional and large mononuclear forms of the blood, in the sense of Ehrlich, and it is chiefly in the hematological literature that monocytes are analyzed, in spite of the fact that it may well prove that, in function, they are more conspicuously a cell of the connective tissues than are the other white blood-cells. We are, however, giving our evidence regarding the formation and development of the monocytes in connection with a study on the origin of all of the white blood-cells, which appears in this same volume.

LITERATURE.

In following the work on the free cells of the connective tissues, developed during the last 30 years, it has become clear that it has progressed along the lines of certain ideas. These ideas are the relative degree of speed of locomotion possessed by different cells, the relative degree of phagocytic ability (including the great mass of experimental work on vital dyes), the reactions to supravital stains, and the origin of the cells. These concepts have been mutually helpful in the course of their development and have been, to a considerable extent, intermingled; together they have resulted in a mass of evidence of the greatest interest and importance.

DIFFERENTIAL MOTILITY OF THE CELLS OF THE CONNECTIVE TISSUES.

The first concept that gave any very specific impetus to an exact definition of the cells of the connective tissues was the work of Maximow (1902) on their rates of motility. He found that when he introduced two sterile cover-slips under the skin in rabbits, and classified the cells according to the time it took them to migrate between the covers, he could clearly define certain types of cells. The first to enter the space between the cover-slips were the polymorphonuclear leucocytes; these appeared in about 4 hours. After about 16 to 18 hours, certain large mononuclear cells appeared between the covers; these cells Maximow called *resting wandering cells* or *polyblasts*, and they correspond to the cells we shall call *clasmatocytes*. Finally, much later (after 4 days), typical fibroblasts appeared between the cover-slips. These studies of Maximow on the rates of migration of the cells of the blood and connective tissues have been confirmed by Cunningham (1922a) in the case of cells entering the peritoneal cavity in response to the presence of irritants.

DIFFERENTIAL PHAGOCYTIC CAPACITY OF MONONUCLEAR CELLS.

The concept that certain cells are more especially concerned in the phago-cytosis of particulate matter we owe primarily to the pioneer and classical re-searches of Metchnikoff (1883, 1892) on the reactions of free cells to bacteria and débris. He called the large mononuclear, phagocytic cell the *macrophage*, and differentiated it functionally from the polymorphonuclear leucocyte on the evidence that, except for the tubercle bacillus and the bacillus of leprosy, the mononuclear cell disregarded bacteria and took up only cellular débris. He also laid much stress upon the differences in the ferment content of these cells, but the recent work of Opie (1909–10) has shown that the large mononuclears also have proteolytic ferments, though these are slightly different from those of the polymorphonuclears. Many other observers have noted the phagocytosis of bacteria, pigments, cellular débris, and foreign particles by the mononuclear cells and this capability has become a very general criterion in pathological literature.

Of especial importance from the standpoint of cellular differentiation has been the development of the use of vital stains. We owe the inception of this work to the search for specific chemicals for use in treating bacterial and protozoan diseases. Bouffard (1906), under the stimulation of Mesnil in Paris, found that when large doses of certain benzidene dyes, especially isamine blue, were adminis-tered intravenously, there were certain large cells of the connective tissues which were specifically stained. These cells Bouffard called interstitial cells. Inde-pendently, Goldmann, working with Ehrlich, made very similar observations with another benzidene dye, pyrrol blue. Goldmann's work, published in two mono-graphs (1909, 1912) gives a detailed study of all the cells of the animal organism which he found took up pyrrol blue after intravenous injections. Goldmann showed that the areolar tissue under the skin, the intermuscular septa, and the connective-tissue framework of organs contained two major types of cells—pyrrol cells (clas-matocytes or wandering endothelial phagocytes), staining massively, and fibroblasts which took up but little of the dye. Goldmann concluded that the pyrrol blue had stained certain preformed intracellular structures or bodies in quite the same way as other dyes had been shown to stain cytoplasmic structures.

Soon after the publication of Goldmann's first monograph, many workers began studying the effects of vital dyes, and before long they were divided into two groups—those who supported Goldmann's idea of the staining of specific, preformed cytoplasmic structures and those who considered the storage of the benzidene dyes to be quite similar to the phagocytosis of any foreign material. Tschaschin (1912) believed that the structures which were stained in his preparations with isamine blue and trypan blue were mitochondria. On the other hand, the theory that the staining with vital benzidene dyes was a similar reaction to that of the phagocy-tosis and storing of particulate matter was advanced by Evans and Schulemann (1914, 1915). These authors found that the ability of cells to store benzidene dyes had no relation whatever to the chemical nature of the dye, but that instead it was associated with the physical size of the colloidal particles. They suggested

that the taking up of pyrrol blue, trypan blue, and other dyes of the benzidene series, as well as the storage of carmine, colloidal solutions of metals, and other substances, took place by a diffusion of the particles through the cell membranes and the subsequent aggregation of these submicroscopic particles into masses readily visible with the microscope. Schulemann (1912, 1921) further experimented with a large series of dyes of various particulate sizes and found that those which diffused readily from a watery solution into gelatin would also stain an animal diffusely after they had been introduced into the subcutaneous tissues; while those whose particulate size was so large that they could not diffuse into the circulation through the endothelium were only taken up locally. This demonstrated that there is a very wide difference in permeability of cell-membranes to different dyes, but that when taken up the dyes are stored in the same way. Finally, Shipley (1919–20) has brought the most specific proof that this reaction is similar to the phagocytosis of larger particles and débris, by showing that when vitally stained (trypan blue) cells are brought into contact with a supravital stain, e. g., neutral red, the latter dye will enter the same area previously occupied by the trypan blue. Thus the conclusion became wholly certain that the staining of cells with benzidene dyes was a process of the diffusion of the dye particles into the cell and a subsequent aggregation of these particles into masses.

On the basis of this concept of phagocytosis and aggregation of the particles into zones in the cytoplasm, which we call vacuoles, Evans and Scott (1920), in a beautiful and finished monograph, have given us further proof of the separation of clasmatocytes (macrophages or our wandering endothelial phagocytes) from fibroblasts. They showed, with a large series of dyes, that there were several points of distinction between these two types of cells. In the first place, with mild acute staining the fibroblasts took up much less dye than the clasmatocytes. Secondly, there were distinctive ways in which the two types of cells stored the dyes, the droplets being much larger in the clasmatocyte than in the fibroblast. And finally, they showed that if animals were subjected to a long-continued staining with these dyes the fibroblasts could be forced to take up as much dye as the clasmatocytes, but they also found that the clasmatocytes gave up their load of dye to the circulation much earlier than the fibroblasts, thus permitting its excretion by the organism. This is probably a very fundamental distinction, taken in connection with the known temporary storage of material in the Kupffer cells, a cell-type which we regard as entirely analogous to the clasmatocyte, and the action of the kidney in secreting these dyes. That is, it indicates that the clasmatocyte is concerned with ridding the body of foreign matter, while the fibroblast is only forced to react to an abnormal condition. Phagocytosis is a very general property of cells, but certain cells, such as clasmatocytes and monocytes, have this property accentuated for a physiological purpose of the organism as a whole. It is obvious that the use of insoluble particles to demonstrate the phagocytic power of these cells is only a means toward finding out how they actually deal with those soluble substances which they meet in normal physiological conditions or in the course of their reactions to disease. These experiments signify that the clasmatocyte, in contrast

to the fibroblast, is normally concerned in dealing with débris and possibly, also, as Shipley suggests, in digesting material for further or special use in the body.

Further distinctions between the clasmatocytes and the fibroblasts have been brought out by Cunningham (1922b) in a study of the cells of the omentum of the rabbit which had been subjected to mild forms of irritation. He found that during mild irritations the fibroblasts put out very extensive branching processes and that the vital dyes were to be found in enlargements of these processes, often at their very tips. The distribution of the dye was uneven in the cytoplasm nearer the nucleus, but here there was always much less than in the corresponding clasmatocytes. Generally, under irritation the clasmatocytes were rounded up, but when they had long processes the tips of these were usually free from phagocytized particles.

It is in connection with the specific reactions of cells of the connective tissues to phagocytized material that we have obtained much of the information that enables us to discriminate the clasmatocytes from certain other cells of the connective tissues, as well as from the fibroblasts. Among these are the serosal or mesothelial cells lining the body-cavities. As is well known, the question of the origin of the cells found in peritoneal exudates has an extensive literature; this has recently been analyzed in detail elsewhere (Cunningham, 1922a).

In this paper we will present further evidence that the serosal cell is a distinct type which does not become transformed into a free, living phagocytic cell when it desquamates. The majority of authors who have studied the question have concluded that it can become so transformed; among these are Heinz (1902), Weidenreich (1907), Schott (1909), Szecsi (1912), Lipmann and Plesch (1913, 1915–16), Szecsi and Ewald (1914), and Karsner and Swanbeck (1921). Marchand (1921) derives the majority of the free cells from the adventitial cells, but also concludes that the serosal cells are responsible for some of the free phagocytic cells. On the other hand, Pappenheim (1913) and Pappenheim and Fukushi (1914), by means of their beautiful hematological technique, have sharply differentiated the serosal cell from all other cells appearing in the exudate and maintain its specificity, despite even the experiments with thorium-X reported by Lippmann and Plesch. In these experiments it was found that animals subjected to a treatment with thorium-X became aleucocytic. In such animals, in which the total number of leucocytes in the circulating blood had been reduced to almost zero, Lippmann and Plesch introduced foreign substances into the peritoneum and found that the ensuing exudate contained numerous cells which they believed must have come from the mesothelium. Pappenheim suggested, and this seems to be especially significant in the light of our observations on the cells found in the omentum and in the exudate during peritoneal irritations, that there are stem-cells of primitive type in the tissues adjacent to the peritoneum, and that under stress these can give rise to the free mononuclear phagocytes. These stem-cells he considered as more resistant to the thorium-X.

Finally, Cunningham (1922a) found that, on the basis of reactions to trypan blue and the subsequent study with neutral red, the serosal cell always remained

a distinct entity and never simulated the wandering endothelial phagocyte or any other free cell in peritoneal exudates. He failed to note that there were two types of phagocytic cells in these exudates, concluding that all the free phagocytic cells were of the same type. In a preliminary note recently published (1924b) we noted the possibility of the separation of these cells, and it is the object of this communication to extend and confirm, first the entire specificity of the mesothelium, even when considerably irritated, and secondly, the separation of the phagocytic cells into two distinct types.

REACTIONS TO SUPRAVITAL STAINS.

Subsequent to his other work, referred to above, Maximow (1906) added another criterion for the distinction between his resting wandering cell or clasmatocyte and the fibroblast by studying their reactions to vital neutral red. He showed that the two cells, when studied fresh in a dilute solution of neutral red, could be distinguished by their reactions, both of nuclei and cytoplasm, to this dye. The clasmatocytes had sharp cellular outlines and in their cytoplasm were many particles that reacted to neutral red. Their nuclei also stained well in the dye, his figures showing a stained nuclear membrane as well as a fairly abundant chromatin network. The fibroblasts, on the other hand, had larger oval nuclei that stained but faintly in neutral red. The cellular outline was indefinite in contrast to the clasmatocyte, and in the cytoplasm only occasional particles reacted faintly to the dye. We now know that the pictures of the cells given by Maximow can not be considered as the true vital reaction to the dye, because when the nuclei of cells stain at all, it is a sign that the cell has been badly damaged. On the other hand, with more dilute solutions of neutral red, the nuclei never stain and there is only a specific reaction on the part of certain cytoplasmic structures, such as neutrophilic, eosinophilic, and basophilic granules, and of certain areas within the cytoplasm which are either vacuoles or included débris. These reactions are differential so that they help in discriminating cells; moreover, they are so little harmful that they do not inhibit certain activities like cell-division and motility for considerable periods of time. Thus we consider that true vital staining is a major factor in the differentiation of types of cells in living tissues.

ORIGIN OF CERTAIN FREE PHAGOCYTIC CELLS FROM ENDOTHELIUM.

In this connection the work of Mallory (1898) takes precedence, for as early as that year he presented the idea of the phagocytic mononuclear as an "endothelial leucocyte." We believe there is considerable evidence, though perhaps not entirely complete, that the cell which is most markedly phagocytic, the clasmatocyte or wandering endothelial phagocyte, is derived from endothelium and that it is thus related in point of origin to the Kupffer cell. The clasmatocyte is also the same as the "adventitial cell" of Marchand (1890, 1901) and the "trailer cell" of Buxton and Torrey (1906). It now becomes important to analyze what evidence we have for the origin of clasmatocytes from endothelium. Exactly what type of free cell does come from endothelium and on what evidence? Where and how do clasmatocytes arise in the embryo? Where do they arise in the adult? Do they

actually differentiate from endothelium generally in the adult, or only in certain limited places, and how far are they derived in the adult from the division of pre-existing clasmatocytes? These points must be analyzed both under abnormal conditions and in normal tissues, and we propose to give what evidence we think we have to bear on these points.

Since the first observations of Mallory on the phagocytic cells, which he thought were derived from endothelium, a large number of experiments have been conducted in examination of his theory. McJunkin (1919) evolved the theory that particles of India ink of a certain size are phagocytized by endothelial cells practically alone, and hence may be utilized as a specific criterion for identifying derivatives of endothelium when they have become free in the tissues. Using this method in conjunction with other studies, Foot (1919, 1921) and Permar (1920, 1921) have concluded that the origin of a very large part of the free phagocytic cells of normal and pathological states is from the general vascular endothelium. From their studies, made with vital dyes, on the so-called specific endothelia, Evans, Bowman and Winternitz (1914), Evans (1915), Evans and Scott (1921), and Simpson (1922) have concluded that the clasmatocyte is derived from certain peculiar endothelial cells of the spleen, liver, lymph-glands, and bone-marrow.

We wish to mention here, from some unpublished studies of one of us (Cunningham), that under conditions of very prolonged stimulation in rabbits clasmatocytes may be found in excessive numbers anchored within the sinuses of the splenic pulp. In these experiments these clasmatocytes were anchored within the sinuses where they had arisen and were also free in the pulp to which they had wandered. The anchored clasmatocytes were in every way identical with the Kupffer cells of the sinuses of the liver. The method by which the Kupffer cell probably arises, as an anchored endothelial phagocyte, became very clear to one of us in the study of developing vessels in the area vasculosa of the living chick (Sabin, 1920, p. 238). In the chick of the second and third days of incubation clumps of young red blood-cells cling to the inner walls of the vessels and protrude into the lumen. In the living preparation these clumps of cells can be seen to put out long sprouts which creep along the inner wall of the vessel and attach themselves to the opposite side. The sprouts then make lines along which more red cells develop. The property of sending out long cytoplasmic processes is one of the fundamental characteristics of the angioblasts from which the endothelial cells come. In development, these sprouts are the mechanism by which a vascular plexus is originally formed. The Kupffer cells, then, are daughter endothelial cells, which retain to a marked degree this fundamental property of putting out long sprouts by which the cell is anchored so that it is in the mid-stream of the blood-flow. This position is associated with its heightened power of phagocytosis. That the Kupffer cell is a daughter endothelial cell is evident by the fact that in sections from animals heavily stained with trypan blue the parent endothelial nucleus is often to be seen in the wall of the vessel adjacent to the nucleus of the Kupffer cell itself.

That not all of the excess of clasmatocytes, experimentally produced in splenic sinuses by repeated injections of particulate matter into the circulation, remain

anchored is proved by the showers of macrophages (clasmatocytes) described by Simpson (1921) as occurring in the heart's blood of the right ventricle after repeated doses of trypan blue.

Anchored clasmatocytes have also been found by us in material which we are presenting in another article in this volume (Doan, Cunningham, and Sabin), in patent sinuses in hypoplastic bone-marrow of pigeons which had been chronically stained with trypan blue. They are large cells, in every histological detail identical with clasmatocytes; they have the typical aggregations of trypan blue and the nuclear characteristics of clasmatocytes, and they are apparently attached to the walls of the sinuses by strands of cytoplasm in continuity with the endothelial cells. They are in all respects identical in appearance with the Kupffer cells of the liver. In certain of our studies on pathological human bone-marrow we have also found anchored clasmatocytes with ingested red and white blood-cells in patent ·sinuses.

Aschoff and Kiyono (1913) and Kiyono (1914) have interpreted the relationship between the specific endothelial and reticular cells as being so close that they function as one tissue, and they have expressed this concept by naming the tissue the "reticulo-endothelial apparatus." In essence, their theory is that in spleen and lymph-gland, and to a less extent elsewhere, the endothelium is spread over a framework of reticular cells and the two have become indistinguishable, at least as far as their reactions to vital dyes and their production of free cells is concerned. Aschoff and Kiyono have identified the clasmatocytes and monocytes and have grouped them together under the term "histiocyte." One of us (Sabin, 1921) has published observations on the origin of free cells in the area vasculosa of living chicks of the first few days of incubation, confirming this concept that clasmatocytes and monocytes are identical. The observations were as follows: The area vasculosa of chicks of 3 to 7 days' incubation was cut out and mounted in a hanging-drop preparation, so that the area pellucida could be studied; its vessels could then be seen with great clearness under the oil-immersion lens. Moreover, if these preparations were well sealed and kept at body temperature, the cells lived for several hours. In many of these specimens the borders of the veins, especially in the zone in front of the head of the chick, showed irregular clumps of cells which corresponded histologically to clasmatocytes. They showed the characteristic vital reaction to neutral red, and from time to time one of them became free from the mass and moved very slowly away from the wall of the vein. In these specimens, even though they were living, it was not possible to say that there was complete proof that these cells actually came from endothelium, because their origin from adjoining mesenchyme could not be ruled out. Mesenchyme is, however, very scanty at these stages and the walls of the veins, with the exception of the masses of cells in question, consist either wholly of endothelium or in places of what seems to be a reduplicated endothelium. The primitive veins have a wall of endothelium alone, without any adventitia whatever, so that structurally the vessels are like capillaries with wide lumina; their characterization as veins is a functional rather than a structural one, for the blood is returning to the heart through them.

On the other hand, in these same specimens, an occasional cell, identical in appearance with these adventitial masses, became free from the inner wall of the vessels and dropped off into the lumen. A direct origin of these intravascular cells from endothelium can be regarded with more assurance because they can be found at a time when they make a part of the wall of the vessel and can then be watched as they round up and become free. Moreover, these cells that become free within the vessel have occasionally been found with phagocytized red blood-cells in their cytoplasm, which phenomenon has, indeed, been seen to take place while the cell was still an integral part of the wall of the vessel. This has been illustrated by Maximow (1909), who shows in his figure 4 on plate xviii the phago-cytosis of a nucleated red blood-cell by an endothelial cell that is a part of the vessel, and three desquamated endothelial cells within the lumen of the vessel, all of which have vacuoles and one a phagocytized cell. Therefore, it is with considerable as-surance that we state that the origin of a cell, identical in type with the clasmato-cyte, has been seen to take place by the actual proliferation of endothelium in a living form, thus justifying the term *wandering endothelial phagocyte.*

In these specimens from the chick only a few endothelial cells drop off into the lumen of the vessel; the great mass of similar cells leave the outside wall and pass into the connective tissues. Our judgment is that these observations are our most convincing evidence of the origin of clasmatocytes from endothelium, but that to have called the cells that drop off within the vessels *monocytes* or *histiocytes*, in the sense of Aschoff, was a mistake, in the light of our further observations. The cells within and without the vessels, in the experiments described above, were both of the same type—a type that is identified with the clasmatocyte or wandering endothelial phagocyte, as we now recognize it. We are presenting evidence in this paper to show that the clasmatocyte is not identical with the monocyte, but rather that they are two types of cells from the standpoint of origin and function.

It is thus clear that the history of our knowledge of the cells of the connective tissues centers around three ideas—the discrimination of the different types of cells by histological means, an attempt to analyze their functions, and an effort to determine their origin. It is also obvious that the goal of all these studies is to obtain as much information as possible on the cause of the differentiation of the cells of the connective tissues into types, with reference to their inherent functional potenti-alities under physiological and abnormal conditions.

NOMENCLATURE.

The question of terminology is an important one, as much confusion has arisen from the use of many different names for the same cell. There are two ideas on which to base a nomenclature—clearness and etiological and descriptive correctness. It is, of course, of great advantage when they coincide.

There has been a large number of names applied to the mononuclear phagocytic cells of the tissues. *Macrophage* was first suggested by Metchnikoff and has been reapplied by Evans. It is regarded by many as the better term, but it has not in the past been clearly maintained for one specific type of cell. *Clasmatocyte,*

though first applied by Ranvier to amphibian cells which he thought underwent a fragmentation of their cytoplasmic processes, has the advantage of clearness, because, as far as mammalian tissues are concerned, it has been consistently used for a single, specific cell. The actual cell to which Ranvier applied the term, in amphibian tissues, has, however, not been analyzed with our present methods. The term *histiocyte* was suggested by Aschoff, connoting that both clasmatocytes and monocytes are the same type of cell, a concept that we now think incorrect. The older terms, *adventitial cell, pyrrol cell,* etc., are obviously to be avoided. Mallory's term, *endothelial leucocyte,* seems to be inadvisable, because it relates these cells to the circulating white blood-cells, a relationship which we do not believe exists. *Endothelial cell,* a term which has been used occasionally, is too confusing, as it does not specify any differences between the lining endothelium of blood-vessels and the cells supposed to be derived from it. In general, the phrase *wandering endothelial phagocyte* perhaps best expresses our judgment concerning this type of cell, and we use it as entirely synonymous with *clasmatocyte.* The term is in contrast to *anchored endothelial phagocyte,* which is the Kupffer cell. The term *clasmatocyte* has the obvious advantage of brevity, of being well known in the literature, and of specifically referring to the cell in question. For the other type of phagocytic cell we use the term *monocyte,* and in this category include the *transitional cell* of the blood, in the sense of Ehrlich, the *monocytes* of the blood and tissues, in the sense of Naegeli, and the large mononuclear forms of pathological literature, as far as they are entirely distinct from the clasmatocytes.

METHODS.

In general, the methods which we have employed in this study have consisted in obtaining living cells and studying them on films of vital neutral red alone and on films made with a mixture of neutral red and janus green. The technique for making the films has been given by Sabin (1923) and by Doan, Cunningham, and Sabin (this volume). The animal used was the rabbit. For the most part the living cells that we have studied have been obtained from three sources—punctures of the living spleen, peritoneal exudates, and spreads obtained from the omentum and the subcutaneous tissues. The punctures of the spleen were made with fine glass pipettes while the rabbit was kept under an anesthetic and only while the circulation was normal. The peritoneal exudates were produced by the injection of whole, citrated, or laked blood. The blood was obtained in some cases from animals whose corpuscles and sera had been matched and in others from rabbits in which the corpuscles and sera of the recipient and the donor had been shown to be unmatched. In a few cases cat's blood was used. The experiments on the subcutaneous tissues were performed by introducing whole blood into the loose connective tissue and studying small bits of the tissues on vital films after appropriate intervals. The same varieties of blood were used for the injections. In some cases the area was rendered edematous by injecting neutral red (1 : 10,000) in physiological saline, and bits of the edematous material were studied. In some cases the rabbits were stained with trypan blue before being

used for the acute experiments, and in a few trypan blue was administered throughout the experiment.

The drawings of the living cells have been made for us by Mr. James F. Didusch, artist of the Carnegie Laboratory of Embryology. We are very deeply indebted to him for these drawings, which form an important part of the records of our experiments. We could not bring out in photographs all that we desired to show in these cells; tracings with a camera lucida could not be made because the cells changed too rapidly, so that very accurate and skilled free-hand drawings, with the red blood-cell as the scale of magnification, were our only resource.

EXPERIMENTAL OBSERVATIONS.

DISCRIMINATION OF CLASMATOCYTES, MONOCYTES, AND SEROSAL CELLS

CLASMATOCYTES OF THE SPLEEN.

We have worked out a technique by which the free cells of the spleen can be studied in films that are comparable to films of blood-cells. If a finely drawn out glass pipette be plunged into the spleen of an anesthetized rabbit while the circulation through the organ is active, the free cells will run into the pipette by capillary attraction. The material thus obtained can then be studied with the supravital technique. In such preparations it is easy to identify clasmatocytes and to separate them from monocytes. The clasmatocyte of the spleen has been thoroughly identified by the enormous masses of trypan blue which it engulfs. It was demonstrated in this organ in the first experiments of Goldmann. Moreover, as was brought out in the analysis of the literature, it has become recognized that clasmatocytes are produced from endothelium in the spleen throughout life.

In figure 1 is shown a large clasmatocyte, which is the most characteristic type found in the spleen. Some of these are always to be found in films of cells from the spleen. This particular cell is of enormous size, its diameter being four or five times the diameter of a red blood-corpuscle, thus making its surface area very great in comparison with other cells. It was stained in a mixture of neutral red and janus green and showed no mitochondria whatever. Occasionally, we have seen a clasmatocyte as large as this with a few mitochondria, but the vast majority of these large cells contain none at all. In the cell shown in figure 1 there was a narrow clear zone in the periphery of the cytoplasm, but the rest of the cytoplasm was filled with bodies that stained with neutral red, giving every shade from the bright scarlet of the acid reaction of the dye to a deep maroon, and of every size, from tiny points up to bodies the size of a red blood-cell. Indeed, in this cell there was one red blood-cell, possibly two, and perhaps the débris of many others. The phagocytosis of red blood-corpuscles is a constant function of these cells. When a red blood-cell has just been taken into a clasmatocyte, its color is exactly like that of the free red cells; that is to say, it has the yellow color of hemoglobin, but after the corpuscle has been engulfed for a time, it begins to shrink and its color becomes deeper. Stained in neutral red, it shows a deeper and deeper shade of red as the corpuscle shrinks in size, until finally there is no possibility of identifying a disintegrating red blood-cell from a deeply stained vacuole.

When a clasmatocyte takes in a nucleated cell, as, for example, a leucocyte, the leucocyte will react at first quite normally to neutral red. Soon, however, its nucleus is seen to stain, showing that the cell is dead or dying; then, in a short time, the only differential point is a mass of heavily stained chromatin in the center of a clear zone. Indeed, in such a cell as that in figure 1, our concepts of what has actually stained in neutral red are of the vaguest nature. There are the normal vacuoles of the cytoplasm, which Evans has termed the "segregation apparatus." Into these vacuoles the débris of material which the cell has taken in is collected; if this débris is large in amount or in the size of the individual particles, these vacuoles become enormously overloaded. All cellular material taken in by the clasmatocytes is digestible, so that soon after it has been engulfed it becomes an amorphous mass.

In figure 1 it is impossible to analyze what was the cytoplasm of an engulfed cell and what the original vacuole of the segregation apparatus. We judge that the neutral red has stained the fluid in which the débris was floating and thereby the débris has been wholly obscured or shows deeper colored particles in droplets of red fluid. This much is certain—that if such a cell as that in figure 1 be loaded with trypan blue, a subsequent supravital staining with neutral red will entirely obscure the blue dye. This we interpret as indicating that the fluid of the vacuoles, into which the trypan blue has been segregated, stains enough with neutral red to hide the blue granules within them. This superimposition of the supravital dye upon the vital dye, or rather, the placing of both substances by the cell in the same vacuoles, has been discussed by Shipley and has been referred to in the introduction. From these observations we conclude that a marked collection of round bodies, filled with neutral red, in the cytoplasm of cells stained supravitally, is, in a general way, a sign of phagocytosis. That this can not be a universal conclusion is brought out by the fact that small droplets of neutral red can be found in all red blood-corpuscles at a certain time of their development. In this case it seems assured that the staining with the neutral red is not a true phagocytosis, but an entirely different reaction. What the nature of this especial reaction is it is quite impossible to state; but we feel that it is more akin to the staining of substances of the cytoplasm precipitated by the dye, or to the staining of a nucleus or broken-down cellular débris, than to phagocytosis and the aggregation of stain by the living cell.

It is clear that the forcing of cells to ingest such large amounts of insoluble substances, and such an entirely alien substance as trypan blue or carmine, is an experiment very far from anything that the cell would be likely to meet under normal conditions, and hence we think it significant that a cell showing such signs of phagocytosis as the type in figure 1 should be characteristic of the normal spleen. Certainly this cell from a normal animal is not more complex in its reaction to neutral red than the same cell after it has been stimulated by repeated injections of insoluble foreign particles. This, of course, is not true of the corresponding cell of the normal connective tissue shown in figure 8, which is seen to be much simpler in its content of vacuoles than the splenic cell. This seems to us to mean

that the clasmatocyte of the spleen is normally in a state of high phagocytic activity.

In attempting to make drawings of these large phagocytic cells of the spleen great difficulty was encountered, as many of the cells died before the drawings could be finished.   It was noted that the stain suddenly became diffuse, involving the entire cytoplasm and nucleus alike; then the stain faded out completely and the cells became so changed in their characteristics that they could not be recognized as clasmatocytes.   This indicates that these large cells are especially fragile and suggests that, at the end of a period of digestion of a mass of phagocytized material, they may disintegrate in the body as they do in our supravital films.   This would afford an adequate explanation for their continued origin from the endothelium of the spleen, where they are obviously in continuous function.   The rapid fading of these cells gave us also another suggestion, for we occasionally found in our living preparations very vacuolated cells that did not stain at all, and interpreted them as degenerating forms.   Thus it seems that there are two types of vacuoles—those that react to neutral red and those that take no stain whatever; the latter we regard as a sign of cell death.

Not all of the clasmatocytes are of the large, active type illustrated in figure 1, since occasionally cells like those of figures 2 and 3 are found, both of which were much smaller than the first type.   These two cells are especially interesting, because, coming from splenic punctures, they may be correlated with clasmatocytes such as those shown in figures 8 and 10, taken from the connective tissues.   The clasmatocyte from which figure 2 was drawn was taken from the spleen of a normal rabbit (S 47); it had ingested some nucleated cells and also contained considerable débris.   A still smaller cell is shown in figure 3.   It is the one most like the un-stimulated clasmatocyte of the connective tissues, such as that illustrated in figure 8, except that it is much smaller.   This small clasmatocyte had not taken in any other cell, but had the characteristic bodies, probably vacuoles, that stain with neutral red.   It is, however, the larger clasmatocyte of figure 1 that we regard as most characteristic of the spleen.

We have made a number of differential counts of the free cells obtained from splenic punctures and are surprised at the relative constancy of the percentages. The leucocytes are quite constant at about 29 per cent in an average of 17 counts. The basophilic leucocytes run about 7 per cent and the eosinophilic less than 1 per cent.   In general, monocytes make about 18 per cent.   In regard to lympho-cytes and monocytes, there are two types of counts: (1) those in which lympho-cytes predominate so markedly as to indicate that the needle struck a follicle; in these the lymphocytes ran about 50 per cent and the monocytes about 7 per cent; (2) those with fewer lymphocytes, in which there was an average of 27 per cent monocytes.   In general, we have found from 2 to 3 per cent of clasmatocytes in our counts.   In three normal rabbits there were myelocytes in proportions of 1 to 5 per cent, and occasionally we have found a primitive reticular cell and a primitive white blood-cell.   Thus our average counts of splenic cells showed about 3 per cent clasmatocytes and 18 per cent monocytes.   In studying the free

cells obtained by splenic puncture, it is possible to follow the maturation of the monocyte from a primitive white cell. The full description of these cells and the record of our observations on the maturation of the monocyte we are giving in another paper in this volume (Cunningham, Sabin, and Doan).

Another striking point in the preparations from splenic punctures in the rabbit is the large masses of platelets. These masses are often of really enormous size, filling two or three entire microsopic fields under an oil-immersion lens. Indeed, such masses of platelets are more characteristic of the material from splenic punctures than of that from punctures of bone-marrow. The same point has been brought out recently by Kramer and Drew (1923) from a study of the rat, in which animal they found also more platelets from the spleen than from the bone-marrow.

CLASMATOCYTES OF PERITONEAL EXUDATES.

The range of clasmatocytes to be seen in peritoneal exudates, after injection of blood into the peritoneal cavity, is given in figures 4 to 7. Figure 4 was taken from a rabbit (S 47), which had received three injections of blood into the peritoneal cavity. The first dose was 24 c. c., given on January 9; the second and third doses were given on January 10, one of 20 c. c. in the morning, the other of 20 c. c. in the afternoon, and the cells were studied the next day. On that day the clasmatocytes constituted about 12 per cent of the cells of the exudate, the serosal cells about 5 per cent, monocytes 24 per cent, and the lymphocytes 1 per cent, while the remainder were leucocytes. As may be seen in figure 4, the clasmatocytes had taken up red blood-cells, two of which were very clearly defined. The rest of the cytoplasm was loaded with the débris of nucleated cells and of material which could not be identified. This is the typical clasmatocyte in a high state of activity, comparable with the one from the spleen shown in figure 1. The other three cells make an interesting series. They were taken toward the close of a long experiment (rabbit CD 27), in which the larger clasmatocytes had disappeared, all those found being small cells, every one of which had phagocytized other cells.

Figure 5 represents the smallest type of clasmatocyte that we have seen and, we think, the youngest as well. In striking contrast to the larger clasmatocytes, this cell contained many mitochondria, which we have seen only once or twice in cells as large as those in figures 1 and 4. It had taken in one red blood-cell, which was placed against the nucleus. This we regard as the characteristic position of the first material phagocytized by a clasmatocyte. The granules in the clear cytoplasm were mitochondria, and we think the ones against the phagocytized cell were also mitochondria. The cell from which figure 6 was drawn had ingested 4 red cells, while that of figure 7 contained 2 red cells and numerous vacuoles of various sizes. The rabbit from which these three cells were removed had received 13 intravenous injections of trypan blue, the doses ranging from 2 to 4 c. c. of a 1 per cent aqueous solution of the dye, and had then received several doses of a mixture of blood and trypan blue intraperitoneally. The cell shown in figure 7 was drawn after the sixth intraperitoneal dose. We interpret these three cells as young forms of clasmatocytes which have come into the peritoneal cavity in response to this long-continued irritation. In general, we have found that the large

cell in figure 4 was the type that appeared early in irritations of the peritoneal cavity, while the forms shown in figures 5, 6, and 7 appeared later. The experiments in which animals have been given trypan blue, first intravenously and then intraperitoneally, were carried out to test whether monocytes, as well as clasmatocytes, phagocytized the dye. On the day after the eighth intraperitoneal injection of trypan blue three differential counts were made:

	I.	II.	III.
	p. ct.	*p. ct.*	*p. ct.*
Neutrophilic leucocytes.......	53	56.5	54.8
Eosinophilic leucocytes.......	1	0	.5
Lymphocytes, small..........	8.5	7	7.8
Monocytes, young...........	4	15	9.5
Monocytes, mature..........	14	11	12.6
Clasmatocytes..............	19.5	11	15.3

Since, in these supravitally stained preparations, the neutral red obscured the trypan blue, a fixed smear, stained in carmine, was counted and gave the following: Neutrophilic leucocytes, 49 per cent; lymphocytes, 8 per cent; mononuclear cells without trypan blue, 33 per cent; mononuclear cells with trypan blue, 10 per cent. As we studied these fixed films, we regarded all of the cells with massive granules of trypan blue as clasmatocytes. There was one cell classed with the group negative to trypan blue that did have one tiny granule, quite different from the massive amounts in the clasmatocytes. This cell we think might have been a monocyte. From many studies like the one just quoted, we conclude that monocytes may occasionally take a little trypan blue, but that their reaction to this dye is generally negative in acute experiments. We feel sure that the monocyte does not show the clumping of the engulfed dye, which is so characteristic of the clasmatocyte.

CLASMATOCYTES OF THE CONNECTIVE TISSUES.

The characteristics of the clasmatocytes of the resting connective tissues are now well known to every histologist who uses vital methods. The way to demonstrate such a cell as that of figure 8, the typical clasmatocyte of normal areolar tissue, is to inject a dilute solution of vital neutral red (1 : 10,000 in physiological salt solution) into the aorta of an animal until the connective tissues become edematous. For damaged areas a local injection of the dye may be better. Tiny bits of the edematous, jelly-like connective tissues are then cut out and studied at body-temperature. The slides may have films of janus green on them if this dye also is desired. The cell shown in figure 8 was taken from such a gelatinous bit of tissue in the region of the kidney of a rabbit. It was a large cell and showed the vital reaction to neutral red, in which there was a range in the shade of the dye from a bright scarlet of the acid reaction to a deep maroon color. We did not find the yellow reaction of the alkaline phase of the dye in the vacuoles. There was considerable variation in the size of the bodies that stained with neutral red in this cell, but much less than has been seen in similar cells after they had been experimentally stimulated. This particular cell had two nuclei and at one end of the cell was a group of unstained mitochondria.

The first reaction of the clasmatocyte to stimulation is shown in figure 9; it consists of an increase in the size of the vacuoles, which also obviously contain some débris. This cell was taken from the groin of a rabbit 24 hours after the injection of blood. The experiment was as follows: The rabbit (S 50) was anesthetized and 20 c. c. of blood was drawn from the heart and injected into the subcutaneous tissue of the groin. The next day the tissues were made edematous by the local injection into the groin of a solution of neutral red (1 : 10,000 in salt solution), and then small bits of the tissue were studied on the film of neutral red and janus green. A clasmatocyte from the subcutaneous tissues, showing the phagocytosis of red blood-cells, is illustrated in figure 10, from a similar experiment (rabbit S 51), taken also 24 hours after the injection of blood. It contained 2 red blood-cells which we think had just been engulfed, as they still had the color of hemoglobin, and another that had probably been taken up earlier, because it stained heavily with neutral red. Besides these phagocytized cells, the cytoplasm contained also many stained vacuoles which reacted in various tints to the neutral red. The same correlation between the normal clasmatocytes and the cell after stimulation is to be followed in figures 11 to 13, taken from the omentum.

In figure 11 is shown the typical cell as it can be obtained by spreading any fresh omentum on a slide and staining with dilute neutral red. Figures 12 and 13 represent clasmatocytes from a rabbit that had received 13 intravenous injections of trypan blue and 8 doses of blood, mixed with trypan blue, intraperitoneally. In both of the cells the trypan blue has been obscured by the neutral red, but still shows as darker masses in the stained vacuoles. The cell of the type of figure 12 is comparable to the one in figure 9, and is merely one of the adult cells of the omentum which has phagocytized trypan blue. The smaller cell, on the other hand, shown in figure 13, corresponds to the stage which has been described so many times in the literature as a clasmatocyte that has rounded up under irritation. We are inclined to regard this rather as a young clasmatocyte which has resulted from the division of a larger form as the result of the irritation of the experiment. We have not found clasmatocytes actually in division, as we have the monocytes, and do not regard their rate of multiplication as at all comparable to that of the monocytes, since the clasmatocytes remain comparatively few in number. However, the types of small clasmatocytes shown in figures 5, 6, 7, 10, and 13 agree so well with our concept of young forms of cells (some of them certainly having mitochondria) that we are led to suggest the hypothesis that they have arisen by the division of the larger cells rather than by a transformation of the larger cells into small, round forms.

These small cells appear in the omentum, as shown in figure 13, at the same time that they occur in the peritoneal exudates, for the cells in figures 5 and 6 were drawn from the same animal on the same day. We regard the omentum as the source of these free clasmatocytes of the peritoneal cavity. These smaller cells occur, then, in the omentum, in the peritoneal cavity and in the subcutaneous tissues at a time when many of the original large clasmatocytes have probably disappeared by disintegration. From the evidence at hand it seems to us that the

most likely conclusion is that a mild irritation stimulates some of the large clasmatocytes to an increased phagocytosis and others to cellular division, thus giving rise to small forms that are adult types in a physiological sense and hence are immediately phagocytic. We wish, therefore, to emphasize the fact that we have found two types of clasmatocytes in our experiments: the large, highly phagocytic forms of the normal tissues, and the small, equally phagocytic types present normally, in small numbers, in the spleen and occurring generally in the early stages of mild irritation. It is, of course, this smaller type of clasmatocyte with which the monocyte has been confused.

MONOCYTES.

We turn now to the type of cell which we identify as the monocyte, the characteristics of which will be seen in figures 14 to 19. The most striking point about the monocytes is that the bodies which react to neutral red are characteristically arranged in a rosette around a central clear spot, the centrosphere, which is itself opposite the *hof* of the nucleus. There is a tendency for larger bodies, probably vacuoles, to appear in the periphery of the neutral-red rosette and, beyond these vacuoles, mitochondria, which are practically always present. In the monocyte the color of the bodies that stain in neutral red and make the rosette is quite characteristic; it is a salmon tint and is always quite uniform. We find that there is very much less variation in this reaction to neutral red in the monocytes of different species of animals than, for example, in connection with neutrophilic granules. We found no monocytes in any of the animals studied that did not stain well in neutral red; on the other hand, the neutrophilic granules of human blood stain in neutral red, while those of dog's blood hardly react at all. We do not know the nature of the stainable substance that forms the rosette of the monocyte. These bodies are fairly uniform in many cells, both in size and in color, but when the cells have been very markedly stimulated, all of the staining consists of rather large bodies approximately equal in size, which we interpret as vacuoles. If they are vacuoles, it is interesting that they lack the marked variation in color of the vacuoles of the clasmatocyte. In regard to mitochondria also, the monocytes differ markedly from clasmatocytes. As was said, many of the clasmatocytes do not show mitochondria, but the monocytes practically never lack them. They are to be found in the periphery of the rosette and along the border of the nucleus opposite to the rosette. They are more numerous in the younger forms, but may still be very numerous indeed in mature forms.

It was in preparations from splenic punctures that we first made the discrimination between clasmatocytes and monocytes. From the spleen one can always get examples of monocytes, both the young form and the more mature cell. Figure 14 is from a mature monocyte from the spleen, taken while the cell was moving. In motion the cell is stretched out and tends to advance with a somewhat irregular lateral movement. This is different from the motion of the lymphocyte, which is always nucleus first, and of the leucocyte, which usually has its nucleus in the rear. In this monocyte the rosette was slightly obscured, as it usually is when the cell

moves. The cell shows the typical indented nucleus of the monocyte and the characteristic mitochondria.

In figure 15 is the typical monocyte of an early stage of irritation of the cells of the peritoneal exudates. The nucleus has the characteristic indentation, and opposite its *hof* is a rosette consisting of a few neutral-red bodies and outlying mitochondria. We have seen a monocyte in which the rosette consisted of a single row of neutral-red bodies around a central clear space, and are giving in another paper in this volume (Cunningham, Doan, and Sabin) the criteria by which the young monocytes may be discriminated from the earliest myelocytes, in which the specific granules form in a clump and not in a rosette.

In following both peritoneal exudates and the tissues from beneath the skin after the injection of blood, the monocytes were as characteristic as the clasmatocytes, and from these experiments we have been able to define certain characteristics of this group. The first striking point is the frequency of an amitotic type of cell division in the monocytes.

In every survey that we have made of the cells of peritoneal exudates and of subcutaneous exudates after the administration of mild irritants, we have found dividing monocytes. The process of amitotic cell division of the monocytes is shown in figures 16 and 17. The former was taken from the subcutaneous tissues, but we have identical pictures from the monocytes of peritoneal exudates. The cell has all the characteristics of the monocytes, the rosette and the mitochondria. In this cell there are a few of the larger vacuoles in the edge of the rosette. The striking point, however, is that the nucleus has divided and the rosette is between the two nuclei. The next step of amitosis is shown in figure 17, which was taken from a cell from a peritoneal exudate, and it shows that the centrosphere divides. This is the essential point of amitosis, that the division of the centrosome follows nuclear division, while in mitosis it precedes nuclear division. When this cell was first seen the centrosome had already divided and it took 15 minutes for the cell to complete the process of division, which took place along the line indicated by the arrows.

The immature monocyte, such as the one in figure 15, does not show any evidence of phagocytic ability, but after it has become more mature it develops this capacity to a marked degree, as can be seen in figure 18. This cell was from a rabbit (CD 27) which had received 13 intravenous injections of trypan blue and 8 intraperitoneal injections of a mixture of blood and trypan blue. It is the same animal from which the cells of figures 5, 6, and 7 were taken and, like them, this cell also was from the peritoneal exudate. It had phagocytized 8 cells, one of which was certainly a red cell.

The second striking contrast between clasmatocytes and monocytes is in the manner in which they deal with phagocytized material. In the monocyte engulfed cells are all placed in the periphery of the cell, i. e., in the region of the larger neutral-red aggregations of the rosette, while the first material taken in by the clasmatocyte is placed adjacent to the nucleus. In the monocyte this central position in the *hof* of the nucleus is always occupied by the rosette, which is so

wholly characteristic of this type of cell. In the stage when the monocyte is actively phagocytic (fig. 18) there is more variation in the shade of color of the vacuoles than appears in the less stimulated phase, so that it tends to approach the clasmatocyte in the appearance of its included material; but the monocyte of figure 18 could be discriminated with certainty from the clasmatocyte by the peripheral position of phagocytized material and the persistence of the rosette. In our experience we have never found more phagocytized cells in a monocyte than are shown in this one, while in clasmatocytes it is not uncommon to find 20, 30, or even more phagocytized red blood-cells, as has long been known to pathologists.

It is true that the monocyte does approach the clasmatocyte in appearance during the phase of great phagocytic activity. Such a cell is shown in figure 19. This cell was taken from a peritoneal exudate of a rabbit (CD 50) 48 hours after an injection of whole blood into the cavity. As will be seen, the bodies that have stained in the neutral red are very large as compared with the less active monocytes in figure 14 and the young form in figure 15. There is still, however, a suggestion of the pattern of the rosette. A cell almost as stimulated as this one was shown by Sabin (1923, fig. 5, plate 35), a monocyte of human blood in a case of Malta fever. In this instance all of the monocytes of the peripheral blood were markedly changed after three injections of an autovaccine. They became filled with large vacuoles, which varied somewhat in color, but the clear centrosphere remained a prominent feature of the cells. In figure 19, on the other hand, the centrosphere is very indistinct. Along the outer margin there were a few mitochondria, as shown in the drawing. The exudate from which this cell was taken showed 79 per cent of monocytes, of which only 2 were immature forms; the rest were nearly evenly divided between fully mature monocytes and the very actively phagocytic types of our figure. In this specimen, then, we had every opportunity to see transitions between the monocytes in which the centrosphere was clearly evident and the cells like the one in figure 19, in which it was indistinct. It may be that a monocyte that has wholly lost the central clear space has actually begun to degenerate, but we are by no means sure, at the present time, as to how to distinguish between the phase of most active function and the beginning of the phase of degeneration. In a general way, however, we think that the type of cell shown in figure 19 can be discriminated as a highly active monocyte, as contrasted with clasmatocytes of the same state of activity shown in figures 1 and 4. In a still later phase of activity or degeneration it may become impossible to tell the two types apart.

SEROSAL CELLS.

It remains now to describe the vital characteristics of the serosal cells of peritoneal exudates. They have already been described, but not illustrated, by Cunningham (1922a). He has also recently (1924) shown how very wide the variation of the serosal cells may be under different experimental conditions. Under irritants they may vary all the way from thin, flat cells to the tallest cylindrical cells, and vacuoles may develop in especially characteristic zones. The normal cell of an unirritated type is shown in figure 20. It was scraped from the anterior

wall of the body-cavity of a normal rabbit (S 49).   This serosal cell, which is not more than the average size for the type, is enormous in surface area, as is shown by the accompanying red cell, and the cytoplasm has a dull, mottled appearance. Often nothing at all reacts to the neutral red, but in this cell there were four or five small bodies that did take the stain. We have never found any mitochondria at all in serosal cells. We have studied them many times on the janus-green films and have never seen them react in any way to this dye. There are three different reactions to janus green that have been observed by us: first, the clear blue color of the definite mitochondria; second, a dull green body that appears in platelets; and third, when a cell with basophilic cytoplasm (like that of the primitive white blood-cell) dies, the cytoplasm has a gray tone in janus green. None of these three types of reactions has ever been seen in the serosal cells.

In the experiments in which the serosal cells were more irritated, the free cells showed very characteristic refractive droplets; these are shown in figures 21, 22, and 23.   The first two were taken after 4 injections of blood into the peritoneal cavity and the third after 5.   The nature of these refractive bodies has been discussed by Cunningham (1922a). As far as we have observed, they occur in by far the greatest numbers in desquamated serosal cells, and it is our belief that they develop within the cytoplasm of these cells and are not taken in by them by the process of phagocytosis. When an exudate contains disintegrating serosal cells which have within their cytoplasm numbers of these refractive bodies, they become free in the exudate and are also found in the monocytes, appearing in the peripheral vacuoles of these cells. They occur also in fibroblasts when these are subjected to injury.   Figure 24 shows them in a fibroblast which was taken from the subcutaneous tissue of the abdominal wall of a rabbit after it had been punctured many times.

From our studies of peritoneal exudates, it is our opinion that the serosal cells, which desquamate from their fixed position, retain their characteristics until they degenerate and die in the exudate. Serosal cells from exudates never show the signs of phagocytosis such as are characteristic of the other two types we have been studying. An occasional vacuole may appear in a serosal cell, such as those in figure 21, and stain with neutral red, but the drawing of this cell was started at 11:15 and there were no stainable vacuoles in the cell until 11:45; these vacuoles, therefore, may have been signs of degeneration rather than of function. With great irritation the serosal cells may have an occasional neutral-red vacuole, just as repeated injections of trypan blue finally cause these cells to become lightly stained (Cunningham, 1923). The highly refractive droplets shown in figures 21 to 23 are, we think, a sign of irritation of the cell, of which three degrees are shown. This material is given into the fluid of the exudate as the serosal cells disintegrate, as is evidenced by their presence in the phagocytic monocytes at that special time.   In such an experiment it may be noted that the clotting time of the exudate is markedly increased. From this suggestion, it is clear that when such an exudate is obtained it should be studied chemically with reference to this property.

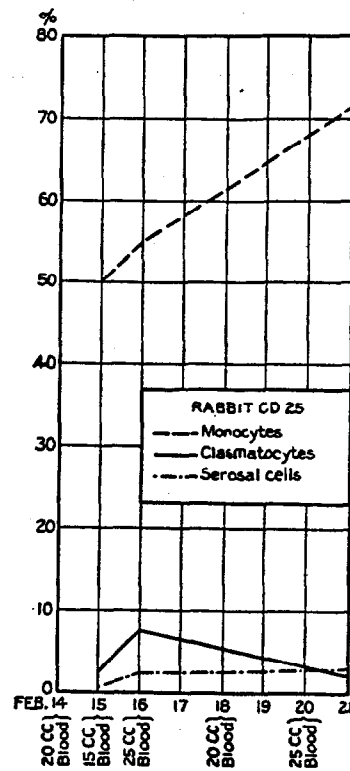## GENERAL SURVEY OF THE THREE TYPES OF CELLS UNDER EXPERIMENTAL CONDITIONS.

In the preceding pages we have given a description of the three types of cells in terms of their reactions to vital dyes and the reasons for regarding them as three different strains of cells. If such a discrimination of the types of cells be sound, it must prove that it is of functional significance. The discrimination of the serosal cell as a functional type is not difficult to make, since its characteristics will readily be seen to be tied up with its position as a lining cell. Its modifications as a lining cell may be very marked, but when it desquamates it loses its functional position and its changes are not great nor difficult to follow in the vital technique. It does not develop into a new type of cell, but degenerates in the exudate, without showing any signs of taking on that phagocytic power which would equip it to deal with foreign substances of the exudate. It shows no transitions either toward clasmatocytes or toward monocytes.

There are, however, two groups of phagocytic cells in subcutaneous tissues and peritoneal exudates, and to separate them is, of course, a time-honored problem which obviously concerns the reactions of tissues to injury and many other pathological problems. It is clear that there are four great types of cells, which function in the connective tissues, to be considered apart from the fibroblasts— leucocytes, clasmatocytes, monocytes, and lymphocytes. The full story of the reaction of tissues to injury may well be retold, with the chance we now have to follow living cells. In general, it is obvious that an individual clasmatocyte deals with much more débris than any other one cell, certainly with far more than a single monocyte under the conditions of mild irritation which we have been following. A clasmatocyte often engulfs 20, 30, or even more red cells, while the maximum number of cells engulfed by the monocytes, within the range of our experiments, is 10. Moreover, the clasmatocyte reacts strikingly towards the phagocytosis of red cells, but certainly this is not an exclusive property, for our studies show that after hemorrhage the monocytes also take up red corpuscles. It is the clasmatocyte in the spleen that is constantly and physiologically concerned with the destruction of whole red blood-cells, but the monocytes may be concerned with the red cells after they have undergone fragmentation, as shown by Rous and Robertson (1917, a, b). Granting that the single clasmatocyte does phagocytize more than a single monocyte, nevertheless, when the remarkable power of the monocyte to divide is taken into consideration, giving comparative numbers so greatly in favor of the monocyte, it is impossible to say that clasmatocytes are actually more important in relation to the clearing away of débris than are the monocytes. The point that must now be subjected to analysis is, what is the actual difference in what the two cells do, in order to account for their differentiation.

With the power to discriminate between two types of phagocytic cells, it now becomes necessary to restudy the subject of peritoneal exudates. The complete story will take many experiments, followed through with the vital technique and then correlated with a study of fixed tissues of all the organs involved. For such complete studies, however, we have at the present time certain points that will

serve as a guide.   We have followed peritoneal exudates in a series of 10 rabbits. In two experiments, in which we injected blood taken from the heart into the peritoneal cavity of the same animal, we obtained but few blood-cells from the cavity after 24 hours.   Moreover, these animals were autopsied and the peritoneum was normal, demonstrating that a considerable hemorrhage can be cared for with ease.   In general, in the experiments with peritoneal exudates, we have obtained the cells by punctures with glass pipettes and studied them on vital films.   In the counts we have included from 400 to 1,500 cells, the average counts including 600 to 800 cells.   In three differential counts of the cells of the normal peritoneal cavity, obtained directly or after the injection of a little normal salt solution, we found that the polymorphonuclear leucocytes varied from 65 to 95 per cent, the monocytes from 1 to 20 per cent, the clasmatocytes from 0 to 2 per cent, the lymphocites from 0 to 8 per cent, and the free serosal cells from 0 to 4 per cent.   Occasionally, also, there are a few basophilic leucocytes.
These inadequate surveys of the normal content of the cavity have not included any experiments in which the cavity was opened and washed out, in order to collect the cells and get more complete surveys, but in general these records indicate that when cells occur in the normal peritoneal cavity they are the same types as those occurring in the blood-stream.   To the cells like those of the blood are added a few clasmatocytes and a few serosal cells.

Three of our experiments on peritoneal exudates were from animals that had received a series of 12 to 13 doses of trypan blue into the blood-stream, starting with doses of 2 c. c. and running up to 4 c. c.   In the first experiment (rabbit CD 25) three counts of the cells of the peritoneal cavity were made.   The first count was made 24 hours after an injection of blood and trypan blue into the peritoneal cavity; immediately after these studies were made blood was again injected, and the next day the cells were counted and blood again injected.   Within the next 4 days there were three different injections and a final study was made 24 hours after the last injection.   The percentage of monocytes, of clasmatocytes, and of serosal cells, is shown on graph 1.   As in all of our counts, the monocytes far
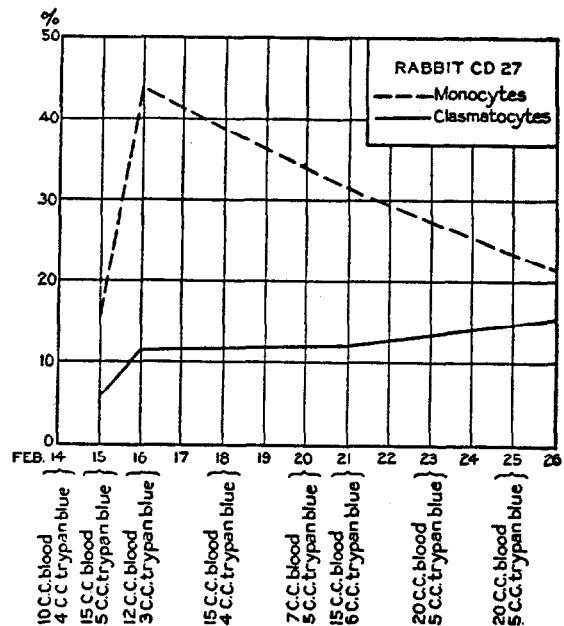

GRAPH 1.—Relative proportions of monocytes, clasmatocytes, and serosal cells in the peritoneal exudates of a rabbit (CD 25) after injections of blood into the peritoneal cavity.

exceed the other cells, ranging from 50 to a final 71.5 per cent.   The clasmatocytes and serosal cells were at all times under 10 per cent, with a slight increase in the clasmatocytes on the day of the second count and of the serosal cells on the day of the third count.   On the first day the striking point was that all of the clasmatocytes were markedly phagocytic, while, on the other hand, the monocytes were strikingly young cells, with many mitochondria and no signs of phagocytosis.   Five monocytes out of 600 cells counted were dividing; that is, 5 in 300 monocytes, making

nearly 2 per cent. On the next day most of the clasmatocytes had phagocytized other cells; a few of the monocytes were becoming phagocytic, and others were in division. Ultimately, the monocytes far outnumbered the other cells, even the leucocytes, for at the beginning of the experiment there had been 42 per cent leucocytes and 50 per cent monocytes, while at the last study there were 18 per cent leucocytes and 71.5 per cent monocytes.

The next experiment was similar in type (rabbit CD 27, graph 2). After 13 intravenous injections of trypan blue, and intraperitoneal injection of a mixture of 10 c. c. of whole blood and 4 c. c. of dye was made. The vital studies of peritoneal exudate, made 24 hours later, showed 5.3 per cent clasmatocytes in 800 cells. There were a few red cells free in the exudate and the clasmatocytes had taken up others. The monocytes were again markedly young, with mitochondria and no phagocytosis. Three were found in division. On this day leucocytes preponderated, there being 77 per cent, with 15.6 per cent monocytes. On the next day, after a second intraperitoneal dose, the monocytes had increased to 43.5 per cent, the clasmatocytes to 11.3 per cent, with phagocytosis in both groups. Five days later, after three more injections, there were 11.5 per cent clasmatocytes, many of which were of the small types shown in figures 5, 6, and 7, while the monocytes, which made 31.5 per cent of the cells, were markedly phagocytic and were represented by the cell in figure 18. On the last count, after three more injections, there was another shower of young, non-phagocytic monocytes, while the clasmatocytes were very phagocytic. The proportions were, leucocytes 54 per cent, eosinophiles 5 per cent, lymphocytes 7.7 per cent, monocytes 22 per cent, and clasmatocytes 15.2 per cent. Of the monocytes it was recorded that fully half were young cells.

GRAPH 2.—Relative proportions of monocytes and clasmatocytes in the peritoneal exudates of a rabbit (CD 27) after injections of blood and trypan blue into the peritoneal cavity.

The third experiment, with an animal chronically stained with trypan blue (rabbit CD 28, graph 3), had 7 successive studies of the peritoneal exudate, with 6 injections of blood into the peritoneal cavity. The experiment was begun on January 7 with an injection of blood into the peritoneal cavity; 24 hours later no exudate was found. Blood was again injected and in 24 hours there were very few clasmatocytes, while the monocytes constituted 54.5 per cent of the free cells. Of the monocytes, only 2 per cent were found to be phagocytic, while on January 12, when the next peak of the monocytes was reached, 13 per cent of them were found with other cells engulfed. On this day the phagocytosis of red blood-cells was
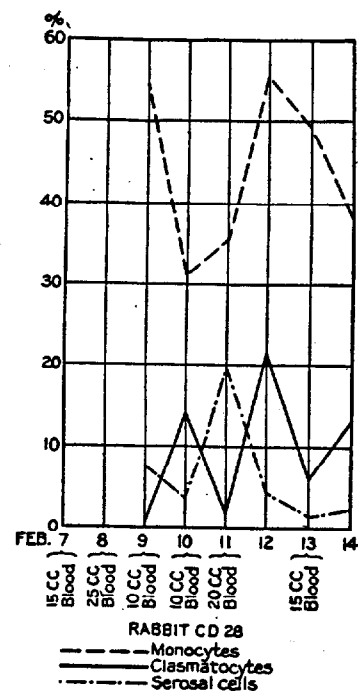
quite marked, many of the monocytes having from 2 to 5 red cells within them. On the last day of the study, when the monocytes were 38.5 per cent of the total free cells, about one-third of them were very young cells, one-third were mature but not phagocytic, and the other third had phagocytized whole cells. Amitosis of the monocytes was noted on every day and was marked throughout the experiment. There were two showers of clasmatocytes, one after 3, the other after 5 injections of blood. In both instances about half of the cells were the large types, with much phagocytized material, and half were the small types of clasmatocytes.

The trypan blue of these experiments was used, of course, to test whether the monocytes could be distinguished from the clasmatocytes by this dye. It was well known that clasmatocytes do take up trypan blue. We studied both vital films and fixed smears stained with carmine. By both methods it appeared that the monocytes did not react to the dye in more than a very minor way. For instance, in an unstained preparation 22 per cent of the cells had trypan blue, while 21.3 per cent were clasmatocytes in the vital-red differential. In the smears stained with carmine, the clasmatocytes could be seen containing both trypan blue and phagocytized cells. The trypan blue was often present in considerable masses in the clasmatocytes, while only rarely did a monocyte contain any, and then only as minute granules. Thus it is specifically the clasmatocyte, not the monocyte, that is the pyrrol cell of Goldmann, at least so far as acute reactions are concerned.

In an experiment in which blood alone was injected into the peritoneal cavity (rabbit S 47, graph 4), we made a number of interesting observations. The blood was, in every case, from other rabbits whose blood matched that of the recipient. The first dose was 24 c. c. plus 3 c. c. of a 20 per cent sodium-citrate solution and 10 c. c. of distilled water. The second and third doses (22 c. c. and 20 c. c. of blood respectively),

GRAPH 3.—Relative proportions of monocytes, clasmatocytes, and serosal cells in the peritoneal exudates of a rabbit (CD 28) after the injection of blood into the peritoneal cavity.

both with 2 to 3 c. c. of citrate, were given in the morning and afternoon of the second day. The differential count from exudate material 24 hours later was as follows: neutrophilic leucocytes 57.2 per cent, eosinophilic leucocytes 0.25 per cent, lymphocytes 1 per cent, monocytes 24.7 per cent, serosal cells 4.7 per cent, with 400 cells counted. The clasmatocytes on this day were markedly phagocytic; the cell in figure 4 was drawn from one of them. It was a very large clasmatocyte and contained the maximum load of débris, red blood-cells, and leucocytes, characteristic of the clasmatocyte of the early stages of irritation of the peritoneal cavity. On the next day vital studies were again made with no further intervening injection of blood. The clasmatocytes were still phagocytic, but the striking change was in the monocytes, which had increased to 34.5 per cent, and which
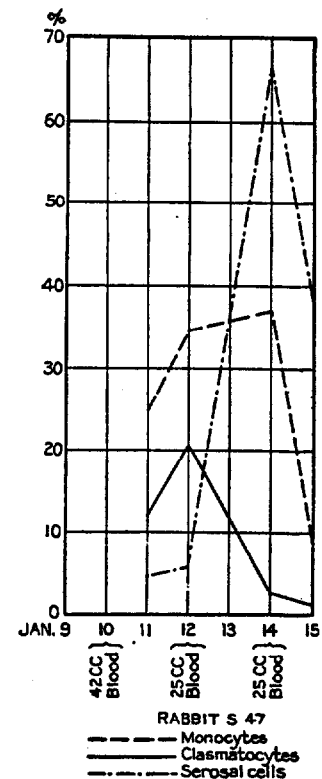
could be divided into two groups—one of young cells with marked mitochondria and very little phagocytic activity, the other (making about one-third of the total) of older cells, so markedly phagocytic that they approached in type the clasmatocyte. On this day it was noted that there was marked amitotic division of the monocytes.

In regard to the monocytes that were phagocytic, it is striking that many of them contained, besides the vacuoles that stained in neutral red, highly refractive unstained droplets. These droplets were like those that have been described as especially characteristic of the highly stimulated serosal cells. It will be noted that the number of serosal cells was high in this animal from the start; in the next count, made 2 days later, 24 hours after the fourth injection of blood, there was a very marked desquamation of serosal cells, often in great sheets, reaching the astonishing percentage of 66.5 per cent of the free cells. On this day 2 free serosal cells were drawn (figs. 21 and 22) to show the stage in the irritation of this type of cell. These cells are to be compared with the cell in figure 20, which was the typical non-irritated serosal cell scraped from the body-wall of an animal that had not been irritated; and with a still more stimulated cell (fig. 23) drawn from the exudate from this same animal on the following day. On January 24 the dividing monocyte of figure 17 was drawn. On the following day the serosal cells had dropped to 38 per cent, showing their rapid disappearance, and the monocytes were recorded as so highly phagocytic as to approach the appearance of clasmatocytes.

GRAPH 4.—Relative proportions of monocytes, clasmatocytes, and serosal cells in the peritoneal cavity of a rabbit (S 47) after injections of blood into the peritoneal cavity.

It was also noted that the monocytes contained many refractive granules, but no red blood-cells. The clasmatocytes, on the other hand, were few in number, but all of them contained both red and white blood-cells.

In the preceding experiments we have often studied the exudates 24 hours after the injection of blood. In three experiments we injected blood into the peritoneal cavity of an animal which had no other irritation of the cavity, and studied the exudate after an interval of 48 hours. In all of these experiments the clasmatocytes were loaded with red blood-cells. The monocytes, however, were the striking cell of the exudate and in two of the experiments they were recorded as being of two types—young, non-phagocytic forms and older phagocytic forms in about equal proportions. The third experiment (rabbit CD 50) was especially interesting. This was referred to on page 144 in connection with the highly stimulated monocyte of figure 19. Out of 79 monocytes in 100 cells, 2 were immature, 39 were mature, and 38 were markedly phagocytic. The point of especial interest was that 12 of the mature forms, or 15 per cent of the monocytes, were in division; one of them is shown with two nuclei and the centrosphere in figure 16.

These surveys of the cells of the peritoneal exudates, when taken together, give the following story of events: In the peritoneal cavity there may be a considerable number of neutrophilic leucocytes in rabbts that are apparently entirely normal. Lymphocytes play but little rôle, either normally or in mild irritations of short duration. There are usually a few large clasmatocytes in the cavity, and these cells become engorged with red and white cells within 24 hours after a first injection of blood into the cavity. These large clasmatocytes soon become loaded with débris to their maximum capacity, and in that state tend to disappear from the exudate. Normally, there are few monocytes, like those of the blood, in the body-cavity, but early after the injection of blood, large numbers of very young monocytes, much younger than those usually found in the blood, appear in the peritoneal cavity. In one of the animals in which there was a shower of young monocytes in the peritoneal cavity on the last day of the experiment, the omentum was studied and was found to have such large masses of young monocytes as to indicate that the omentum was the source of the showers of young monocytes which appeared in the peritoneum. Amitosis is the most striking feature of these young monocytes, as well as of the more mature ones, so that the percentage of monocytes quickly goes over that of any other type of cell in the exudate. The average percentage of division of the monocytes has been from 1 to 3, but in one instance 15 per cent of the monocytes were dividing. After 3 or 4 days of continued irritation, the large clasmatocytes of the early stages are replaced by younger and smaller clasmatocytes, which are, however, so phagocytic that our records repeatedly show that every single one of them contained a few phagocytized cells. At the same time the monocytes of these later stages have matured and become phagocytic. Monocytes take up a few red cells, but it is the clasmatocyte that uniformly engulfs them in large numbers.

<div align="center">SUBCUTANEOUS TISSUE.</div>

In the light of these studies of the cells of peritoneal exudates, we can analyze some of the results of the injections of blood into the subcutaneous tissues, though here, also, our studies are far from complete. It is a difficult matter to get a complete survey of the cells around an injection of blood into the subcutaneous tissues by the vital method, for there is such a large amount of tissue to be covered that the cells die before the entire area can be studied, even when three individuals are working. In two experiments made with the injection of cat's blood into the subcutaneous tissues of a rabbit, we found only large masses of leucocytes after 24 hours, and practically no reaction of the tissue-cells. Near the clot there were the normal clasmatocytes, but they did not seem to be stimulated in any way. In connection with the leucocytes, it was striking that they were young active cells and had well-stained mitochondria in their cytoplasm. Since the leucocytes of the circulating blood have usually lost their mitochondria, it seemed as if the leucocytes in the tissues must have been young cells called into the circulation by the injury. We therefore studied the blood and found that the leucocytes had nuclei that were markedly horseshoe-shaped, indicating a shifting to the left in the Arneth pattern; but we found only an indefinite reaction to janus green in the leucocytes of the

blood-stream, rather than the blue-staining mitochondria of the leucocytes around the exudate. In another rabbit (CD 39), studied 3 days after the injection of cat's blood into the subcutaneous tissue, we found the tissues filled with neutrophilic leucocytes and basophiles, but neither clasmatocytes nor fibroblasts near the clot.

We then took blood from the heart of a rabbit (S 50) and injected it into its own subcutaneous tissues and found that many of the clasmatocytes near the blood-clot were of the type drawn for figure 9, which shows what we consider to be the first sign of stimulation, namely, an increase in the size of the vacuoles of the cell. This can be seen by comparing the cell with the unstimulated form in figure 8. The clasmatocytes of the type in figure 9 had not taken up any of the red blood-cells, but in another area in this animal there were numerous clasmatocytes that had begun to engulf the red blood-cells. In this area there was perhaps a slight increase in the number of clasmatocytes over the normal number, but no monocytes were to be seen.

In the next experiment we injected blood taken from the heart of a rabbit on two different days into the right and left axillae, and then from matched rabbits into the right and left groins, also on different days, and finally made studies of each of the four areas. In the zone studied at the end of 24 hours, we found around the area leucocytes which were not much stimulated, that is, they did not show marked vacuoles or signs of phagocytosis. None of them showed mitochondria; that is, they were not the especially young forms that we had seen around the clot of cat's blood. There was also a considerable number of monocytes like those of the blood-stream.

At the end of 48 hours there were three striking changes: (1) The leucocytes had disappeared. (2) Monocytes were the predominating cell, and the notable thing about them was that they were actively dividing. The type is shown in figure 16, which was taken at this time and is a mature monocyte in division. In the same preparation we found 6 cells in division, which shows how active the process was. Besides these mature monocytes in active division, there were areas in which the monocytes had been markedly phagocytic and were either in a phase of maximum phagocytosis or even beyond that, in the phase of beginning degeneration. (3) The clasmatocytes were small and all phagocytic. In films of the tissues that had received the blood 4 days previously, the clasmatocytes were loaded with blood-cells. Monocytes were still present and fibroblasts were noted. In the zone that had been injected 7 days previously, the fibroblast was the predominating cell. These studies indicate that in the subcutaneous tissues, as well as in the peritoneal exudates, the monocytes, as well as the clasmatocytes, play a rôle as phagocytic mononuclear cells.

GENERAL CONCLUSIONS.

In the preceding pages we have given criteria, by means of supravital staining, for the separation of two types of phagocytic cells, clasmatocytes and monocytes. The endothelial phagocyte, both in its anchored form, the Kupffer cell, and in its free form, the clasmatocyte, has the greatest power of phagocytosis of any cell in the body. For this reason it was termed the *macrophage*. In the living cell the cytoplasm is very mottled, and when stained with neutral red is filled almost to the edge with bodies of varying size and color. In contrast to the monocyte, in which there is a definite pattern in the cytoplasm, there is no constant arrangement of any sort in the cytoplasm of the clasmatocyte. The first cells to be phagocytized are placed centrally, near the nucleus, and by this criterion a clasmatocyte can often be identified in fixed tissues; when much material has been engulfed, however, no pattern whatever can be made out.

With the monocyte, on the other hand, all of the substances we distinguish in the cytoplasm show a definite pattern. There is first the rosette of small bodies that surround the centrosphere; then the vacuoles, and later the phagocytized cells are in the periphery of the rosette, and beyond them the mitochondria. The mature clasmatocyte, in its active phase, seldom has any mitochondria, while the monocyte always has them, unless it be in the stage of degeneration. At the extreme phase of phagocytosis, toward the time of its death, the monocyte tends to approach the clasmatocyte in appearance, but the long period of distinction between the two types indicates a functional difference.

As has been said, it was from the study of splenic punctures that we first began to discriminate clasmatocytes and monocytes. Here the large and very actively phagocytic clasmatocytes are always to be found. It must be noted that in both spleen and liver the endothelial phagocytes appear to be especially capable of handling large amounts of foreign materials, particularly degenerating leucocytes and red blood-cells. But if Rous and Robertson (1917a, 1917b) are correct in concluding that the normal method of the destruction of erythrocytes is by a fragmentation of their cytoplasm, then it seems possible that monocytes might also play some rôle in this physiological process. The ability of cells to phagocytize cellular débris, bacteria, and other particulate matter seems, in the case of the clasmatocyte, to run parallel to the staining with vital dyes. But it must be noted that this correlation may not always be the case with other cells. For example, the question has been raised as to whether the polymorphonuclear leucocyte, a cell which is known to take up bacteria, has also the more general power of phagocytosis of which the taking up of trypan blue may be the symbol. In studying the group of leucocytes of the circulating blood, it is quite clear that there are at least two different substances that react to vital stains: first, some of the specific granules, such as the neutrophilic granules; second, certain round bodies which we have called vacuoles. Neutrophilic leucocytes, for example, may show vacuoles which react with varying shades to neutral red. In a general way, the neutrophilic leucocytes in a given specimen of blood all tend to react alike; when any of them contain vacuoles it will probably be true of all of them. Vacuoles appear in

leucocytes with such active motility that we interpret them as signs of function rather than of degeneration, and have assumed that they indicate that the leucocytes have been taking up débris. This is, however, only an assumption, for the matter has not been put to the test of forcing leucocytes to take up bacteria or trypan blue and then staining them supravitally with neutral red to ascertain if the phagocytized material could be seen through a red-stained fluid, thus constituting a typical vacuole of phagocytosis. The observation of these vacuoles in the leucocytes has been limited to the neutrophilic types, for we have seldom if ever seen them in either the eosinophilic or basophilic forms.

The point has also necessarily arisen with regard to the monocyte. Here none of the substances which can be stained vitally have been correlated with the granules of fixed smears. First, in the vitally stained monocytes, there are the small bodies of salmon tint in neutral red that make the rosette. These vary in number, from a single row in the youngest cell identifiable as a monocyte to very great numbers, far beyond the number we have illustrated in any of our drawings. Such monocytes are to be found in tubercles, soon to be referred to. Second, besides these small bodies there are the larger, so-called vacuoles of the periphery of the rosette, in the zone where we know that the cell places phagocytized whole cells. The peripheral vacuoles are not present in the immature forms; if, then, the small bodies of the rosette are vacuoles, it is not clear why they appear so long before the cell seems to function. In the mature form, also, the relationship of the bodies of the rosette to the vacuoles is not clear, but in the cases in which our experiments have made the cells more and more active, the two types of stainable bodies have approached each other in appearance until the entire cell has been filled with bodies like the large vacuoles. This is the type of cell seen in the case of Malta fever, already referred to, in which the centrosphere was still visible, and is the form seen in figure 19. In the production of such a type there must have been a great reduction in the number of the smaller bodies of the rosette. These observations suggest at least that the smaller bodies of the rosette may be tiny functional vacuoles that enlarge and coalesce.

We have not carried out any experiments in which trypan blue, alone or mixed with blood, has been administered intraperitoneally over very long periods of time. Cunningham (1922a) found that, in the cat, after very long exposure of the peritoneal cavity to doses of trypan blue, practically all of the free cells contained some blue dye. He was not able to confirm these findings on the rabbit, since this animal was less resistant to such experiments. In the light of Downey's (1917, 1918) findings, that all the blood-cells take trypan blue when exposed to it in static blood (i. e., between ligatures of a vein), the matter can be put to the test. Furthermore, Cunningham (1922a) found that in an occasional animal the polymorphonuclear leucocytes, and even the lymphocytes, contained trypan blue, and now we have seen neutrophilic leucocytes loaded with trypan blue in regenerating tissues. It must therefore be recognized that there are possible conditions in which the various hematological cells take trypan blue, but whether this is only after an injury to the cells, as H. M. Evans (1915) claims, can not be wholly settled

at present. F. A. Evans (1916, *a, b*) has shown that the mononuclear cells that give a positive oxidase reaction never show any carmine in their cytoplasm when the animal has been stained with lithium carmine, and he thinks that the "transitional cell," which is the monocyte of the blood-stream, always shows oxidase granules.

The significance of the efforts to distinguish the exact reactions of different types of cells to experimental stimulations is bound up with the effort to analyze the functions of these cells.   Taking the general group of the free cells of the connective tissues in the larger sense, that is, including the white cells of the blood, a study of the exact nature of the phagocytosis of whole cells, of cellular débris and of foreign bodies, such as bacteria and insoluble particles, is of great significance.   In the first place, substances developed in these cells from phagocytized material may be of functional significance.   That these cells have chemical, physiological functions has been shown, in that certain of them produce proteolytic ferments.   Recently a still more striking example was given by Carrel (1924) in the demonstration that leucocytes give out growth-producing substances.   In the second place, the power of taking up such foreign bodies as bacteria may be, as Metchnikoff has indicated, a part of the mechanism by which animals have been able to survive.

The subject of the presence of mitochondria in these cells has been an interesting one.   As has been said, the very active clasmatocytes have not shown them. On the other hand, in the cell in figure 8, which was a typical clasmatocyte from areolar tissue, there was a clump of mitochondria showing as unstained granules at one end of the cell.   It has occurred to us, especially in connection with the monocytes, that the forms of cells capable of division tend to have mitochondria often in large numbers, so that their presence may be a sign of a young or of a mature form, and their absence may be correlated either with a very undifferentiated stage or with a senile stage in which there is little or no capacity to divide.   Certainly, mitochondria are always present in monocytes and we have found them in division in the peripheral blood of normal animals, as well as in the exudates which we have produced experimentally.

The actively phagocytic clasmatocyte of the spleen has proved so fragile in our preparations as to suggest that it might also disintegrate readily in the body. On the other hand, the clasmatocyte of the subcutaneous tissues has been considered a cell of long life, on account of the experiments with trypan blue.   With repeated injections of the animal, this cell becomes engorged with trypan blue and may be demonstrated in so chronic a reaction as to preclude its being a cell of short life. Indeed, it is highly probable that the clasmatocyte of the diffuse connective tissues is a cell of long life and of a low rate of increase by cell division.   In the spleen, however, the clasmatocyte is constantly dealing with material, not like the insoluble, artificial dyes of the experimental irritations, but with substances which it can and does dissolve, and it is therefore not unlikely that in this organ the cell does disintegrate when it has dissolved a great mass of phagocytized material.   If this concept be correct, that the large clasmatocyte of the spleen is constantly disin-

tegrating, it would be easy to understand why it is continuously being formed directly from endothelium in this organ. This idea of their ready disintegration would also fit with the findings of Simpson (1922) that in animals in which the endothelium of the splenic sinuses is being irritated by repeated injections of particulate matter, so that there is a very unusual production of these cells, showers of clasmatocytes pass into the circulating blood and the cells are found in the blood of the right ventricle, but are absent or nearly so from that of the left ventricle. Moreover, Simpson emphasized that these showers of macrophages are very transitory, for they may constitute 70 to 80 per cent of the white cells in the right heart at one time, while 15 minutes later the blood of the same ventricle may show only 1 to 3 per cent of them. We have also found that typical clasmatocytes, the endothelial phagocyte, may occur in the peripheral blood-stream in our experimental animals. It is now known that the megalokaryocytes may also occur in the blood (Minot, 1922), and so these two different types of very large cells must be carefully discriminated in preparations of abnormal blood.

· We believe that the weight of evidence indicates that clasmatocytes come from endothelium. In the first place, in early embryonic life there is a time of a very general origin of free cells from the walls of vessels. This has been observed by Schmidt (1892), Schridde (1907), Danchakoff (1907), Maximow (1909), and Sabin (1920). It is practically certain that in the spleen the origin of these cells from endothelium is a constant process throughout life. Whether there is a widespread origin of clasmatocytes from the endothelium of the peripheral capillaries is not yet certain. Mallory, McJunkin, Foot, and Permar believe that, in certain pathological states, such a derivation occurs, but this conclusion is not yet generally accepted.

In this connection we wish to emphasize our two groups of clasmatocytes, the large branched ones and the smaller round ones. We suggest that these smaller clasmatocytes are not simply the original cells rounded up, but that they may have come from the division of the larger forms. It may well be that a mild irritation stimulates certain of the clasmatocytes to phagocytosis and certain younger forms to division. One possible sign of this is that some of the large clasmatocytes of the tissues contain mitochondria and many of the smaller forms have them. We have not actually seen the division of clasmatocytes, but we have found clasmatocytes with two nuclei 24 and 48 hours after the injection of blood into the subcutaneous tissues. Certainly the clasmatocytes have no such rate of division as monocytes, where we often find from 1 to 3 per cent, or even as many as 15 per cent of them, dividing. It is a striking point, however, that the small clasmatocytes, which we think may be cells that have just divided, are highly phagocytic, so that if we are correct in our assumption that they have resulted from the division of the large forms, it is clear that they represent the division of a mature cell and function at once. The small, active clasmatocytes that appear in peritoneal exudates could easily have come from the cells of the omentum, and those of the subcutaneous tissues from the surrounding and preexisting, quiescent clasmatocytes. In such a place as the spleen, where it is certain that

clasmatocytes are constantly arising from endothelium, it would be interesting to study those within the sinuses and actually attached to the wall, with regard to the matter of size and phagocytic power.

When we turn to the monocyte, on the other hand, one can readily find in splenic punctures all the steps of their origin from primitive cells. We are illustrating this origin in another paper in this volume (Cunningham, Sabin, and Doan). The tracing of the origin of the blood-cells by interpreting a series of types as showing their origin has been the main evidence of the maturation of the blood-cells from the time of Ehrlich. While recognizing its weakness, we also are using it, necessarily; but we are at the same time giving a proof of a quite different order concerning the earliest cells of our series. We wish here to state that the primitive cell which we found in depleted and simplified bone-marrow, and which we have identified as the forerunner of the white blood-cells, we have seen also in the connective tissues of the lungs, in the omentum (associated with clumps of young monocytes), and, most interesting of all, in the liver. The specimens from the latter were from a rabbit in which an active tuberculosis of the liver had been produced by Dr. Arnold Rich and Dr. C. Promas, in order to study, by the vital technique, the cells that make up the tubercle. Through their kindness we were permitted to study their material and found in several bits of tissue, not distorted by technical procedures, clumps of young monocytes, so young that the rosettes had only one row of neutral-red bodies; these young monocytes were touching the primitive cell, on the one hand, and more mature monocytes, the characteristic epithelial cell of the tubercle, on the other. Thus we consider that there is some proof that the monocyte has this very striking characteristic: it can and does develop *in situ* very widely over the body, but considerable time is required for its maturation.

In some of the peritoneal exudates in which there were showers of young monocytes we had exceptional opportunities to see all the stages of the development of monocytes, without the presence of any other cells that might complicate the picture. This was true because all other cells of the exudates were wholly mature forms. We therefore are convinced that the monocyte develops like a blood-cell—that it maturates from a primitive form. A clasmatocyte develops like a tissue-cell; that is, it comes from the division of a mature cell and functions at once after division. This is our interpretation, since we find that every single small, young clasmatocyte in the peritoneal cavity has phagocytized other cells, while monocytes drawn into the peritoneal cavity take 2, 3, or 4 days before they become phagocytic. Thus the monocyte of the tissues arises like a blood-cell and has to undergo a maturation before it becomes a typical adult type.

Normally, the place where monocytes can always be found developing is the spleen, and perhaps this organ is the more usual place of origin of monocytes of the blood; but we believe that the monocyte may arise locally in wide areas of the body and that its remarkable power of cell division often makes it the predominating functional cell after it has had time to mature. We thus think that it has a much wider zone of origin than the leucocyte. The leucocyte, as has long been known,

comes to the tissues from the blood-stream and is the first cell to reach a damaged area; it has great power of rapid motion, but has wholly lost its power to divide and hence it dies out. The clasmatocyte is the next cell to react, and being a highly phagocytic cell, can take up a great deal of débris; soon, however, the large clasmatocyte is replaced by smaller ones, half the size of the original ones. These small clasmatocytes are all phagocytic. The question of their origin in adult connective tissue is not wholly clear. We make the suggestion that they arise in loco in adult tissues from the division of preexisting clasmatocytes, since this would cover the known facts of normal tissues. This leaves the question open as to whether there is a new differentiation of clasmatocytes from endothelium in adult subcutaneous tissues under conditions of disease. That there is a continued differentiation of clasmatocytes from the endothelium in certain places, notably in the splenic sinuses, is practically certain. Ultimately, in an irritated area, clasmatocytes are far outnumbered by monocytes, which are much slower to become phagocytic because they have to maturate before they become functional. We consider that, normally, monocytes are forming all the time in the spleen and in small numbers in bone-marrow, but that they may arise locally, under conditions of irritation, from primitive cells that are to be found in small numbers in the connective tissues everywhere.

Thus we consider that the weight of evidence points to two separate strains of phagocytic cells of the connective tissues: clasmatocytes, which are of endothelial origin and come into the blood-stream only occasionally and abnormally, and monocytes, which are a constant type of blood-cell, arising largely in the spleen, but also a specific cell of the diffuse connective tissues, where they both arise and function. By our vital technique we can discriminate the endothelial phagocyte both in its large active or large inactive form and in the small, very phagocytic types that occur in irritated areas. From these three phases of the endothelial phagocyte we can distinguish the monocyte and tell whether it is in the young phase, too immature to function, or whether it is the mature form. We can discriminate the monocyte in a highly active state of phagocytosis, but there may be a stage of beginning degeneration in which we can not distinguish the two types of cells. In experimental work, records of these types of cells may be made by the vital technique and then correlated with the study of the fixed tissues. In this way we may learn to discriminate these different types in fixed tissues. The goal of these surveys is to open up a method to test the different phases of function that must have been the stimulus for the differentiation of these types of cells.

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DESCRIPTION OF PLATES.

PLATE 1.

All of the cells on this plate are clasmatocytes, drawn while the cells were living. They were all taken from the rabbit. The cells of figures 1 to 3 were from the spleen; of figures 4 to 7, from peritoneal exudates; of figures 8 to 10, from areolar tissue; of figures 11 to 13, from the omentum. All of the cells are given at the same magnification, which is about 2600. A red blood-cell of a rabbit drawn at the same magnification is given for comparison.

FIG. 1.—Typical large clasmatocyte from a splenic puncture of a normal rabbit (S 46). Drawn January 10, 1924. This cell represents the type to be found in small numbers in every puncture of the spleen; it makes about 2 to 3 per cent of the free cells. Stained with neutral red and janus green.

FIG. 2.—Smaller clasmatocyte from the spleen of a rabbit (S 47). Drawn January 16, 1924. This rabbit had received 5 injections of blood into the peritoneal cavity for the sake of stimulating peritoneal exudates. This was probably without effect on the spleen and the cell may be regarded as normal for that organ. It had engulfed one nucleated cell. Stained with neutral red alone.

FIG. 3.—Smaller clasmatocyte from the spleen of a normal rabbit (S 45). Drawn January 10, 1924. The differential count of the cells from this puncture showed 2.5 per cent myelocytes, 1.5 per cent clasmatocytes and 22 per cent monocytes. This cell is the smallest clasmatocyte we have found in the spleen. It had not phagocytized any whole cells. Stained in neutral red.

FIG. 4.—Large clasmatocyte from the peritoneal cavity of a rabbit (S 47). Drawn January 11, 1924. This animal had received 3 injections of citrated rabbit's blood into the peritoneal cavity, one 2 days before and two the day before the studies were made. This cell shows the marked stimulus toward phagocytosis under the conditions of the experiment. The cell shown in figure 14 is a young, unstimulated monocyte drawn from the same exudate. This clasmatocyte had engulfed both red and white blood-cells, which are seen in every stage of being digested. The reaction to the stain shows the marked variation in color characteristic of clasmatocytes. Stained in neutral red.

FIG. 5.—Clasmatocyte from the peritoneal cavity of a rabbit (CD 27). Drawn February 26, 1924. This cell was taken after prolonged irritation of the peritoneal cavity. This animal had received 13 doses of a 1 per cent solution of trypan blue intravenously and 8 doses of rabbit's blood and trypan blue intraperitoneally. This is a young clasmatocyte containing mitochondria in its cytoplasm. It had, however, phagocytized one cell, which is seen in characteristic position against the nucleus. Stained with neutral red and janus green.

FIG. 6.—Clasmatocyte from the peritoneal exudate of the same rabbit (CD 27) as figure 5. Drawn February 26, 1924. It is also a young clasmatocyte; it had phagocytized 4 red blood-cells. Stained in neutral red.

FIG. 7.—Clasmatocyte from the peritoneal cavity of the same rabbit (CD 27) as figures 5 and 6, but taken earlier in the experiment. Drawn February 23, 1924. The rabbit had received 13 doses of a 1 per cent solution of trypan blue intravenously and 6 doses of blood and trypan blue intraperitoneally. All of the clasmatocytes on this day were like this one and all of them had phagocytized red and white blood-cells. This cell is to be contrasted with the cell of figure 18, which is a monocyte taken from the peritoneal cavity of the same animal two days later to show the actively phagocytic monocyte. This clasmatocyte had phagocytized 2 red blood-cells, and they are in the characteristic position in the cell. Stained with neutral red.

Fig. 8.—Clasmatocyte from the peri-renal connective tissue of a rabbit (S 45). Drawn January 10, 1924. The connective tissue had been made edematous by the injection of neutral red (1 to 10,000) into the aorta. This is the typcial clasmatocyte of the normal connective tissue, identical with the type to be found in the subcutaneous tissue and in the intermuscular septa. There is a group of unstained mitochondria in one end of the cell. The cell had two nuclei. Stained with neutral red alone.

Fig. 9.—Clasmatocyte from the groin of a rabbit (S 50). Drawn January 22, 1924, 24 hours after the injection into the groin of 12 c. c. of blood taken from the heart of the same animal. The cell shows the first reaction to the injection of blood—an enlargement of the bodies that stain in neutral red, which we consider to be vacuoles of phagocytosis. Several of the vacuoles in this cell contained débris. This cell is to be compared with the unstimulated type of figure 8. Stained with neutral red.

Fig. 10.—Clasmatocyte taken from the subcutaneous tissue of the axilla of a rabbit (S 51). Drawn Januray 1, 1924, 24 hours after the injection of 12 c. c. of blood from a matched rabbit into the axilla. This cell had just taken in a red blood-cell, whose color is still like that of the normal red cell. Another red cell had been engulfed earlier and appears intensely stained with neutral red. Besides these red cells there are many large stained vacuoles. Stained with neutral red.

Fig. 11.—Clasmatocyte from the omentum of a normal rabbit (S 49). Drawn January 18, 1924. This cell shows the typical unstimulated type, entirely comparable with the clasmatocyte of figure 8. Stained with neutral red.

Fig. 12.—Clasmatocyte from the omentum of a rabbit (CD 27). Drawn February 26, 1924. This cell is to show the effect of prolonged irritation. The rabbit had received 13 doses of 1 per cent trypan blue intravenously and 8 doses of blood and trypan blue intraperitoneally. The vacuoles of the cell contained masses of trypan blue which have been obscured by the subsequent staining with neutral red. This is the large cell of the omentum to be compared with the small clasmatocyte of the omentum, taken at the same time and shown in figure 13. It may also be contrasted with similar small clasmatocytes found free in the peritoneal cavity at the same time, shown in figures 5 and 6. Stained with neutral red.

Fig. 13.—Clasmatocyte from the omentum of the same animal as the cell of figure 12 (CD 27). Drawn February 26, 1924. This is the small type of clasmatocyte. It is like cells found free in the peritoneal cavity at the same time and shown in figures 5 and 6. Stained with neutral red.

PLATE 2.

The cells on this plate are monocytes, serosal cells and a fibroblast. They were all taken from the rabbit and were drawn while the cells were living. The cells of figures 14 to 19 are monocytes; of figures 20 to 23, serosal cells; figure 24 is a fibroblast. All of the cells are given at the same magnification, which is about 2600. A red blood-cell of a rabbit drawn at the same magnification is given for comparison.

Fig. 14.—Monocyte from the spleen of a normal rabbit (S 46), the same from which the clasmatocyte of figure 1 was taken. Drawn January 10, 1924. It represents the elongated shape which the monocyte assumes when in motion. The rosette of bodies stained in neutral red is obscured, as shown here, when the cell moves. The mitochondria are shown in green. Stained with neutral red and janus green.

Fig. 15.—Monocyte from a puncture of the peritoneal cavity of a rabbit (S 47). Drawn January 11, 1924, on the third day of the experiment. The animal had received 3 injections of blood, one 2 days previously and two on the day before the cell was taken. It is to show the young, non-phagocytic monocyte which is characteristic of the early stage of a mild irritation of the peritoneal cavity. It has the simple resette of bodies staining with neutral red and the outlying mitochondria. Stained with neutral red and janus green.

Fig. 16.—Monocyte from the subcutaneous tissue of a rabbit (S 51). Drawn January 1, 1924, 48 hours after the injection of blood from a matched rabbit into the axilla. This cell is to show the first phase of amitotic division which is characteristic of the monocytes. It is a fully differentiated monocyte, with a well-marked rosette, a few outlying vacuoles, and many mitochondria. The nucleus has divided and the centrosphere is in the center of the cell between the two nuclei. Stained with neutral red and janus green.

Fig. 17.—Monocyte from a peritoneal exudate of a rabbit (S 47). Drawn January 14, 1924, after four injections of blood into the peritoneal cavity covering a period of 5 days. The cell was a mature monocyte and was drawn to show the second phase of amitotic division, namely, the division of the centrosphere. The centrosphere had divided when the cell was found and the subsequent division of the cell in the line of the arrow was completed 15 minutes later. Stained with neutral red.

Fig. 18.—Monocyte taken from the peritoneal cavity of a rabbit (CD 27). Drawn February 21, 1924. This cell is to show the monocyte in the phase of active phagocytosis. The rabbit had received 13 doses of 1 per cent trypan blue intravenously and 8 injections of blood and trypan blue intraperitoneally. The drawing was made 7 days after the first intraperitoneal injection. The cell had phagocytized at least 8 cells and shows that material taken into the monocyte is placed peripherally. The characteristic rosette is plain in the *hof* of the nucleus. Stained with neutral red and janus green.

Fig. 19.—Monocyte taken from the peritoneal cavity of a rabbit (CD 50). Drawn May 26, 1924. This rabbit had received blood into the peritoneal cavity 48 hours previously. This cell shows a stage of activity which may be bordering on degeneration. The centrosphere is still visible but obscure. Stained with neutral red and janus green.

FIG. 20.—Serosal cell scraped from the anterior body-wall of a normal rabbit (S 49). Drawn January 18, 1924.
This cell shows the characteristic mottled cytoplasm of the normal serosal cell. It had no mitochondria
and only three small bodies that reacted to neutral red. Stained with neutral red.

FIG. 21.—Serosal cell from the peritoneal exudate of a rabbit (S 47). Drawn January 14, 1924. This rabbit had
received four injections of citrated blood into the peritoneal cavity and the drawing was made on the
fifth day after the first injection. On this day serosal cells made 66.5 per cent of the free cells. This
cell shows the beginning of the development of the refractive droplets characteristic of an irritated
serosal cell. The bodies that reacted to neutral red appeared only after the cell had been watched for
half an hour and so they may be a phenomenon of degeneration. Stained with neutral red.

FIG. 22.—Serosal cell from the peritoneal exudate of a rabbit (S 47). Drawn January 14, 1924. This cell was taken
at the same time as the cell in figure 21 and is to show a still greater number of the refractive droplets.
Stained with neutral red.

FIG. 23.—Serosal cell from a peritoneal exudate of a rabbit (S 47). Drawn January 15, 1924. This cell was taken
from the same rabbit as the cells of figures 21 and 22, but after the fifth injection of blood. It was
taken 24 hours after the fifth injection and 6 days after the first injection. It is to be compared with
the cells of figures 21 and 22 drawn the preceding day. It shows a still greater reaction of a desquamated
serosal cell to irritation. Stained with neutral red.

FIG. 24.—Fibroblast from the subcutaneous tissue of the body wall of a rabbit (S 47). Drawn January 15, 1924.
It was taken from the area through which the peritoneal cavity had been punctured many times. It
is to show that the refractive bodies that occur in such large numbers in serosal cells, occur also in the
fibroblast under irritation. Stained with neutral red and janus green.

PLATE I

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Rabbit red cell

J. F. Didusch fec.

A.Hoen & Co.

PLATE II

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Rabbit red cell

J. F. Didusch fec.

A.Hoen x Co.