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A STUDY OF SPLENIC FUNCTIONS WITH RESPECT TO RED BLOOD CELLS

by

Seh Hoon Song, M.D., M.A.
Department of Biophysics

Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
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Seh Hoon Song

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University of Western Ontario,
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ABSTRACT

It has been known since the 1920's that one function of the spleen in mammals is to store red cells which can be discharged into the circulation in an emergency, and another is to cull and destroy abnormal or aged red cells. If this be true, the site of storage for these two different purposes (reservoir vs destruction) could be different. Therefore the red cell washout might show a different pattern from that of a single compartment.

By perfusing isolated feline spleens with oxygenated Ringer's solution, the characteristics of red cell washout were studied. The kinetics showed that the red cell washout corresponded to that from a three-compartment model with desaturation half times, 54 min, 8 min, and 30 sec, equivalent to perfusion by 0.2, 9.8 and 90% of the total splenic blood flow, respectively. These compartments were referred to as _slow_, _intermediate_ and _fast_ respectively.

The red cell washout was also studied from spleens contracted by norepinephrine or epinephrine, and the kinetics corresponded to a three-compartment model. However, the total store of red cells in the spleen decreased significantly (equivalent to 12% of the total mass of the animal). This

was due almost entirely to a 16-fold reduction in size of the intermediate compartment. This compartment may, therefore, be identified as the reservoir from which cells are discharged in an emergency.

As this model was derived merely from a mathematical analysis, further experiments were carried out using histological techniques and observing the spleen under the microscope. When 50 ml of Ringer's solution were perfused, free cells in vascular lumens disappeared. This confirmed that the fast compartment represents red cells in the vessels. After 600 ml, few free cells remained in the red pulp and thereafter the number of cells adhering to reticulum cells or sinus walls decreased gradually. It is concluded that the intermediate compartment represents free cells and the slow compartment bound red cells in the red pulp, respectively.

By collecting the venous outflow at three particular stages of the washout, pure samples (>85%) of cells from each compartment were obtained. Density and volume measurements showed red cells of the slow compartment (SV_S) were lighter and larger ($P<0.01$) than those of circulating cells (PA) suggesting that they are young red cells. Furthermore, reticulocyte counts of the venous outflow using supra-vital stains showed that the slow compartment consists entirely of reticulocytes. The ratio of reticulocytes to rubricytes in the spleen (75:1) suggested that the reticulocytes were not produced in the spleen but were accumulated from the circulating

blood. With this evidence and observations of rubricytes in the spleen in different stages of the denucleation process, it is concluded that immature red cells released from the bone marrow are sequestered and matured in the spleen.

I. INTRODUCTION

The mammalian spleen has been considered by many biologists as primarily a lymphoid organ. It is well known that germinal centers, which consist mostly of lymphoid cells, can be seen even with the naked eye when the organ is cut open through the stroma. However, quite apart from the lymphoid structures the spleen contains abundant red blood cells in the red pulp, and the vascular system in the organ is very complicated. The abundance of red cells in the spleen has drawn the attention of many investigators since 1854 when Gray first described the presence of capillary meshes in the red pulp. It is now generally accepted that the spleen is an erythro-lymphoid organ rather than just a simple lymphoid organ.

Many questions arise from this interrelationship between the spleen and red cells. Why should the spleen retain such a large fraction of the total red cell mass and yet transport only a small portion of this into the venous blood at a steady rate? Are the red cells altered in any way when they pass slowly through the spleen? We do not yet know the answers to these questions.

In the early 1920's, Barcroft and his associates (Barcroft & Barcroft, 1923; Barcroft & Poole, 1927) began to think of this organ as a reservoir of red cells. Using several different procedures, e.g. exercise, bleeding, CO inhalation or epinephrine injection, in cats, dogs, rabbits and sheep, they demonstrated conclusively that the spleen is indeed a reservoir of red blood cells. However, Barcroft could not eliminate the possibility that other functions might be involved, for he observed that the hemolytic behavior of red cells obtained from the splenic pulp was different from that of cells taken from peripheral blood (Barcroft, 1925). Only if red cells in the spleen could maintain normal physiological conditions during the storage would it be perfectly reasonable to infer that the spleen is nothing but a simple physiological "BANK" of red cells.

Meantime, Rous (1923) and Mellgren (1939) suggested that the spleen might also be a place of red cell destruction, i.e. a "GRAVEYARD". This hypothesis was well supported by their observations that splenic red cells were more osmotically fragile and were apt to be easily destroyed in the spleen. Since then, it has been generally assumed that old or abnormal red cells can be culled and destroyed, or else phagocytosed by macrophages in the spleen (Crosby, 1957; Weiss, 1962; Simon & Burke, 1970).

Axiomatically, any organ which is rich in macrophages is regarded as a filter against foreign materials and is known to be a part of the reticuloendothelial system (RES). With

respect to the filter system of the human spleen, it has been proposed that aged cells would be removed from the general circulation if the organ contained a filter equivalent to a system of cylinders 3.7μ in diameter. The exact size of this "mini-cylindrical diameter" was derived from a laborious statistical treatment of the areas and volumes of 1016 red cells taken from peripheral blood (Canham & Burton, 1968).

In addition to the filtering or culling of red cells, the spleen is also thought to have the capacity of producing new red blood cells (erythropoiesis) during early fetal life (up to five or six months in man) until the bone marrow takes over the hematopoietic function (Thiel & Downey, 1921). Furthermore, it has been recently reported that the spleen even in adult mice and rats is able to produce red cells (Gurney & Rosett, 1968). This is an interesting contrast to the ideas of a "BANK" or a "GRAVEYARD". There may, of course, be differences among mammals in the function of the spleen with respect to red blood cells but, nevertheless, a series of interesting problems confront us; how could this organ carry on a triple role at once? What kind of a structural organization of the splenic pulp could allow it both to produce and also to destroy cells?

It is quite disappointing to find that the structure of the splenic microcirculation has remained almost completely hidden in mist, so to speak, in spite of the hard efforts of many investigators. The first reliable observation regarding the scheme of the splenic intermediary circulation was that of

Knisely (1936). His closed circulation theory, derived from direct observations on transilluminated spleens, claims that there are closed channels between the ends of the arterioles and the beginnings of the venules. On the other hand, the open circulation theory, revived by MacKenzie, Whipple & Wintersteiner (1941) from examination of transilluminated spleens in the same species studied by Knisely proposed a diffuse passage of blood through the red pulp from the terminal end of the arterioles (considered as opening on to the sinuses), the blood draining to the venules without passing through any well defined vascular channels in between. In this way, the vascular architectonic theories have been divided into two contradictory themes---open versus closed. To seek a compromise, Snook (1958) suggested that the intermediary circulation could be open anatomically, but physiologically closed, and recently, electron-microscopists seem to have accepted that a combination of the two theories best describes the nature of the splenic microcirculation in the red pulp (Weiss, 1962; Koyama, Aoki & Deguchi, 1964; Burke & Simon, 1970).

Harris, McAlister & Prankerd (1958) recorded radioactive counting rates over the surface of the spleen in man, after injection of 51 Cr-labelled red cells intravenously, and observed two distinct components of the mixing kinetics, rapid and slow. At the same time, Motulsky, Casserd, Giblett, Brown & Finch (1958) reported similar results from experiments on normal human subjects, on patients with hereditary spherocytosis, and in animals (mice and rats). These findings also

agreed with that of Barcroft and Barcroft (1923) who demonstrated a slowly-mixing phase of CO-combined red cells in the spleen following a rapid phase and explained it in terms of a very large pool of stored red cells. On the other hand, Motulsky *et al*. (1958) hypothesized that the slow compartment might be the result of splenic destruction of red cells since its size could vary with the hemolytic conditions of various patients. The results of radio-isotope studies of mixing kinetics are in fact highly suggestive of a combination of both "BANK" and "GRAVEYARD" in the red pulp and also of a combination of the "open" and "closed" circulation schemes.

If the mixing kinetics depend on both the condition of the red cells passing through the splenic sinuses and the paths taken through the red pulp, then measurement of the transit times of red cells through the spleen may throw an important light on what the spleen does to red cells. For instance, if the sites of retention of red cells for reservoir purposes and for culling or destruction were separated, then whether the spleen is a simple "BANK" or a "GRAVEYARD", or both, could perhaps be demonstrated by the time courses for red cells to traverse the different loci where these functions are carried out. It is possible, of course, that the particular locations involved might be quite distinct from the ordinary vascular channels. If these assumptions be true, then the washout behavior of red cells from the spleen, when the whole organ is perfused with cell-free Ringer's solution, would not correspond to a single exponential decay, but would

be equivalent to that from a system of two or more compartments.

Therefore in this thesis the author has had the following aims; (1) to determine the kinetics of red cell washout from isolated, perfused feline spleens, (2) to investigate the morphological counterparts of the corresponding compartments, by examination of histological sections obtained from spleens perfused by different volumes of the perfusate, (3) to characterize certain physical properties of red cells sampled at different times during the course of washout. This would show whether the red cells from different compartments are different in any way and whether they are physiologically normal or not.

II. HISTORICAL BACKGROUND

The phrase "Mysterii Plenum Organon" stated first by Galen early in 150 A.D. has been a common cliché used to describe splenic structure and function (quoted from Krumbhaar, 1926; Dameshek & Welch, 1953; Burke & Simon, 1970). In fact, all investigators who have engaged in a study of splenic anatomy and physiology have left one aspect or another unexplained or incompletely explained in their treatises. Nevertheless, our knowledge about the spleen has been vastly increased since the old Goulstonian lecture given by William Stukely in 1722 (quoted from Prankerd, 1963). The whole developmental chronicle can be divided into eight distinct sequences which are shown in Table I. For the purpose of further discussions in detail, this chapter will be presented with two major separate subjects, (1) The spleen, and (2) Red blood cells in relation to the spleen.

(1) The spleen

The spleen is found in all vertebrates (Klemperer, 1938; Romer, 1970). Its function and/or the equivalent structure may differ among species (Klemperer, 1938; Kampmeier, 1969; Romer, 1970) and it has been recognized that the organ may differ as regards both morphology and function in young

TABLE I

A chronological development of investigations on the mammalian spleen

	Special Topics	References
1)	An initial period of vigorous morphological studies	Gray, 1854 Billroth, 1860, 1862
2)	A speculative stage on the functions of the spleen	Krumbhaar, 1926
3)	A period of intensive study on the reservoir function	Barcroft, 1930
4)	A period of investigating the splenic sequestration of red cells	Björkman, 1947
5)	A period of studying the pathological and clinical aspects of the spleen	Dameshek & Welch, 1953
6)	A period of studying the hemolytic function of the spleen	Crosby, 1957
7)	A stage of assessing the quantitative relation between the splenic pooling of red cells and destruction of red cells	Prankerd, 1963
8)	A thorough review on the splenic structure and hemolysis	Wennberg & Weiss, 1969

versus old individuals of the same species (Gross, 1919).

(A) Morphology of the spleen

All mammalian spleens have certain general structures in common, such as a capsule, trabeculae, lobules, red pulp and white pulp. However, the circulatory channels through the capillary network in the red pulp have been described in a confusing way---closed or open, perhaps a combination of the two, in different species (Snook, 1958) and even in one particular animal (Weidenreigh, 1901; Helly, 1902-3).

It was as early as 1860 when the intensive anatomical investigations on the microcirculation of the splenic red pulp started (Gray, 1854; Billroth, 1860 & 1862). The following is the presently accepted picture of the circulatory pathway, the arrows indicating the direction of blood flow: from the splenic artery → the central artery → passing through the germinal center → surrounded by sheath of Schweigger-Seidel (1862) and passing through the Billroth cord → then draining into sinusoids or venous capillaries leading to collecting venules → the splenic vein.

This pathway was apparently observed by early investigators for Billroth wrote as follows (Billroth, 1860):

"Nachdem durch die Arbeiten von Ecker, Gray, Hlasek, Reichert, Förster, Leydig, Kölliker, etc. die schon früher ausgesprochene Ansicht aufs Neue bestätigt und weiter ausgeführt war, dass die rothe Milz pulpe aus einem cavernösen Venennetz bestehe, in welches die Arterien einmunden, und das hier das Capillarysystem besondere Eigenthümlichkeiten Zeige und nicht wie in andern Organen des Körpers eingerichtet sei, wurde ich durch die Arbeiten von Fuhrer veranlasst, den merkwürdigen Resultaten dieses Forschers näher nachzugehen".

The problem thus seemed to be so simple that just five arrows could indicate all the directions of the blood flow within the spleen, as mentioned above; however this is not true. When Billroth observed the intermediary circulation of the human spleen between the arteriolar end and the splenic sinusoid, he described it as being "open" ("mit offener Mündung") and his name is still used to describe the system of "Billroth cords". This discovery of an "open" circulation later attracted the attention of many anatomists, hematologists, and pathologists.

Around the same time in 1861, Axel Key presented the opposite findings in the same journal in which Billroth reported. Using the injection method he observed a ramified capillary network which did not communicate with the sinuses in the red pulp. Therefore, Key thought the sinuses must be separated from the vascular channels by definite "closed" walls. Since this observation, the debate regarding the "open" and "closed" circulation theories has continued without agreement yet being reached. The debate became hottest during the 1940's when Knisely (1936) observed the closed circulation while MacKenzie et al. (1941) described the open system again. Some anatomists now believe that both types of circulation are indeed present (Whipple, Parpart & Chang, 1954, Snook, 1958). Whether or not the circulation is "open" or "closed" is very important from the viewpoint of splenic function with respect to red blood cells, especially the destruction of abnormal or aged cells.

Table II - Groups who proposed "open" circulation theory

Authors	Methods	Subject
Billroth (1860, 1862) Stieda	Histological Sections Injections	Man
Müller (1865)	Injections and Histological Sections	Man. Apes to Fish
Frey (1874) Warth (1891) Laguesse (1891) Hoyer	Histology	Man Dog
Weidenreich (1901)	Injection Avian RBC	Rabbit Dog
Mall (1903)	Injection (Na-nitrite)	Dog
Neubarth (1922)	Isolated perfusion	Swine, Dog, Cat
Thoma (1924)	Dye Injection	Dog
Mills (1926) Foot (1927)	Indian Ink	Man
Robinson (1930)	Perfusion	Cat
McNee (1931)	Transillumination	Mouse
MacKenzie et al. (1941)	Transillumination	Mouse
Whipple et al. (1954)	Transillumination	Mouse
Parpart et al.(1955)	Transillumination	Mouse

Quoted from Billroth (1860, 1862), Weidenreich (1901), Foot (1927), Robinson (1930), MacKenzie et al. (1941), Björkman (1947), Whipple et al. (1954), Parpart et al. (1955).

TABLE III - Groups who proposed "closed" circulation theory

Authors	Methods	Subjects
Key (1861, 1862) Gray (1854)	Injection	Calf, Sheep, Swine, Man
Kölliker (1849, 1867)	Injection	Man
Kyber (1880), Retzius (1886) Krah (1877), von Ebner (1889)	Injection	Man
Thoma, *et al*. (1895)	Injection	Dog
Sokoloff (1888)	Artificial Congestion	Rabbit
von Kalenkiewicz (1892) Wicklein (1888) Panski (1890)	Histological Sections	Dog Cat
Helly (1902, 1903)	Histological Sections	Dog, Rabbit
Mollier (1910, 1911)	Perfusion	Man, Ape, Dog, Cat
Knisely (1936)	Transillumination Living Spleen	Mouse, Rat, Cat
Snook (1950)	Histological Sections	Rabbit
Peck & Hoerr (1951)	Transillumination	Rabbit

Quoted from Gray (1854), Knisely (1936), Björkman (1947), Snook (1950), Peck & Hoerr (1951).

Summaries of these two different schools of thought are presented in Tables II (open circulation theory) and III (closed circulation theory). These tables show a trend in that rabbits were generally considered to have a "closed" system, while mice were considered to have an "open" system. In this regard, Snook (1950) studied the comparative histology of splenic vascular arrangements in several species of mammals using a graphic reconstruction method. He classified the 11 species into two different groups, (1) sinusal and (2) non-sinusal, from differences in the degree of vascularization in the red pulp. Moreover, he accepted the theories of both Knisely and MacKenzie _et al_. as well, maintaining that the spleens of guinea pigs, rabbits, rats, dogs and man fit into Knisely's schema, while spleens of mice, moles, cats, horses and cows corresponded to that of MacKenzie _et al_.

If Snook's observation (1950) was right, the rabbit's spleen must have closed channels with powerful sphincters as Knisely (1936) described. Later Snook (1958) studied the rabbit's spleen again and described the intermediary circulation of the organ as being predominantly of the "open" type. Another confusing example which was reported by Weiss (1957, 1962) is also worth noting. In 1957 Weiss suggested that the classically accepted Billroth's cord does not exist, but was made artificially by collapsing the sinusal structures, and later in 1962 he wrote that the cord is a genuine structure. I believe this unfortunate contradiction was not the investigator's fault, but arose from the

complexity of the organ and the use of different techniques.

Reviewing the methods presented in Tables II and III it should be noted that unnecessary distortion can easily be introduced in any study. For instance, if an indicator dye were to be injected through the splenic artery into the red pulp, the amount of injected material will determine the portions of the area to be shown, and the injection pressure may well distort the normal pattern of the blood flow. This was pointed out by Tait and Cashin (1925) who perfused the spleen with Ringer's solution and then injected dyestuffs into the splenic artery and as well as intravenously in dog, cat, and rabbit. They found it necessary to maintain the temperature of the infusate at 39°C, the perfusion pressure at 80 mm Hg, and to remove large particles from dyestuffs in order to obtain consistent results from perfused spleens.

Even the famous transillumination method (Knisely, 1936; MacKenzie _et al._, 1941) for studying the microcirculation through the spleen of the living animal has its inherent artefacts. Whipple _et al._ (1954) pointed this out and quoted from MacKenzie _et al._ (1941) that "Transillumination carries with it as serious limitations as does any other technique. Optic difficulties are due to the different refractivity of the parenchymatous organs,----".

In addition to the structural complexity of the spleen the role of environmental factors in controlling the status of the individual microvessels as being "open" at one

time and "closed" at another, or even intrinsic properties of the smooth muscle within the red pulp of the spleen could constitute additional regulating factors. We do not, however, have any definite evidence on which to base this notion except for the overall splenic rhythms discovered by Barcroft and his colleagues (1932).

(B) Functional aspects of the splenic red pulp

Prankerd began his Goulstonian lecture (1963) by referring to that given by William Stukely in 1772. Stukely mentioned that the sponge-like spleen was usually filled with blood and can aid digestion during mealtime by throwing blood into the circulation whenever the diner is cheered. Apparently in the eighteenth century most physiologists believed that the spleen was the place where emotion and digestion were controlled. The term, SPLEEN, originates from the Greek and the Latin SPLĒN, which means 'ill temper' or 'violent mirth' (Britannica World Language Dictionary, 1958).

In 1926, when Krumbhaar reviewed about 185 references, it was recognized that the spleen has a variety of functions (Table IVA). Although Krumbhaar presented evidence taken from the original papers to support each particular aspect of splenic function, he commented as follows; "it is surprisingly disappointing that the results obtained in many directions have not been more conclusively indicative of its functions". Furthermore, his section entitled "general conclusions" contained many such phrases as "in some way as yet unknown, it seems to be...., supposed antagonistic relations, or there is good reason to consider....,". Moreover, the ideas which presumed possible effects of the spleen on digestion and metabolism or, perhaps, endocrine functions are very speculative, far beyond the limit of

TABLE IV

A list of splenic functions

	A. (Krumbhaar, 1926)	B. (Wennberg & Weiss, 1969)
1.	Contractility and reservoir function	Storage of red cells and delayed circulation
2.	Blood formation	Questionable except in hypoxic rats and mice
3.	Blood destruction	Conditioning, fragmentation, and phagocytosis
4.	Immunity to antigens and tumors	Antibody production. Immunity to tumors?
5.	Digestion and metabolism	?
6.	Endocrine functions	?
7.	?	Sequestration of lymphocytes and monocytes and transformation into plasma cells and macrophages
8.	?	Sequestration of platelets, particles, and colloid foreign matters

? means unknown or unexplained

conclusive evidence.

Two aspects of a possible endocrine function of the spleen have been reported; (1) a humoral substance produced by the living cells of the shielded, implanted, or injected splenic tissues (Jacobson, 1952) and (2) a neuro-humoral substance from the splenic nerve endings (Peart, 1949). The former was suggested as a non-cellular factor(s) which was capable of protecting hematopoietic cells from radiation damage; it was thought to be related to the control of erythropoiesis (Giovannini & Biagioni, 1968). However, the identity of the humoral factor is not yet known. The latter was known as "splenic sympathin" and this was shown to be norepinephrine (Ottis, Davis & Green, 1957; Gillespie & Kirpekar, 1966; Klein & Thureson-Klein, 1971), which had been later studied extensively in connection with regulation of the splenic blood flow (Celander, 1954; Green, Ottis & Kitchen, 1960; Ross, 1967). Besides humoral substances from the spleen, Tomaya, Mora & Montfort (1960) also suggested that unknown factors released from other organs might cause hypersplenism, but this has not been proved yet.

It has been recognized that the spleen does have "contractility" and "a reservoir function of red blood cells" (Barcroft, 1930). The rhythmical nature of splenic volume changes was first described by Roy (1881), and has since been studied by Schäffer & Moore (1896), Hargis & Mann (1925), Barcroft, Khanna & Nisimaru (1932), Ferguson, Ivy & Greengard (1936), Grindlay, Herrick

& Mann (1939), Fleming & Parpart (1958) and Greenway & Stark (1967). This functional aspect was very well supported by morphological findings on the lobular nature of the splenic pulp (Mall, 1903) and the innervation of the spleen (Utterback, 1944). Utterback (1944) found that in the cat the splenic nerves arise exclusively from the celiac plexus (i.e. are sympathetic in nature) but these were later shown to be sympathetic cholinergic nerves (Burn & Rand, 1960). Since that time the function of the spleen with respect to red cells has been investigated extensively.

By 1969, Table IVA had been changed into Table IVB with revisions contributed by many investigators between the 1930's and 1960's (Wennberg & Weiss, 1969). In IVB the sequestration of platelets, particles and colloid foreign matter is worthy of note, for if the spleen is a part of the RES, it must be capable of filtering out foreign particles or bacteria which have invaded into the animal body. It has been reported that not only bacteria (Wyssokowitsch, 1866, quoted from Robinson, 1928; Mudd, 1922) but also foreign red blood cells (Opie, 1925), platelets (Bedson, 1926; Penny, Rosenberg & Firkin, 1964; Aster, 1966) and possibly all the abnormal material present in the blood (Voorhoeve, 1923) can be trapped and treated by the spleen. Jacob, MacDonald & Jandl (1963) and Jandl (1959) have provided firm evidence that the spleen is very efficient in removing particulate matter from the circulation, but how it does this is not yet properly understood.

If the splenic filter consisted of a system of cylindrical apertures, a simple mechanical separation of particles from the menstrum would be possible. Now, from the viewpoint of an "open" type of circulation, it is difficult to presume the presence of such a cylindrical system in the splenic pulp. On the other hand, the "closed" theory would allow the possible existence of multiple cylinders in parallel which could retain particles of diameter larger than that of the cylinders. The problem arises, however, how particles such as platelets or bacteria which are even smaller than normal red blood cells can be selectively recognized and retained in the splenic red pulp?

The process of filtration seems not to operate exclusively by the relative size of the pores in the spleen to that of foreign bodies, but perhaps by other physico-chemical forces between the splenic microcirculation and the particles. Regarding this particular subject, Robinson (1928) investigated the characteristics of the filtration process of different colloidal particles by injecting them into a peripheral vein of an animal (in vivo), or into the splenic artery of the isolated spleen (in vitro). He found that the spleen filtered out negatively charged particles (colloidal solutions of platinum and silver) while positively charged particles (of copper) were readily passed through the splenic pulp. His fascinating results led him to conclude that electrical forces between the particles and the pulp cells must play the important role in the filtration process.

Unfortunately, this ingenious theory has never been confirmed or even pursued by later scientists.

If Robinson's interpretation were valid, we might apply his hypothesis to explain the relationship between the splenic pulp and red blood cells. In other words, as red cells become older, and the electrical charge on the membrane changed, they could perhaps be selectively witheld by the pulp cells and hemolyzed or destroyed. Unfortunately the evidence does not support this idea, for as red cells get older their negative surface charge decreases (Danon & Marikowsky, 1969) and yet these older cells are thought to be culled and destroyed in the spleen (Jacob & Jandl, 1962a; Wagner, Razzak, Gaertner, Cain & Feagin, 1962; Weiss, 1962; Crosby, 1963).

(C) Hypersplenism

Studies of splenic function in man have been restricted largely to that in pathological states, for in health the spleen is a rather 'silent' organ which produces no dramatic effects (Dameshek & Welch, 1953).

The term, splenomegaly (megaly; originating from the Greek, megas meaning excessive or abnormal size) has been used to describe an abnormal enlargement of the spleen with or without hyperfunction, while hypersplenism merely designates a state of exaggerated splenic function (Dameshek & Welch, 1953). Blaustein (1963) stated that:

> Hypersplenism is a pattern of reaction in which the peripheral blood is lacking in an element whose bone marrow precursors are present in normal or increased number, in which

removal of the spleen leads to restoration of normal levels. Any or all of the formed elements of the blood may be affected and the state may be present in any disorder affecting the spleen, with or without splenic enlargement.

Von Haam and Awny (1948) suggested that hereditary spherocytosis, idiopathic thrombocytopenic purpura, splenic neutropenia and pancytopenia could also be considered under the category of primary hypersplenism. In these various cases the normal, healthy spleen can in fact bring about the destruction of all the formed elements in the blood. However, it is not yet known whether the hypersplenism is developed from a merely exaggerated normal function or from a pathological condition. It is too risky to conclude that the pathological spleen carries out the same functions as the normal spleen but to a greater degree, because hypersplenism has been known to bring about not only panhemocytopenia (Doan & Wright, 1946), but also neutropenia alone (Wiseman & Doan, 1942). A patient with hypersplenism can be cured symptomatically by surgical removal of the spleen, but whether he can survive indefinitely without incurring further trouble is not entirely clear. Certainly splenectomy results clinically in a state of hyposplenism.

(D) Hyposplenism

If splenic function is reduced below the normal level by means of regressive changes (Blaustein, 1963) or congenital anomalies (McLean & Craig, 1922; Adler & Van Slyke, 1953) the condition is known as hyposplenism (Crosby, 1963). A patient with congenital absence of the spleen could, if

free of other severe complications, survive for a normal life span (Boggs & Reed, 1953). Furthermore, patients survive well after splenectomy provided postoperative infections are controlled (Mayo, 1926; King & Schumacker, 1952; Smith, Erlandson, Schulman & Stern, 1957).

However, there is much evidence that blood components go wrong after splenectomy in the normal man and in patients as well as in normal animals (Krumbhaar, 1932; Singer, Miller & Dameshek, 1941; Miller, Singer & Dameshek, 1942). Krumbhaar (1932) made a thorough review of the literature on red cell counts following splenectomy; in normal mammals splenectomy induces a mild degree of anemia varying in duration and time of onset in different species and individuals. The anemic state then leads to an increase in reticulocyte count and an increase in osmotic resistance. Cellular hyperplasia develops in the bone marrow and this certainly represents a compensatory response to anemia but may also reflect the loss of an organ that had performed an important hemolytic function, for hyperplastic cells are also capable of destroying red cells. A marked increase in platelet count occurs after splenectomy, but Krumbhaar did not indicate whether this might be due to the removal of a platelet 'death-chamber' or not.

Krumbhaar's review (1932) was thorough but his conclusions were vague. In contrast to this, Singer, Miller & Dameshek (1941) provided very definite conclusions about five particular aspects; 1) appearance of target cells,

2) changes in hypotonic fragility, 3) increase in platelet count, 4) destruction of red cells, 5) alterations in metabolism of lysolecithin. More recently, it has been recognized that red cells can show quite bizarre shapes as a result of splenectomy (Slater, Muir & Weed, 1968; Smith & Khakoo, 1970).

At present, we cannot be sure whether splenectomy shortens the life of the normal man or not, because the spleen may involve other unknown factors essential for life and any of the factors mentioned above can, if severe enough, lead to a fatal condition. In fact, the relationship between the spleen and red cells is so important that we cannot afford to consider their functions separately.

(2) Red blood cells in relation to the spleen

It may be just a coincidence that in general red blood cells (erythrocytes) are found in all vertebrates and these animals all possess a spleen, or it may be that the spleen is a necessary organ for animals which have red cells.

(A) Equilibrium shape of the red cell

The shapes of red cells are not the same in different species (Schermer, 1967) although in all vertebrates red cells function as a carrier of O_2 (Prankerd, 1961; Bishop & Surgenor, 1964). In the higher classes of mammals (feline, canine, apes, and man, etc.) the mature red cell is round, biconcave and non-nucleated. Many biologists have been attracted to the study of red cells because of the problem posed by their unusual biconcave shape (Ponder, 1948; Lehmann & Huntsman, 1961; Fung & Tong, 1968).

It was once suggested that when the biconvex red cell precursor lost its nucleus, the viscous material would sink in to reform a non-nucleated cell as a biconcave disc (Howell, 1891...see Lehmann & Huntsman, 1961).

Teleological explanations for the shape were later sought in terms of ease of O_2 diffusion or the ease with which the cell could squeeze through microcirculatory channels. Prankerd (1961) emphasized that while the biconcave shape might appear to facilitate the diffusion of O_2 into red cells, theoretical calculations suggest very little advantage for this shape over others.

In this department different theories on the

biconcavity of red cells have been proposed by different investigators. Rand (1964) and Shrivastav (1968) agreed that the membrane of the red cell is stiff and homogeneous in a mechanical sense. Rand claimed that the pressure inside the red cell is slightly greater than that which exists outside. Because the membrane appears to be homogeneous and isotropic, Shrivastav suggested that the dimple could be due to molecular chains, between the opposite faces of the cell, which attract the membranes together. Canham (1970) proposed yet another theory, that of minimum bending energy of the membrane, to explain the biconcavity of the red cell. As yet, however, no particular explanation has been accepted generally. Certainly the high surface area to volume ratio of the cell and the deformability of the membrane are very important for survival of the red cell as it is buffeted around in the microcirculation (Merrill, 1969; Weed, 1970; Braasch, 1971).

(B) Different shapes of red cells

In the book written by Bessis and translated into English by Ponder (1956), there are fine chapters on techniques for studying blood cells and detailed descriptions about each blood cell series. Bessis presented five different forms of normal red cells which all occur in different media, viz. crenated sphere, crenated disc, flat form, cup-shaped and hollowed disc. Wintrobe (1967) described in detail the different shapes of red cells encountered in various hematological disorders, e.g. anemia

(reduced Hb content), polycythemia (increased red cell count), sickle cell anemia and hereditary spherocytosis, etc.

Recently, Weed (1970) reviewed disorders of the red cell membrane and summarized the causes of red cell fragmentation, based on references from 1864 to 1969; not only external physico-chemical stimuli but also genetic errors (thalassemia, sickle and hemoglobin H cells) could lead to fragmentation of red cells, particularly in the spleen. It is thought that a decrease in cellular deformability, due to rigidity of either the membrane or the cellular contents, is responsible for trapping and hemolysis in the spleen, but we do not yet have conclusive evidence about this.

(C) Inclusion bodies of the red cell

In blood smears we do not usually see any special granules within the cytoplasm of normal, mature red cells; using Wright or Giemsa stains the cytoplasm appears homogeneous and red in color. Sometimes, however, bluish stained, basophilic granules may appear in the cytoplasm. One example of these "inclusion" bodies is "Heinz bodies", which are generally thought to represent toxic hemolysis due either to oxidative agents, e.g. phenylhydrazine, (Rothberg, Corallo & Crosby, 1959; Jandl, Engle & Allen, 1960; Rifkind & Danon, 1965; Jacob, 1970; Simpson, 1971) or to lead poisoning (Mcfadzean & Davis, 1949; Engelbreth-Holm & Plum, 1950). Red blood cells containing Heinz bodies are known to be sequestered in the splenic pulp and destroyed there, possibly because of the presence of rigid particles within

the cell (Azen & Schilling, 1963; Acevedo & Mauer, 1963; Rifkind, 1965; Jacob, 1970).

Other examples of inclusion bodies are Howel-Jolly bodies, Cabot ring, refractile bodies which are believed to exist in immature or abnormal red cells, and iron dots giving the Prussian blue reaction, Azurophil granules, etc. (Bessis, 1956). In all these cases, the chemical nature of the particles, the cause of their occurrence and their pathological significance are still unknown; moreover their interrelationship with splenic function has not yet been established.

(D) Hemolysis

According to Stedman's Medical Dictionary (1966), hemolysis (G. haima, blood + lysis, destruction) means the alteration, dissolution, or destruction of red blood cells in such a manner that hemoglobin is liberated into the medium in which the cells are suspended. In essence, hemolysis means the leakage of hemoglobin out through the red cell membrane.

In the 1920's it was recognized that there are several ways in which hemolysis may occur in vivo (Rous, 1923). The most important mechanism proposed at that time was erythrophagocytosis by the RES. Later, the importance of hypotonic fragility was emphasized in connection with erythrostasis in the spleen (Castle & Daland, 1937; Ham & Castle, 1940a, b). The 1960's ended with a quite complicated set of hypotheses, such as the antibody reaction (Jandl &

Kaplan, 1960), pH and temperature (Murphy, 1967) and blockage of sulfydryl groups on the red cell membrane leading to metabolic inhibition (Jacob & Jandl, 1961, 1962a, b). Recently, the metabolic aspect of red cells has been regarded as the primary factor in the maintenance of both the biconcave shape and the cellular deformability (Weed, LaCelle & Merrill, 1969).

Those who regard the spleen as a graveyard of red cells are agreed that either the hemolytic process or erythrophagocytosis must be responsible for the destruction of red cells in the splenic pulp. Several mechanisms have been suggested, e.g. low pH and pO_2, antibodies (Jandl, Richardson-Jones & Castle, 1957, Cutbush & Mollison, 1958), hypotonic stress (Baker, 1967), low glucose level (Jandl, 1968) and low cholesterol level (Murphy, 1962). Although all these factors are known to contribute to the "conditioning" of red cells (Wennberg & Weiss, 1969), none of them has yet been proved to participate in the destruction of red cells in the spleen. Electron microscopists have concentrated on the study of erythrophagocytosis (Essner, 1960; Pictet, Orci, Forssmann & Girardier, 1969; Simon & Burke, 1970) and their findings raise such questions as; how many red cells can be destroyed per day by this mechanism? Are the red cells hemolyzed already before phagocytosis or after being in contact with phagocytes? Is the red cell destroyed or in fact remodelled by RES cells? Such questions cannot be answered from "static" pictures, but require investigation

by more powerful "dynamic" techniques. This field really needs the genius of new ideas and new experimental approaches.

(E) Regulation of red cell production and destruction

The fact that human red cells cannot survive longer than 120 days (Berlin, 1964) and yet the total number of circulating red cells is maintained at a steady level, strongly suggests that there must be a regulating mechanism which matches the rates of production and destruction of red cells.

It is widely accepted that in the fetal stage of mammals and in most lower vertebrates, the spleen carries out erythropoiesis (Klemperer, 1938; Romer, 1970). In the more mature stages of the animal, the erythropoietic function is taken over exclusively by the bone marrow. In the adult it is the bone marrow which must produce each day a quantity of red cells equal to that destroyed. When the balance is broken, a state of anemia or of polycythemia ensues.

The concept of the 'erythron' (Boycott, 1929) is that..."the whole blood is an organ". In other words, the erythron indicates the combined mass of immature and mature red cells, including all cells in the red cell series, intra-vascular as well as extra-vascular, circulating as well as fixed. Boycott proposed that hypertrophy of the erythron might be induced by low O_2 concentration (polycythemia at high altitude) and that atrophy of the erythron could result from a high O_2 environment. This concept is interesting from the viewpoints of the blood as a tissue or an organ, and of O_2

as the possible regulator of the total mass of blood cells.

During the last two decades the endocrine function of the spleen has been studied extensively in relation to bone marrow function (Altman, Watman & Salomon, 1951; Dameshek & Welch, 1953), but all these efforts led only to indirect suggestions without any solid evidence (Hayhoe & Whitby, 1955). In this regard Crosby (1959) wrote:

> "Splenic control of erythropoiesis in the bone marrow is a topic on which good friends fall out. The reason for this is, of course, a lack of valid information which leaves us our hypotheses to argue over and ambivalent data to support them. The bone marrow is not the organ of erythropoiesis; the bone marrow is its housing. The organ of red cell production is the erythroblast".

Crosby summarized the possibilities of splenic control of erythropoiesis as follows: 1) the spleen might influence the enzymatic synthesis of hemoglobin or stromal protein, 2) the spleen might change the number of erythropoietic units in the bone marrow, or 3) the spleen may determine the age at which the young red cells are released from the bone marrow. No firm basis for these three hypotheses has yet been found, the only evidence being indirect (Giovannini & Biagioni, 1968).

Since the 1950's considerable progress has been made in understanding the humoral regulation of erythropoiesis. A plasma factor has been found to increase the total number of red cells, the hematocrit ratio and the reticulocyte count as well as the nucleated red cell population of the bone marrow (Erslev, 1953). It is now

established that this factor, called "erythropoietin", is usually released from the Kidney (Sokabe & Grollman, 1962; Hoffman, 1968). The isolation of this substance in a pure form was recently achieved by Goldwasser and Kung (1968).

These new discoveries bring together hematology and endocrinology, and there is always a possibility that yet other factors than erythropoietin may be involved (Kennedy, Nathanson, Tibbetts & Aub, 1955). According to Boggs (1966), however, there is no convincing evidence so far that this is so. Bogg's elegant analysis has certainly encouraged other workers to become involved in this area (Gordon, 1970; Krantz & Jacobson, 1970).

(3) Summary

The following is a list of unsolved problems, from a review of the historical background, in which the author is most interested:

(i) Is it an "open" or a "closed" circulation in the splenic red pulp that conveys red cells through the micro-vasculature?

(ii) What is the main function of the spleen with respect to red cells, is it a "BANK" or a "GRAVEYARD"?

(iii) How many red cells are stored in the spleen at any time?

(iv) How are the red cells distributed in the spleen and what kind of red cells remain for a longer time in the spleen?

(v) What is the mechanism of storing the red cells in the spleen?

(vi) What are the advantages of having both white and red pulp in the spleen?

(vii) What is the relationship between the spleen and bone marrow and how does it operate?

With regard to these questions, the author intends to examine particularly the red cell store and its distribution in the spleen. A few suggestions will then be offered on each of the remaining topics listed above.

III. METHODS

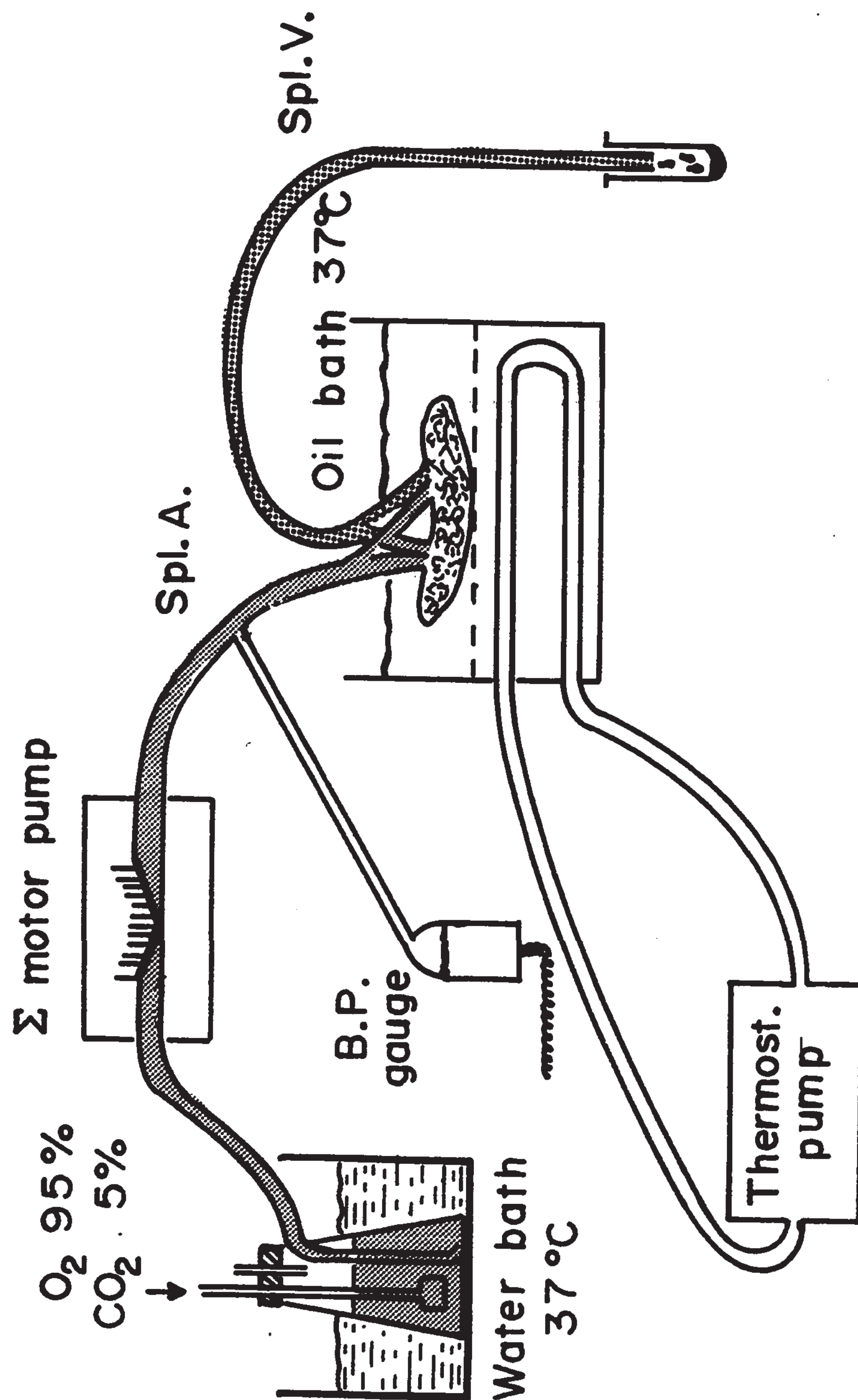
(1) Preparation of the isolated spleen for the perfusion

(A) Normal spleens

The experiments were carried out on cats and dogs. The animals were anesthetized with sodium pentobarbital (cats; 30 mg/kg intraperitoneally, dogs; 30 mg/kg intravenously, "i.v."). After laparotomy through the linea alba, small vessels around the spleen were carefully tied off. Then the greater omentum and the gastrosplenic ligament were cut near to the spleen. After tying off the left gastroepiploic vessels and pancreatic branches, the spleen was isolated vascularly except via the main splenic vessels. The animals were then heparinized (i.v. 500 U.S.P. units per kg). Polyethylene cannulae were inserted into both splenic artery and vein as far from the spleen as possible. After these cannulations the spleen was removed from the animal and placed in a lucite chamber containing 500 ml of mineral oil at 37°C (Fig. 1). At the bottom of the chamber was a heat exchanger in which water at 37°C was circulated by a pump (Haake, Model FS). The spleen was perfused with Ringer's solution by means of a Sigmamotor

FIGURE 1

Schematic diagram of perfusion system. The isolated spleen is immersed in mineral oil at 37°C and is perfused, via a Sigmamotor pump, with Ringer's solution equilibrated with 5% CO_2 in O_2. The pressure at the splenic artery is measured by a Statham transducer, and the venous outflow is collected in a measuring cylinder.


O_2 95%
CO_2 5%
Water bath
37 °C
Σ motor pump
B.P.
gauge
Thermost.
pump
Spl. A.
Oil bath 37°C
Spl. V.

pump (Model TS); the pulse pressure was reduced by addition of air to a small, inverted reservoir in the input circuit. In order to maintain both the flow rate and the inflow pressure within a physiological range, the spleens were perfused at various flow rates between 2.1 and 11 ml/min (2 to 5 ml/min/kg; Frohlich & Gillenwater, 1963). For each spleen the flow rate was maintained constant throughout the experiment.

The perfusion pressure in the splenic arterial cannula was measured continuously using a Statham transducer (P23DC) and was recorded by a Beckman type R dynograph from the beginning of the infusion to the end of the experiment.

The distal end of the venous cannula was set at 15 cm below the level of the spleen and the outflow was collected in a measuring cylinder. In this way the accumulated volume of the outflow up to a given time could be determined. Samples of the venous outflow were collected, at pre-arranged time intervals, for 30 to 60 seconds in graduated centrifuge tubes. The flow rate and also the cellular concentration in each sample could then be determined.

(B) Contracted spleens

In 10 healthy cats weighing 1.7 to 2.5 kg, after surgically exposing the spleen, either l-norepinephrine (0.2% solution, Winthrop Lab.) for 7 cats or epinephrine (0.1% solution, Connaught Lab.) for 3 cats was given i.v. up to an amount of 50γ. Contraction of the spleen was noticed within

less than a minute of the injection. Then the spleen was removed from the animal after usual procedures of cannulation. The infusate also contained 1 γ/ml of the same catecholamine as given previously to the animal and additional doses of the catecholamine were added as necessary to maintain the inflow pressure at a constant level. Especially in the case of epinephrine, more additional doses were required, probably due to rapid degradation of the substance under the influence of high O_2 tension in the Ringer's solution.

(2) Preparation of the perfusate

In most of the experiments a modified Ringer's solution was used, the formula for which is given below:

NaCl	:	9.00 gm/L
KCl	:	0.42 gm/L
$CaCl_2$	:	0.24 gm/L
$NaHCO_3$	:	0.20 gm/L
Glucose	:	1.00 gm/L

to one liter of distilled water and adjust the osmolarities to be 305 mOsm/L approximately.

Sometimes ordinary Ringer's solution without glucose or even the simple physiological saline (0.9% NaCl) was used.

The infusate and the diluting solutions for the Celloscope Counter were prepared free of particulate matter by filtration through Millipore filters (GSWP 04700, 0.22μ) three times before use.

As shown in Fig. 1, the reservoir containing Ringer's solution was kept in a water bath at 37°C. A gas mixture (5% CO_2 in O_2 or N_2) or air was bubbled through the solution for the whole experimental period.

(3) Determination of cellular concentrations in samples of the venous outflow

All data on cellular concentrations in samples of the venous outflow or of arterial blood, presented in this thesis, were determined by means of a Celloscope Counter (Particle Data Inc. series 112).

The diagram of the counter is shown in Fig. 2. Two electrodes (E_o and E_i in Fig. 2) are located in the same electrically conducting medium (in our case it is Ringer's solution), but parted by a glass tube (G). Direct current, from a constant current generator, flows between E_o and E_i through a small aperture (diameters 120, 75, or 48μ, A) made in a Ruby crystal. By means of a vacuum pump, a constant negative pressure was set to allow a steady flow of the medium containing cells from the beaker (B) into the glass tube (G) via the small orifice (A). Any particles or cells that have a different electrical resistivity from that of the suspending medium cause a voltage change, when drawn through the aperture, by changing the aperture impedance. In this way, the cells can be detected and their passage recorded, by an amplifier and an oscilloscope, as a series of pulses whose sizes reflect the individual cell volumes.

The reproducibility of counting was tested by counting all samples three times and also by counting at different dilutions made with cell-free, thoroughly filtered, Ringer's solution. There were always a few particles detected

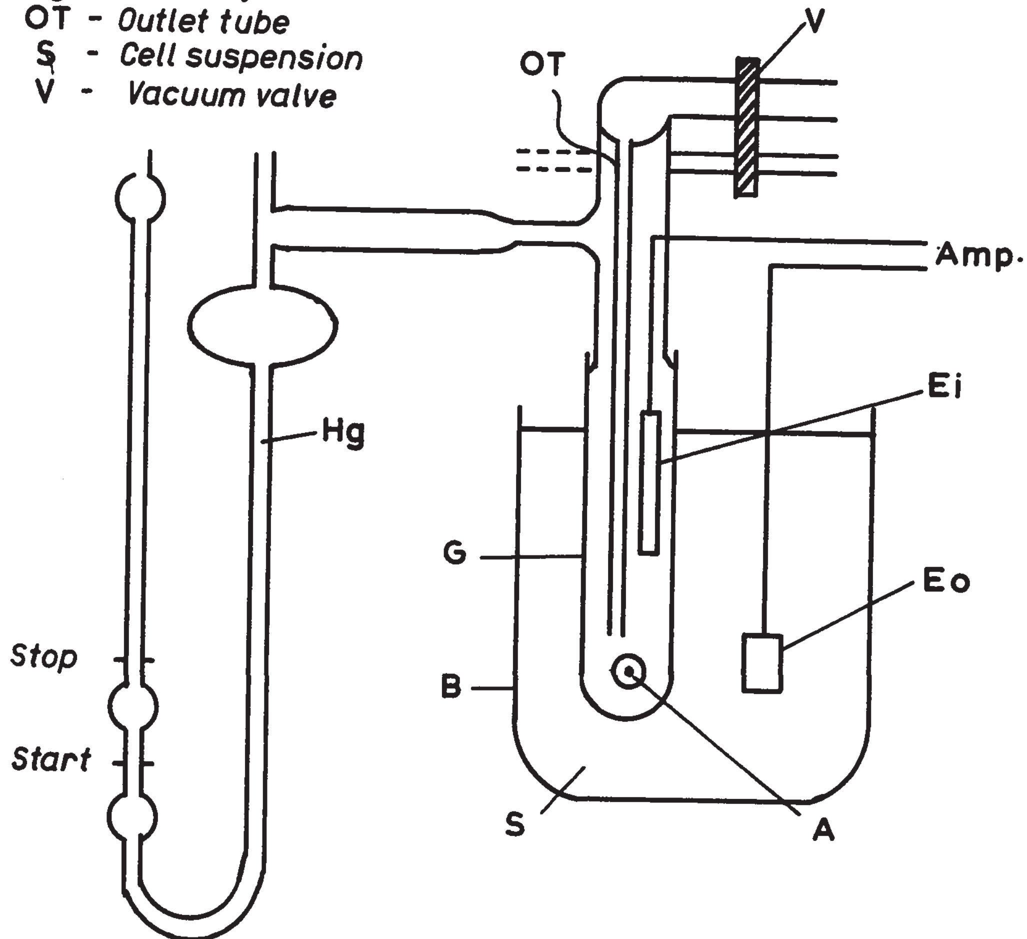


Fig. 2. Schematic diagram of the particle counting system in the Celloscope counter.

in the Ringer's solution, even after three successive filtrations through the Millipore filters. These "background" counts were less than 20 (x64) per 0.5 ml sample and were subtracted from the total counts to give the true sample count.

Because of possible contamination with dust particles from the air, collecting tubes, diluting pipettes and suspending beakers were handled with great care and in an environment as dust free as possible. The volumes of the final diluted samples amounted to between 20 and 50 ml and the dilution was made so as to give not more than 500 pulses (x64) per 0.5 ml of the sample in all cases. Sample counts of cells greater than $10\mu^3$ in volume were assumed to be the numbers of red cells, since above this range the fractions of other blood components, platelets or white cells were negligible in comparison with the red cells.

The distribution of cell volumes obtained by the Celloscope may in fact be distorted, but this will not affect the results obtained for cellular concentrations. However, at one stage of this thesis we shall be concerned with measurements of cell size distribution (section III-7) and therefore the underlying mechanism of the Celloscope will now be considered in a little more detail.

The magnitude, ΔR, of the resistance change when a cell passes through the aperture can be described as a function of the particle volume v (Kubitschek, 1958):

$$\frac{\Delta R}{R} = \frac{v}{V} \left(\frac{\rho}{\rho - \rho_o} - \frac{a}{A}\right)^{-1}$$

where A = area of the aperture normal to its axis,

a = cross-sectional area of the particle normal to the axis of the aperture,

V = volume of the sensing zone,

ρ_o = resistivity of the electrolyte,

ρ = resistivity of the particle.

When red blood cells are being counted $\rho >> \rho_o$ and thus the first term in the bracket becomes equal to unity. Under conditions when $a << A$ the second term in the bracket approaches zero. If this term is not zero then the resistance change will be strongly dependent on the orientation of the cell with respect to the orifice. In the present case the maximum value of $a/A = 0.02$ (i.e. cell normal to the axis of the orifice) so that the dependence on cellular orientation is of minor importance. Under these conditions the equation reduces to

$$\frac{\Delta R}{R} \simeq \frac{v}{V}$$

It was pointed out by Harvey (1969) that, unless the rise time of the amplifier is at least four to five times less than the shortest transit time of particles through the sensing zone, the height of the electrical pulse developed will be dependent on the radial position of the particle within the transducer. The Celloscope (series 112) was designed so as to meet these requirements.

We have therefore confidence to use the Celloscope for determination of cell size distribution.

(4) Compartmental analysis using the washout method

Rather than use radioactively-labelled cells, we have chosen to study the washout of the normal, red blood cells contained in the spleen at the time of cannulation by perfusing with cell-free osygenated Ringer's solution. There are certainly some disadvantages in the use of radioisotope-tagged cells, for they may have been altered in some way as a result of the incubation and labelling procedures and may be treated as abnormal by the spleen, or they may represent primarily the younger cells (Danon, Marikovsky & Gasko, 1966).

If the spleen is considered as a container of red blood cells, then by analyzing the cellular concentrations in the venous outflow as a function of the duration of perfusion, we may obtain information on the kinetics of red cell washout from the spleen. If the spleen confines red cells in a single compartment, the red cell washout will obey the following, simple differential equation:

$$-\frac{dR_t}{dt} = k.R_t$$

where R_t = the red cell content in the spleen at time t, and k = the rate constant of the washout, equal to flow F divided by volume V. The solution is:

$$R(t) = R_o.e^{-kt}$$

where R_o = the initial content of red cells in the spleen.

Again, if the spleen consists of several compartments of red cells, the equation will be the sum of a series of exponential functions with different initial contents of red cells (R_{Oi}) and different rate constants (k_i). Then the general solution will be represented by the following equation:

$$R_T = \sum_{i=1}^{\infty} R_i(t) = \sum_{i=1}^{\infty} R_{Oi} \cdot e^{-k_i t}$$

where 'i' represents the number of the compartment and R_T, the total content of red cells at time t. On the basis that the cellular concentration in the venous outflow is strictly reflected by the red cell contents of the splenic compartments and the flows through each compartment, the washout curve of red cells from each compartment will be exponential. Applying the above mathematical method, we have treated the experimental data of cell concentrations in the outflow, by plotting them on a semi-logarithmic scale against time or the volume of Ringer's solution perfused.

(5) Preparation of histological sections of the spleen

This method was used to study two major subjects; one was to investigate histological characteristics of the distribution of red cells and the other was to observe nucleated blood cells in the splenic red pulp.

Histological specimens were obtained from spleens perfused by various volumes of Ringer's solution, as follows:

(i) less than 50 ml (three spleens)

(ii) 50 to 300 ml (three spleens)

(iii) 0.8 to 3 liters (ten spleens).

The splenic vessels were tied off at the end of the perfusion while the pressure was still maintained and the whole spleen was fixed in 10% buffered formalin solution. After embedding in paraffin, sections 3 to 5μ thick were cut. A variety of staining procedures was used; Giemsa, Wright, Masson's trichrome and H & E (hematoxylin and eosin). Most of the observations were made using sections stained with H & E because this provides the clearest definition of red cells and R.E. cells in the red pulp. The number of red cells/cm^2 remaining in each section was counted visually, at a magnification of 200x or 400x, either from photographic pictures or by direct observation under the microscope. The total number of residual red cells in the spleen was then estimated from measurements of the splenic volume, the thickness of each section, and the number of red cells/cm^2 of the section.

The limits of error for such a single estimate are of necessity large, probably on the order of ± 50%.

In another series, twelve cats and two dogs were used and histological specimens were obtained from spleens perfused by various volumes of Ringer's solution between 1.2 and 3.5 liters. These volumes represented 0.4 to 1.5 liters per kg of body weight, and were sufficient to remove from the spleen the cells representing the fast and intermediate compartments (see IV-1-C). The whole spleen was fixed in absolute alcohol, Bouin's or Zenker's fixatives or in isotonic buffered formalin. Various stains described above were used. Sections 3 to 10μ thick were cut and 3 to 10 serial sections from each spleen were examined under the light microscope.

These histological sections were prepared by Mrs. Z. J. Pattison through the courtesy of Department of Pathology.

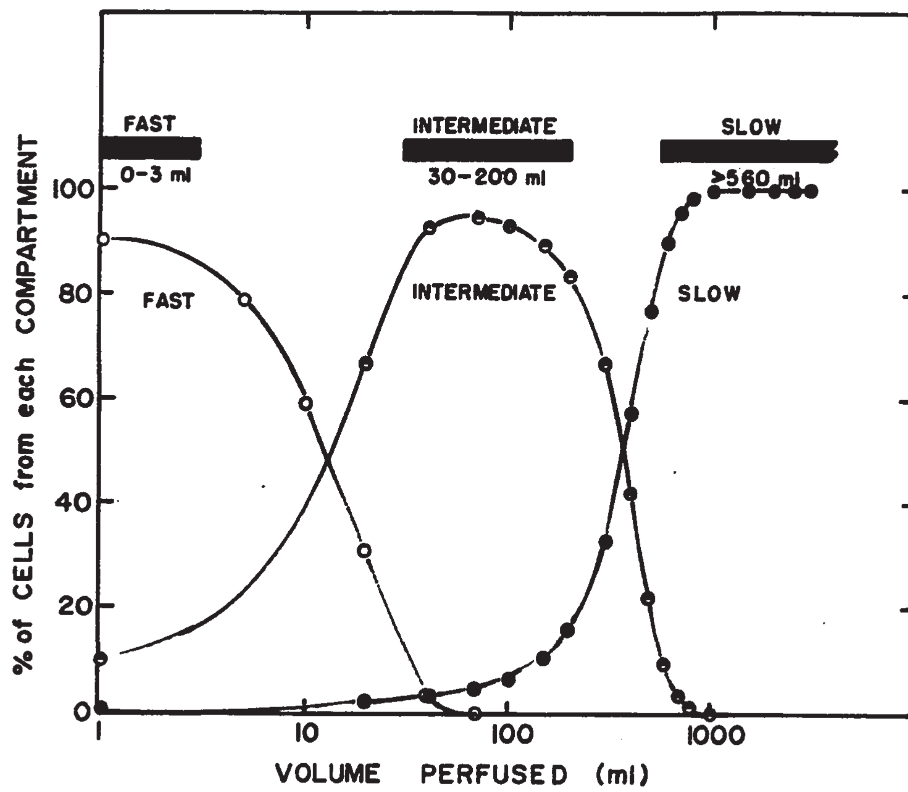
(6) Collection of red cells from different compartments

It is found that the kinetics of cellular washout are equivalent to those from a simple, three compartment model (see IV-1-A, B, C). From these exponential terms we may calculate the fractions contributed by the three compartments to the cell concentration in the outflow at any stage of the perfusion. These contributions, expressed as percentages, are plotted against the volume of fluid perfused in Fig. 3. It is evident that during the very first part of the perfusion the red cells in the outflow have come mainly from the fast compartment, while the contribution of the slow compartment is negligible. This situation changes with further perfusion so that, in sequence, the outflow consists mainly of cells from the intermediate and thereafter the slow compartment. The ranges shown in Fig. 3 indicate stages of the perfusion during which more than 85 percent of the cells in the outflow come from a particular compartment. In this study we collected samples of the splenic venous outflow during these three periods and we denote these as SV_F (fast), SV_I (intermediate) and SV_S (slow) respectively. As a control we also took samples from a peripheral artery (PA) in the same animal.

In ten experiments the spleen was cut open after 0.85 liters of perfusion, and the fluid remaining inside was carefully collected as the organ collapsed. We shall

FIGURE 3

Percentages of the total red cells in samples of the outflow.


FAST
0-3 ml
INTERMEDIATE
30-200 ml
SLOW
≥560 ml
FAST
INTERMEDIATE
SLOW
% of CELLS from each COMPARTMENT
100
80
60
40
20
0
1
10
100
1000
VOLUME PERFUSED (ml)

refer to this as intrasplenic fluid (ISF). Usually between 5 and 20 ml of fluid were collected, depending on how large the spleen was, and the cellular concentration in the ISF was also determined by a Celloscope counter.

(7) <u>Determination of red cell density</u>

The method used was that originally described by Danon and Marikovsky (1964), in which blood cells are separated according to their density using phthalate esters as separating liquids during centrifugation. Measurements of the percentage of red cells above oils of different density yielded the density distribution curve from which the mean value was obtained. Suitable oils were prepared by mixing different proportions of methyl phthalate (S.G. = 1.1890) and di-n-butyl phthalate (S.G. = 1.0416) and the specific gravity of each mixture was measured using a pycnometer. Ten different mixtures were used, with specific gravities ranging from 1.0800 to 1.100.

To determine a density distribution curve at least 0.4 ml of packed red cells was required. However, during collection of the sample SV_S (i.e. during the final part of the washout curve) the volume concentration of cells in the outflow was very low. It was therefore necessary to collect at least one liter of the venous outflow and to separate the cells by centrifugation. Because of this, and in order to avoid any possible artefact, the control sample PA and the splenic venous samples SV_F, SV_I were diluted in one liter of Ringer solution and treated in a similar way to SV_S.

(8) Determination of mean red cell volume

The volume distribution of red cells in each sample (PA, SV_F, SV_I, SV_S) was measured by means of the Celloscope Counter in conjunction with a 128-channel pulse height analyzer (Nuclear Data, N.D. - 110). The output from the multichannel analyzer was shown on an oscilloscope (Telequipment, S 51 B) and recorded, when required, on a step-chart recorder (Dohrmann, SY-850). The red cells of the cat are much smaller than human red cells and therefore we used an orifice 48μ in diameter instead of the 75μ orifice normally used for human cells. The cellular concentrations in the suspensions were kept below 6×10^4 cells/ml to reduce counting coincidences to less than one percent.

The osmolarity of the Ringer's solution for the perfusion ranged from 300 to 310 mOsm/L (Fiske Osmometer) in different experiments. The volume distributions of the samples were compared after suspending the cells in this same Ringer's solution. In addition the cells were suspended in Ringer's solution, modified by changing the concentration of NaCl, at 200 and 400 mOsm/L respectively. The volume distributions were measured at these different osmolarities to study osmotic volume changes. The mean cell volume was determined from each recorded volume distribution by calculating the quotient of the first moment of the area under the curve and the area itself. All such volumes are expressed in units of 'channel number', measured on the pulse height analyzer, at a fixed aperture current and amplification on the Celloscope Counter.

(9) Preparation of slides for reticulocyte counts

During the perfusion each of the venous samples was collected in two different ways. Firstly, one or two drops of the outflow were taken directly on to each of three clean microscope slides. Secondly, the total outflow during a period of 0.5 to 1.0 min was centrifuged to concentrate the cells; one or two drops of the concentrated suspension were then placed on each of three further microscope slides. A minimum of six blood smears, treated with either Brilliant Cresyl Blue or New Methylene Blue stain was thus prepared for each sample; two of these smears were counterstained with Wright's stain. The percentage of reticulocytes on each slide was determined by counting 1000 red cells, except that for the last few samples collected directly from the outflow 500 to 1000 cells were counted.

IV. RESULTS AND DISCUSSIONS

(1) Concentrations of red cells in the venous outflow during the washout process

The aim of this section is (i) to estimate the storage of red blood cells in the spleen of the cat by examining the washout behavior of red cells and (ii) to determine whether the red cell washout corresponds to that from a multi-compartment system or not.

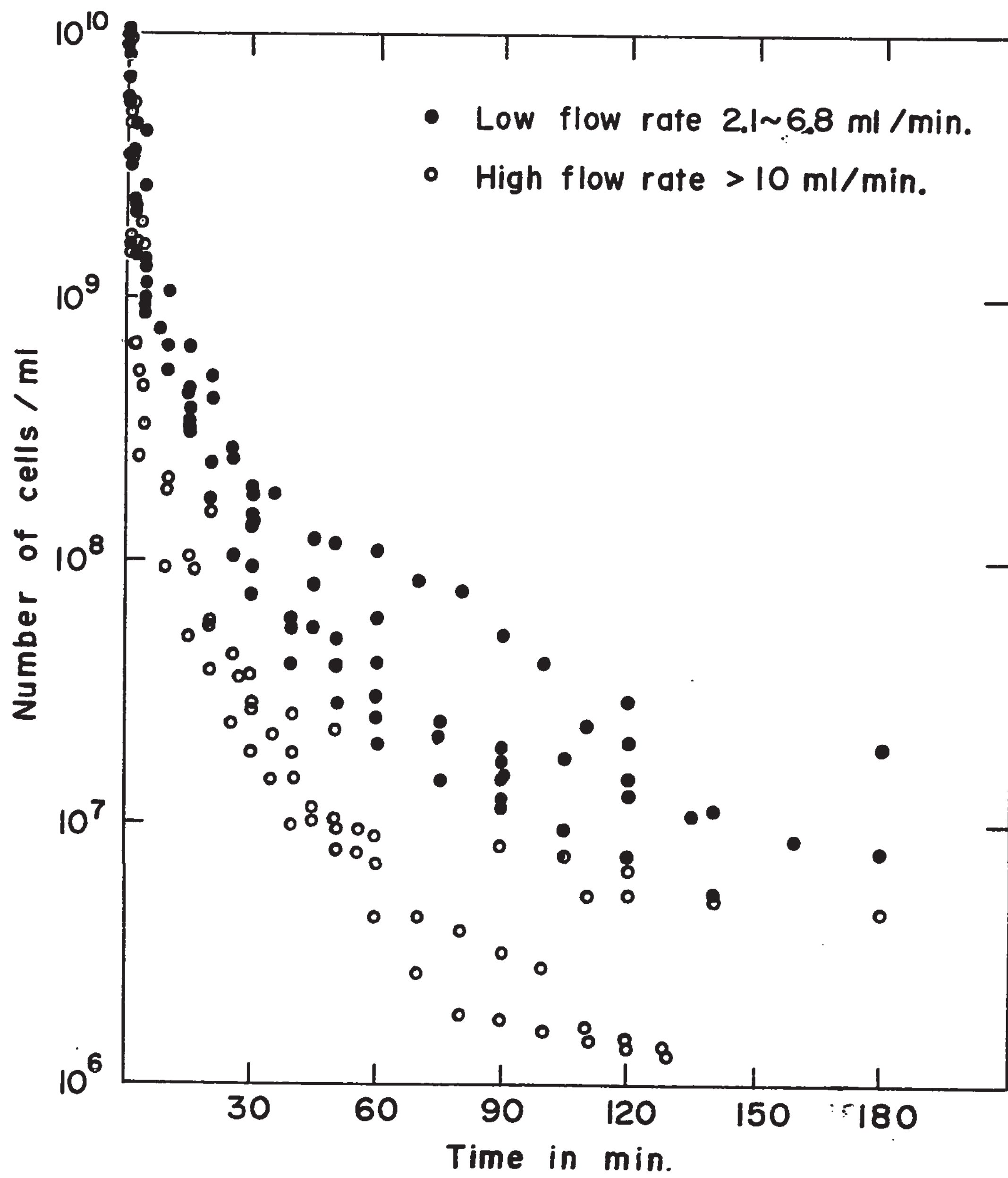
(A) The washout curve from normal spleens

Fifteen healthy cats weighing from 1.7 to 5 Kg were used in these experiments. The red cell counts determined at various times during the washout process were plotted on a semi-logarithmic scale as a function of time (Fig. 4). The initial cell counts at zero time ranged from 5.5×10^9 to 10×10^9 cells per ml of the sample. These values were obtained from either the splenic artery or vein before the infusion was started.

During the first 10 minutes of perfusion, the cell counts decreased rapidly by an order of magnitude. Over the next 30 to 60 minutes a decrease by another order of magnitude occurred and thereafter the counts decreased very slowly indeed. The quite different rates of cell washout against

FIGURE 4

Red cell washout from 15 spleens during perfusion with cell-free Ringer's solution; cell concentrations in the outflow are plotted against the corresponding times of perfusion. Marked differences exist between data obtained at high and low flow rates.



Low flow rate 2.1~6.8 ml/min.
High flow rate > 10 ml/min.
Number of cells / ml
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
30
60
90
120
150
180
Time in min.

time for the low flow and the high flow groups may be seen from Fig. 4.

It is apparent that the concentration of red cells in the outflow at any time is determined by both the flow rate and the initial content of red cells in the spleen (i.e. by the splenic volume). We have therefore plotted, on a semi-logarithmic scale, the cell concentrations as a function of the cumulative volume of fluid perfused (Fig. 5). On such a plot the differences between the washout curves at high and low flow rates disappear and the overall standard deviation of the data is now much less.

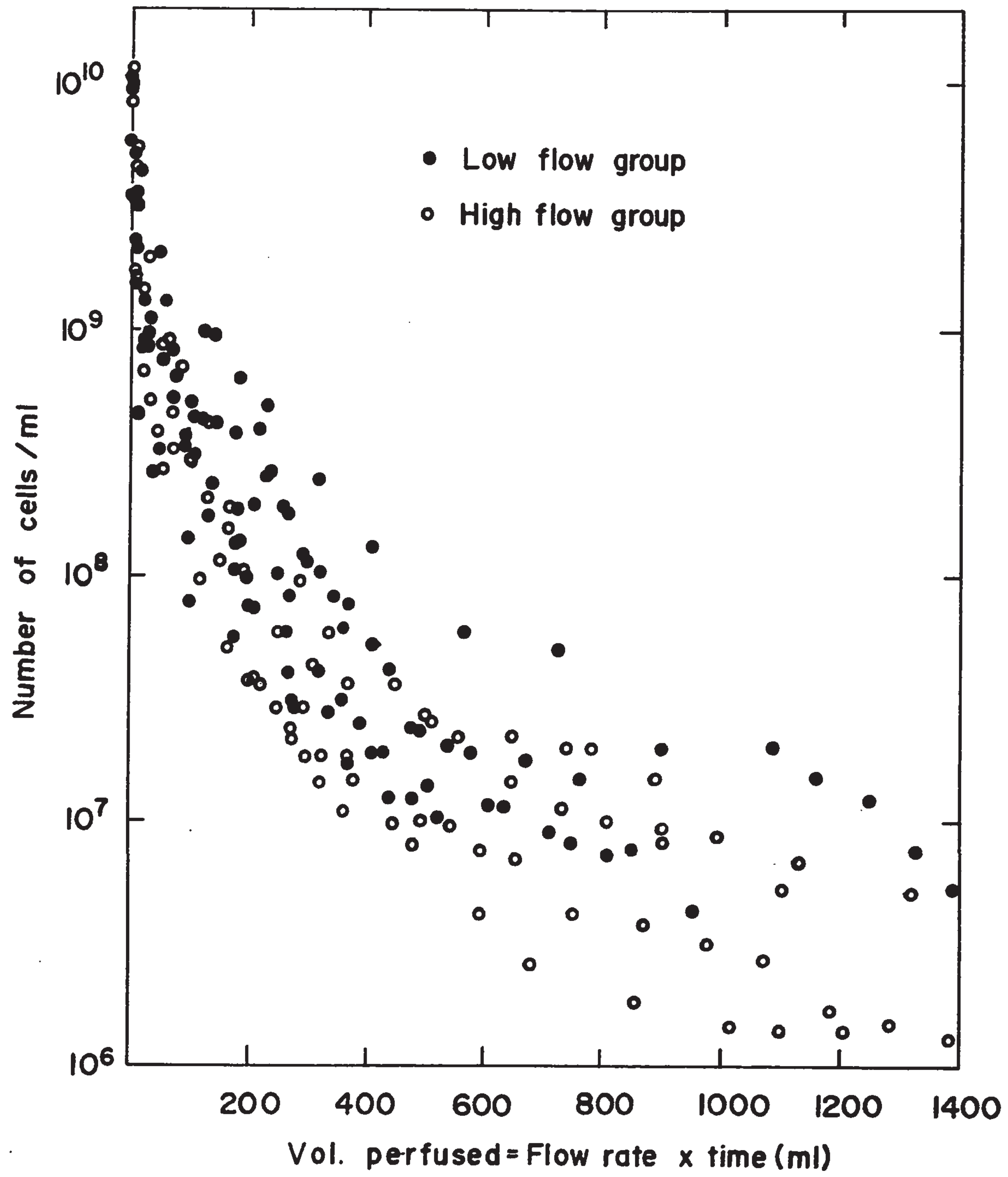
The mean cell washout curve is shown in Fig. 6. Although several continuous functions might be fitted successfully to a cell washout curve, only in the case of a series of exponentials can the equations be derived as the outcome of a set of rational assumptions. A single exponential component represents washout of a single compartment and the cell concentration at time t will be related to that at t = 0 by the equation

$$\log C_t = \log C_o - \frac{F.t}{v}$$

where F = flow rate and v = volume of the compartment. The slope of a graph of log C_t against t will thus depend on both flow rate and volume. However, if log C_t be plotted against the product of flow and time (i.e. volume of fluid perfused) then the curve should become independent of flow rate and the slope should represent the reciprocal of the

FIGURE 5

Red cell washout from 15 spleens during perfusion with cell-free Ringer solution; cell concentrations in the outflow are plotted against the cumulative volumes of fluid perfused. On this basis data obtained at high and low flow rates come together.



Low flow group
High flow group
Number of cells / ml
10^{10}
10^9
10^8
10^7
10^6
200
400
600
800
1000
1200
1400
Vol. perfused = Flow rate x time (ml)

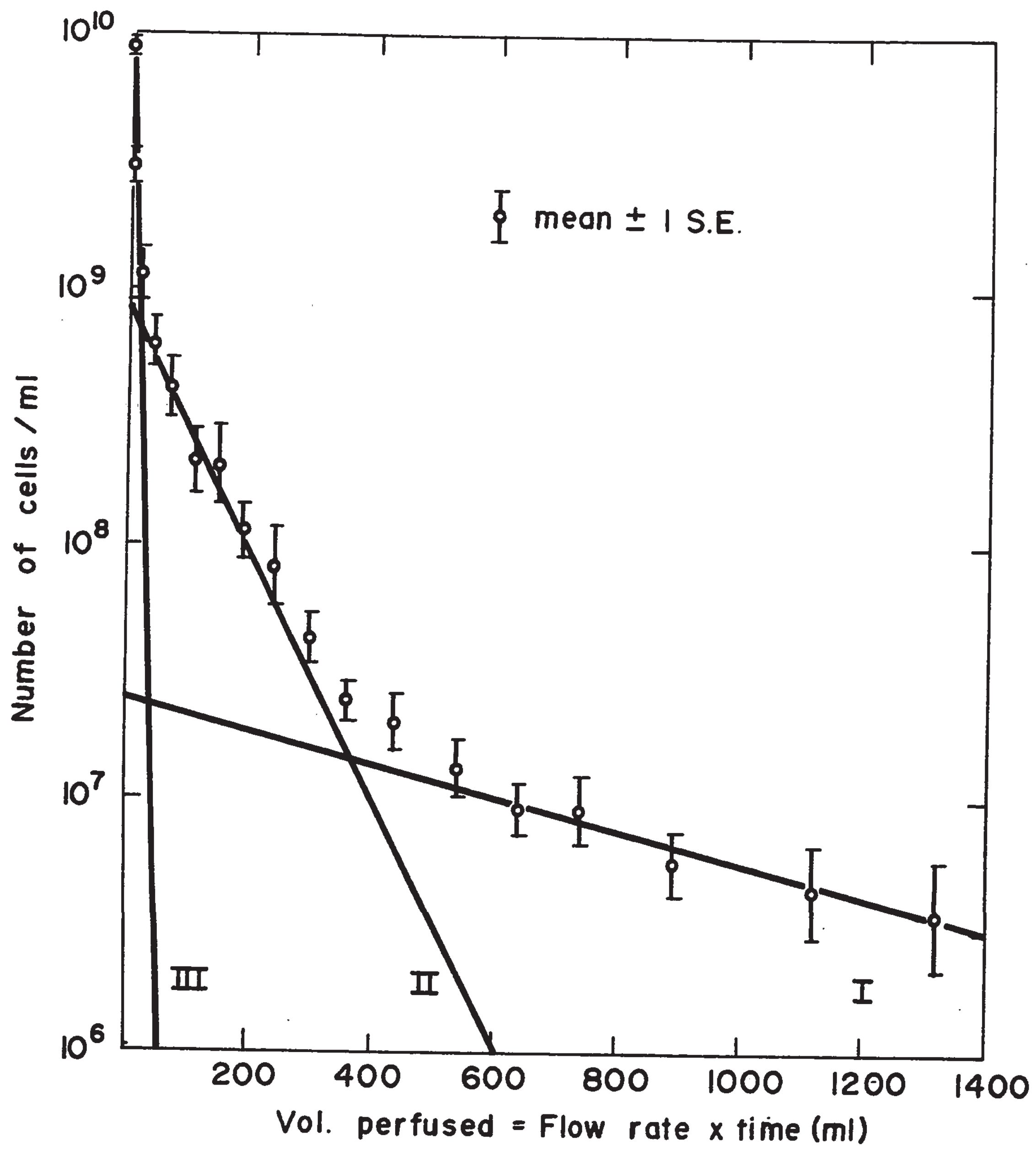
volume of the compartment. Based on this logic the compartmental analysis was carried out in the cases of both normal and contracted spleens.

If such a curve contains two exponential components then the final part of the graph will be a straight line, whereas the earlier part will be curved continuously. To resolve a curve, plotted semi-logarithmically, into its exponential components, a straight line is fitted to the terminal portion (Fig. 6) and the absolute values of the differences between the ordinate values of the original curve and the straight line are then plotted semi-logarithmically. This procedure is repeated until only one straight line remains (Robertson, 1957). The straight lines obtained in this way are the exponential components of the original curve; the intercept and slope of each line represent the constant factor and rate constant of each exponential term. It is obviously important to establish that the washout curve does finally become a single exponential function of the volume perfused. Two of these experiments were continued, for more than three hours, until the volume of fluid perfused exceeded 2000 ml. In both cases the slope, on a semi-logarithmic plot, was constant from 500 ml perfused until the end of the experiment.

When the mean cell washout curve was analyzed in this way (Fig. 6) three components could be clearly identified. The first component, which completely described the washout after more than 500 ml had been perfused, had a desaturation

FIGURE 6

Mean red cell washout curve from 15 spleens; cell concentration (± S.E.) versus volume of fluid perfused. Washout curve may be expressed as the sum of the three exponential components I, II and III (see text).


mean ± 1 S.E.
Number of cells / ml
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
III
II
I
200
400
600
800
1000
1200
1400
Vol. perfused = Flow rate x time (ml)

half-volume $V_{1/2}$ of 470 ml (i.e. the volume perfused to reduce the concentration to one-half) and contributed 0.24% of the initial cell concentration in the blood (Table V). The second component had a $V_{1/2}$ of 70 ml and contributed 9.76% of the initial cell concentration. The third component was evident only in data obtained from 0-40 ml perfused; it contributed 90% of the initial cell concentration in the blood and had a $V_{1/2}$ of 4.4 ml. The average flow rate in these experiments was 8.7 ml/min and, on this basis, the desaturation half-times $T_{1/2}$, corresponding to the values of $V_{1/2}$ previously given, were 54 min., 8 min., and 30 seconds respectively. These values are in the ratio of 100:15:1 approximately and the three components will be referred to by the terms slow, intermediate and fast respectively.

The fact that the sum of three exponential terms can describe completely the washout curve indicates that a simple model, consisting of three compartments, is sufficient to approximate the washout processes from the spleen. The initial rates of cell washout from these compartments will be proportional to their perfusion rates and, on this basis, the percentage of the initial cell concentration in the blood deriving from each compartment represents the percentage of the total flow perfusing that compartment. The total cell store S of a compartment will be given by

$$S = \int_0^\infty c_v . dv$$

where c_v denotes the cell concentration in the splenic venous

outflow, due to washout from that compartment, when v ml of fluid have perfused the spleen. For an exponential function this integral is equal to Co/k where Co is the value of c_v when v = 0 (i.e. Co is the intercept in Fig. 5) and k is the rate constant. In this way the total numbers of cells stored in each of the three compartments have been computed (Table V). The overall splenic red cell store was 15.5×10^{10} cells; the slow compartment contained 11%, the intermediate compartment 56%, and the fast compartment 33% of the total cells.

The initial weight of each spleen was not measured in these experiments and therefore it is not possible to calculate the cells stored per gram of spleen. We may, however, express the mean splenic red cell store in terms of the total red cell volume of the cat, the latter being 15.8 ± 0.7 (S.E.) ml/kg (Groom, Rowlands & Thomas, 1965). In the present experiments the mean body weight was 2.35 kg and the mean red cell volume was therefore 37 ml. The mean hematocrit ratio in cats is 35% (Groom _et al_., 1965, Schalm, 1965) and the initial cell count in these experiments was 8.85×10^9 cells per ml of blood. The total red cell volume therefore amounts to 9.35×10^{11} cells and the cells present in the spleen represent 16.6% of this.

Discussion

Any attempt to analyze the mean curve of log concentration against time, using data obtained at widely different flow rates, will lead to errors in both slope and intercept for each component. Thus results obtained by this

TABLE V - Analysis of washout of red cells from the spleens of cats during perfusion with Ringer's solution. (Means of 15 experiments).

PARAMETER	COMPONENT			TOTAL
	I	II	III	
Intercept C_o (cells.ml^{-1})	$2.5x10^7$	$8.7x10^8$	$8.0x10^9$	$8.9x10^9$
± S.E.	$1.1x10^7$	$2.1x10^8$	$0.3x10^9$	$0.5x10^9$
Rate Constant k (ml^{-1})	$1.48x10^{-3}$	$9.9x10^{-3}$	$157x10^{-3}$	---
± S.E.	$0.53x10^{-3}$	$1.4x10^{-3}$	$18x10^{-3}$	---
Cell Store C_o/k (cells)	$1.7x10^{10}$	$8.8x10^{10}$	$5.0x10^{10}$	$15.5x10^{10}$
± S.E.	$0.9x10^{10}$	$2.4x10^{10}$	$0.6x10^{10}$	$2.6x10^{10}$
% of total flow	0.24	9.76	90	100
Desaturation $V_{1/2}$ (ml)	470	70	4.4	---
Desaturation $T_{1/2}$ (min)	54	8	0.5	---
% of splenic r.b.c.	11	56	33	100
% of total r.b.c. of cat	1.8	9.4	5.4	16.6

means in our earlier experiments, carried out mostly at lower flow rates, suggested that the slow compartment might store 28% of the total cells and receive 2.4% of the total blood flow. In the present results this error has been avoided by basing the analysis on the mean values of log concentration as a function of the volume of fluid perfused. From the mathematical viewpoint the compartmental analysis presented here provides a model "sufficient" to represent the washout processes from the spleen but not "necessary" in the sense of being the only valid model. In particular, Van Liew (1962) has pointed out that if the rate constants of the components of the washout differ by only a small factor, then the system could equally well be represented by a single Gaussian distribution of rate constants having a large fractional standard deviation. In the present case, however, the rate constants are in the ratio 1:7:100 and there is little doubt about the resolution into three quite separate components.

The fact that a very slowly exchanging pool with a $T_{1/2}$ of the order of one hour has not been demonstrated previously in any species almost certainly derives from the methods used hitherto. These involve measuring, by scintillation detectors placed externally, the uptake of radioactively labelled cells in the spleen. It is difficult to quantitate precisely, in this way, the labelled red cells in the spleen due to the geometrical distribution of the radioactivity, to variations in geometry of the organ with

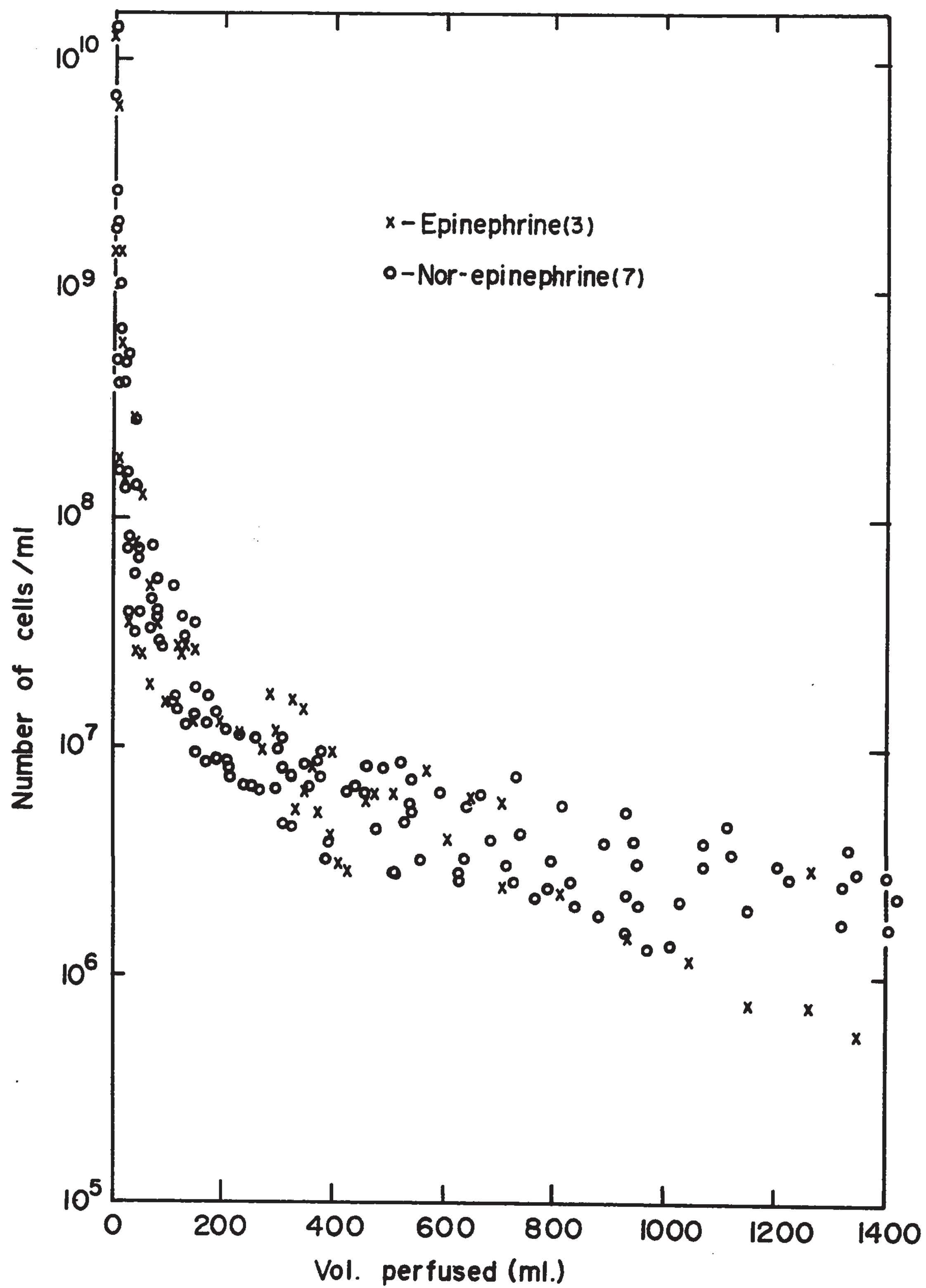
time and, in the intact subject, to radioactivity in the surrounding tissues. In addition, compartmental analysis based on uptake depends upon differences between the values actually measured and the final steady state value. The latter part of any uptake study is therefore limited by the error of the difference between two large quantities and differences less than a few percent of the steady state value cannot be measured. In contrast to this, any compartmental analysis based on washout depends entirely on the values actually measured and therefore the resolution of the method in detecting mixing components representing small fractional flows or small cell stores is far superior to the uptake method. In the present study cell concentrations, in the outflow, could be measured over a range of more than six orders of magnitude, thus allowing the presence of the slow compartment in the cat's spleen to be recognized for the first time.

(B) The washout curve from contracted spleens

Ten healthy cats weighing 1.7 to 2.5 kg were used in these experiments. The objective of this study was to show (i) whether or not the cell washout from the contracted spleen corresponds to that from a three-compartment system similar to the kinetics of the washout from the normal spleen and (ii) to what extent each compartment is responsible for the cells discharged on contraction. In this way we might provide additional evidence for our "three-compartment" model of the spleen.

FIGURE 7

Red cell washout from 10 spleens during perfusion with cell-free Ringer's solution containing NE or E.


x – Epinephrine(3)
o – Nor-epinephrine(7)
Number of cells /ml
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
0
200
400
600
800
1000
1200
1400
Vol. perfused (ml.)

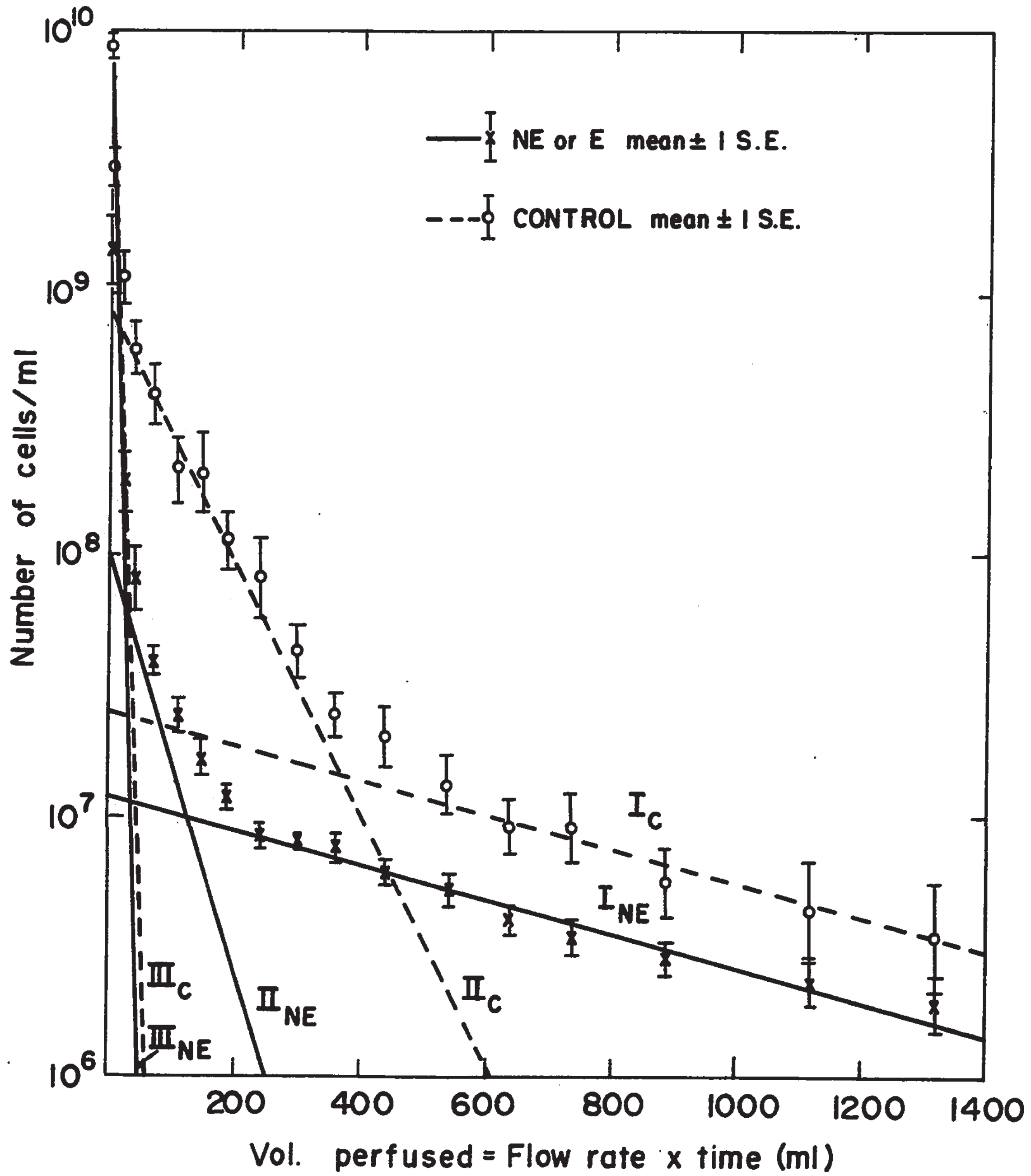
All spleens contracted well in vivo in response to both epinephrine and norepinephrine. However, the spleens to which epinephrine was given (both in vivo and in vitro) could not maintain their initial contraction for the two-hour perfusion period, even though the perfusion pressure was maintained at the initial high level. After 30 minutes of perfusion the spleen gradually relaxed, no matter how much additional epinephrine was given. In contrast to this, spleens which received norepinephrine maintained both their volume and pressure response throughout the experiment. For this reason epinephrine was used in the first three experiments only.

The cellular concentrations in samples of the outflow obtained from ten experiments were plotted on a semi-logarithmic scale as a function of the volume of fluid perfused (Fig. 7). These concentrations cover a range of more than four orders of magnitude. There were no significant differences between data from experiments in which the two different catecholamines were used. The concentrations initially lay in the range (0.70-1.73) x 10^{10} cells/ml and were reduced by 2, 3 and 4 orders of magnitude respectively when 50, 200 and 1700 ml of Ringer's solution had been perfused.

In view of the scatter of the experimental points the data were taken in groups corresponding to successive increments in the volume perfused. Mean values $\pm$ S.E. were calculated for each group and these are plotted in Fig. 8 (lower curve). The graph became linear (i.e. a single

FIGURE 8

Comparison of mean red cell washout curve from 10 spleens contracted by norepinephrine or epinephrine with that from 15 normal spleens. Each curve may be expressed as the sum of three exponential components I, II, and III (see text).


NE or E mean ± 1 S.E.
CONTROL mean ± 1 S.E.
Number of cells/ml
10^{10}
10^9
10^8
10^7
10^6
I_C
I_{NE}
II_C
II_{NE}
III_C
III_{NE}
200
400
600
800
1000
1200
1400
Vol. perfused = Flow rate x time (ml)

exponential function) after 250 ml had been perfused. A straight line was fitted to this terminal portion and the process of analyzing the curve into its exponential components was carried out as described previously (IV-1-A). This analysis was also carried out statistically by determining the regression equations for the appropriate data using the method of least squares.

In fact the regression equation determined for the experimental points (Fig. 7) obtained after 250 ml perfusion, agreed precisely with that derived by fitting a straight line visually through the corresponding mean values (Fig. 8). The same agreement was found in the case of the second exponential component but it was necessary, for this computation, to subtract the concentrations obtained by extrapolation of the first component from the observed values for 50 ml to 250 ml perfused. The correlation coefficients corresponding to these two regression equations were 0.76 and 0.77 respectively.

The analysis demonstrated that the washout curve could be resolved into the sum of three exponential components, (Fig. 8: solid lines I_{NE}, II_{NE} and III_{NE}). Details of the appropriate initial concentrations Co and rate constants k, derived from the three straight lines on a semi-logarithmic plot, are given in Table VI. A consequence of this analysis is that a simple model, consisting of three compartments, is sufficient to approximate the washout processes from the contracted spleen.

TABLE VI - Analysis of washout of red cells from contracted cat spleens. (Means of 10 experiments).

PARAMETER	COMPONENT			TOTAL
	I	II	III	
Intercept C_o (Cells ml^{-1})	$1.20x10^7$	$1.05x10^8$	$8.70x10^9$	$8.8x10^9$
± S.E.	$0.12x10^7$	$0.74x10^8$	$0.3 x10^9$	$0.5x10^9$
Rate Constant k (ml^{-1})	$1.51x10^{-3}$	$18.9x10^{-3}$	$211 x10^{-3}$	---
± S.E.	$0.13x10^{-3}$	$5.2 x10^{-3}$	$20 x10^{-3}$	---
Cells Store C_o/k	$0.8x10^{10}$	$0.56x10^{10}$	$4.14x10^{10}$	$5.5x10^{10}$
± S.E.	$0.22x10^{10}$	$0.43x10^{10}$	$0.40x10^{10}$	$0.6x10^{10}$
% of total flow	0.14	1.2	98.7	100
Desaturation $V_{1/2}$ (ml)	460	36.7	3.3	---
Desaturation $T_{1/2}$ (min)	48	3.8	0.34	---
% of splenic r.b.c.	14.6	10.2	75.2	100
% of total r.b.c. of cat	1.0	0.7	5.1	6.8

Using this model we may now derive additional information from the experimental data. Thus the initial rates of cell washout from the compartments will be proportional to their perfusion rates; the percentage of the initial cell concentration in the blood deriving from each compartment will therefore be equal to the percentage of the total flow perfusing that compartment. For compartments I, II and III these values were 0.14, 1.2 and 98.7 percent respectively.

The total cell store of a compartment is equal to Co/k and this has been computed for each of the three compartments (Table VI). The total red cell store of the spleen was 5.5×10^{10} cells; compartments I, II and III contained 14.6, 10.2, and 75.2 percent of this respectively. The total red cell volume of the cat is 15.8 ± 0.7 (S.E.) ml/kg (Groom et al., 1965) and the mean hematocrit 35% (Groom et al., 1965; Schalm, 1965). The mean body weight in these experiments was 2.05 kg and the initial cell count was 8.8×10^9 cells/ml. The mean total red cell volume therefore amounted to 8.15×10^{11} cells and based on this value the cells contained in compartments I, II and III of the spleen comprise 1.0, 0.7 and 5.1 percent respectively of the animal's total red cell volume.

From a knowledge of the mean flow rate through the spleen in our studies (9.6 ml/min) and the measured desaturation half-volumes $V_{1/2}$ for the three components of the washout we may determine the desaturation half-times $T_{1/2}$ for

the corresponding compartments. These turn out to be 48 min., 3.8 min., and 20 sec. respectively.

Discussion

The fact that the cellular washout curves obtained with the two different catecholamines are not significantly different (Fig. 7) is not surprising, for the initial contraction would have expelled the same volume of cells from the spleen in each case and, during perfusion, the spleen remained in a contracted condition until after compartments II and III had been washed out. Any difference in the slow rate of washout of cells thereafter could well be obscured because of the scatter of the observations.

If the spleen had been perfused with whole blood rather than with cell-free Ringer's solution then the relaxation which occurred with epinephrine would mean the reaccumulation, within the spleen, of a volume of red cells comparable to that expelled during the initial contraction. Whether or not this could occur in man is uncertain for several workers have failed to show, by indirect methods, any change in size of the spleen after injection of epinephrine or norepinephrine (Kaltreider, Meneely, & Allen, 1942; Parson, Mayerson, Lyons, Porter & Trautman, 1948). However, Bierman and his colleagues (1953) observed, after laparotomy, an immediate shrinkage of the normal human spleen when epinephrine was injected into the splenic artery. The possibility that reaccumulation of cells might occur would suggest caution in the use of β-adrenergic stimulants in the treatment of

shock (Ross, 1967).

In the present study we have found certain differences in the rate of red cell washout in contracted versus relaxed cat spleens. These differences may be seen clearly in Fig. 8. Firstly, normal spleens showed a much wider range of cellular concentrations at any given volume perfused than did contracted spleens; this may be seen by comparing the standard errors of corresponding mean values. This situation arises because the sizes of normal spleens vary much more than those of fully contracted spleens. Secondly, statistical analysis showed that, up to a volume of 800 ml of Ringer perfused, the cellular concentrations were significantly different ($p<.02$ to $.001$). Thirdly, the graph became linear much earlier in the contracted spleen (250 ml perfused) than in the normal spleen (600 ml perfused).

When the results of compartmental analysis in the two groups of experiments (Fig. 8: C & NE) are compared statistically it is found that significant differences exist as follows:

(i) Intercept (C_o) of compartment II: $(8.7 \pm 2.1) \times 10^8$ vs $(1.05 \pm 0.74) \times 10^8$. $p<0.005$.

(ii) Cell store (C_o/k) of compartment II: $(8.8 \pm 2.4) \times 10^{10}$ vs $(0.56 \pm 0.43) \times 10^{10}$. $p<.005$. This difference is due solely to item (i).

(iii) Cell store ($\Sigma C_o/k$) of the whole spleen: $(15.5 \pm 2.6) \times 10^{10}$ vs $(5.5 \pm 0.6) \times 10^{10}$. $p<.001$.

When the spleen contracts its size is reduced by 60-70%; the results show that the total red cell store is reduced by a similar amount (iii). Gross differences, between normal and contracted spleens, of cell concentrations in the outflow occurred only during that portion of the wash-out dominated by compartment II. The analysis shows that the reduction in total red cell store, on contraction of the spleen, is almost entirely due to the drastic reduction in size of compartment II (ii above).

Our results agree well with the suggestion of Eisenberg and co-workers (1969), based on their studies of the effects of adrenergic drugs on dog spleens, that the storage function of the spleen might be represented by a "unicompartmental reservoir". The mechanism of emptying this compartment after injection of epinephrine or norepinephrine is not understood. However, Opdyke (1970), from studies of the effects of epinephrine on dog spleens, and Toghill and Prichard (1964), from studies of effects of norepinephrine on human spleens have both suggested that the reservoir is controlled by sphincter mechanisms. If this be true, then these sphincters must control only the size of compartment II in our model.

It is intriguing to find that compartment I (slow) is still present even after contraction of the spleen.

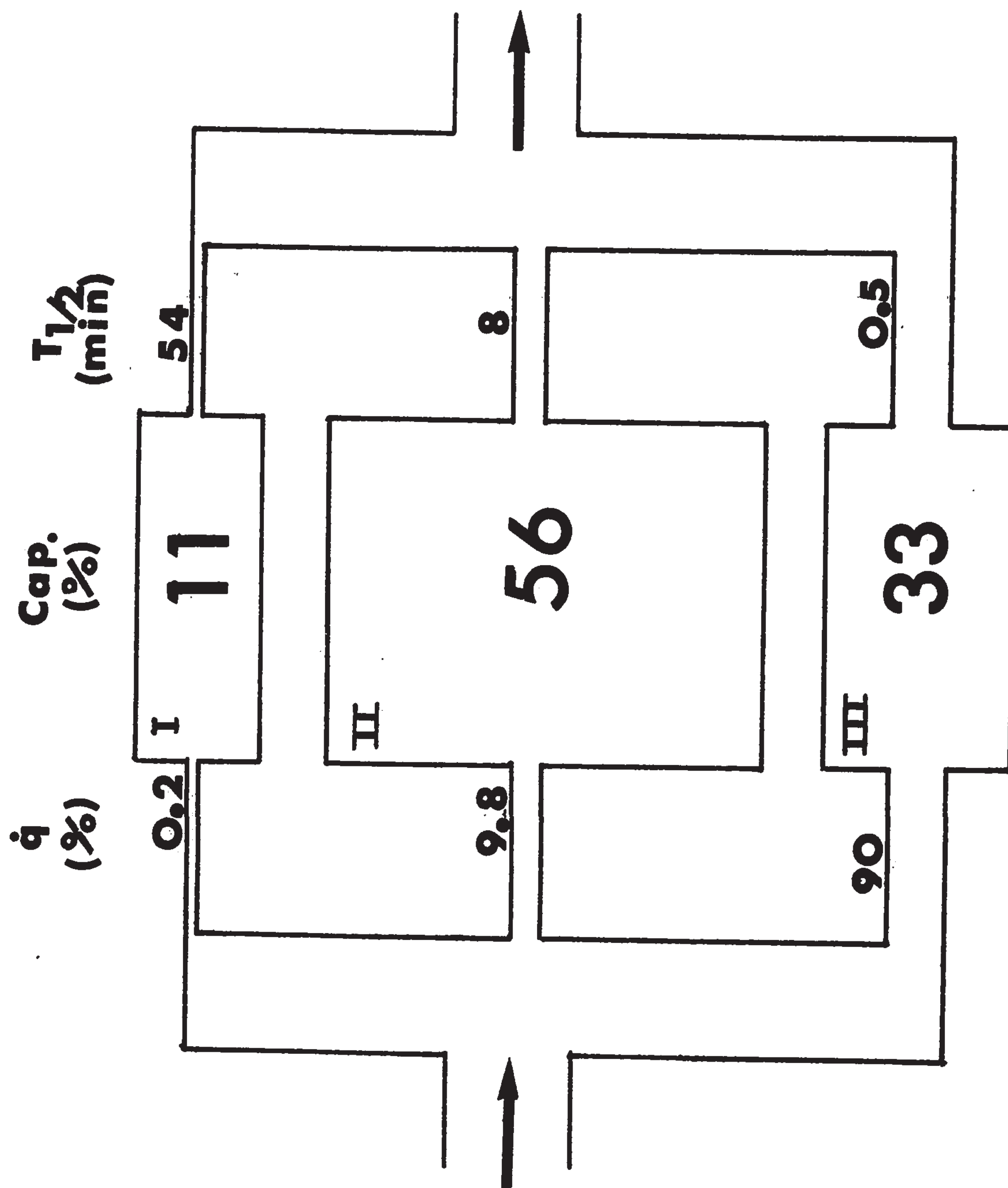
Further, it is interesting that no significant change occurred in either the washout half-time or the cell store. This indicates that the slow compartment has nothing to do with a reservoir function.

(C) Three compartmental model of the spleen

The simple model of the splenic circulation, based on the present results is shown in Fig. 9. This is, to our knowledge, the first time that a three-compartment model has been proposed for the spleen of any species. Barcroft & Barcroft (1923) found, on exposing rabbits, rats and cats to carbon monoxide, that the splenic blood saturated more slowly than the peripheral blood. It was concluded that there must be a slowly exchanging pool of blood in the spleen. More recent experiments have shown that up to 10% of the total red cell volume of the cat can be circulating too slowly to be included in the volume measured by dilution of ^{32}P labelled cells (Groom _et al_. 1965). Further observations (Groom, unpublished) have shown that the mixing time, in the circulation of the cat, for ^{32}P labelled red cells injected intravenously is considerably longer than that of ^{131}I labelled albumin; after splenectomy this difference disappears. The same slow equilibration of red cells in the spleen of the cat has been shown more directly by external counting over the organ following the injection of ^{51}Cr labelled cells intravenously (Prankerd, 1963; Toghill & Prichard, 1964). The uptake curve corresponded to a two-compartment system,

FIGURE 9

Simple three-compartment model for the splenic circulation, derived from the kinetics of the cell washout process. $\dot{q}$ = flow to compartment (% of total inflow); Cap. = red cell content of compartment (% of total splenic red cells); $T_{1/2}$ = washout half-time of compartment (minutes).



q̇ (%)
Cap. (%)
T1/2 (min)
0.2
11
54
I
9.8
56
8
II
90
33
0.5
III

the more slowly exchanging compartment having a half-time of a few minutes and a cell content equivalent to 6-10% of the animal's total red cell volume. These two compartments clearly correspond to the fast and intermediate compartments of the present study.

In the case of man, it has been shown that the normal spleen shows red-cell pooling when abnormal cells are introduced into the circulation (Harris, McAlister & Prankerd, 1957; Toghill & Prichard, 1964). The red-cell pool produced in this way behaves like that in the cat's spleen, for after an injection of norepinephrine the trapped cells are discharged into the circulation (Prankerd, 1963; Toghill & Prichard, 1964). A splenic pool may also be found when the red cells are normal but the spleen is abnormal as a result of disease (Harris, McAlister & Prankerd, 1958; Jandl & Aster, 1967; Toghill, 1964). Studies of the kinetics of red cell uptake under both of these conditions have led to the conclusion that the human spleen also contains a slowly exchanging pool (Bowdler, 1962; Jandl & Aster, 1967), but that under normal conditions the size and fractional blood flow are too small to allow its detection by radioactive uptake techniques. This pool probably corresponds to the intermediate compartment of the cat's spleen but in normal human subjects it appears to be of little importance as a reservoir.

The description of a simple equivalent model for the splenic circulation inevitably raises questions as to what each compartment might represent anatomically. The fast

component, which has a $T_{1/2}$ of only 30 seconds and represents 92% of the total flow, obviously represents blood in a rapid pathway through the spleen. Transit times of approximately 30 seconds have been recorded for ^{51}Cr labelled red cells injected intra-arterially in isolated perfused dog spleens, and similar values have been estimated for the human spleen (Jandl & Aster, 1967).

It is not possible to discuss, on the basis of the model presented, whether the blood in the slowly exchanging splenic pools is confined to a closed vascular system or reaches a diffuse space between the splenic cells. However, from a functional viewpoint the intermediate compartment, containing approximately 60% of the splenic red cells (equivalent to 10% of the total red cell mass of the cat) and having a $T_{1/2}$ of 8 minutes, could represent the reservoir from which cells are discharged in an emergency. Prankerd (1963) has shown that uptake of ^{51}Cr labelled red cells in the cat's spleen occurs much more quickly after administration of norepinephrine.

It is quite remarkable to encounter transit times of an hour or more for normal red cells in any physiological situation. Such long times have been reported, to date, only for abnormal cells in normal human spleens and for normal cells in pathological spleens (Bowdler, 1962; Harris, *et al.*, 1958; Motulsky, *et al.*, 1958; Toghill, 1964). The slow compartment certainly implies long periods of stasis for

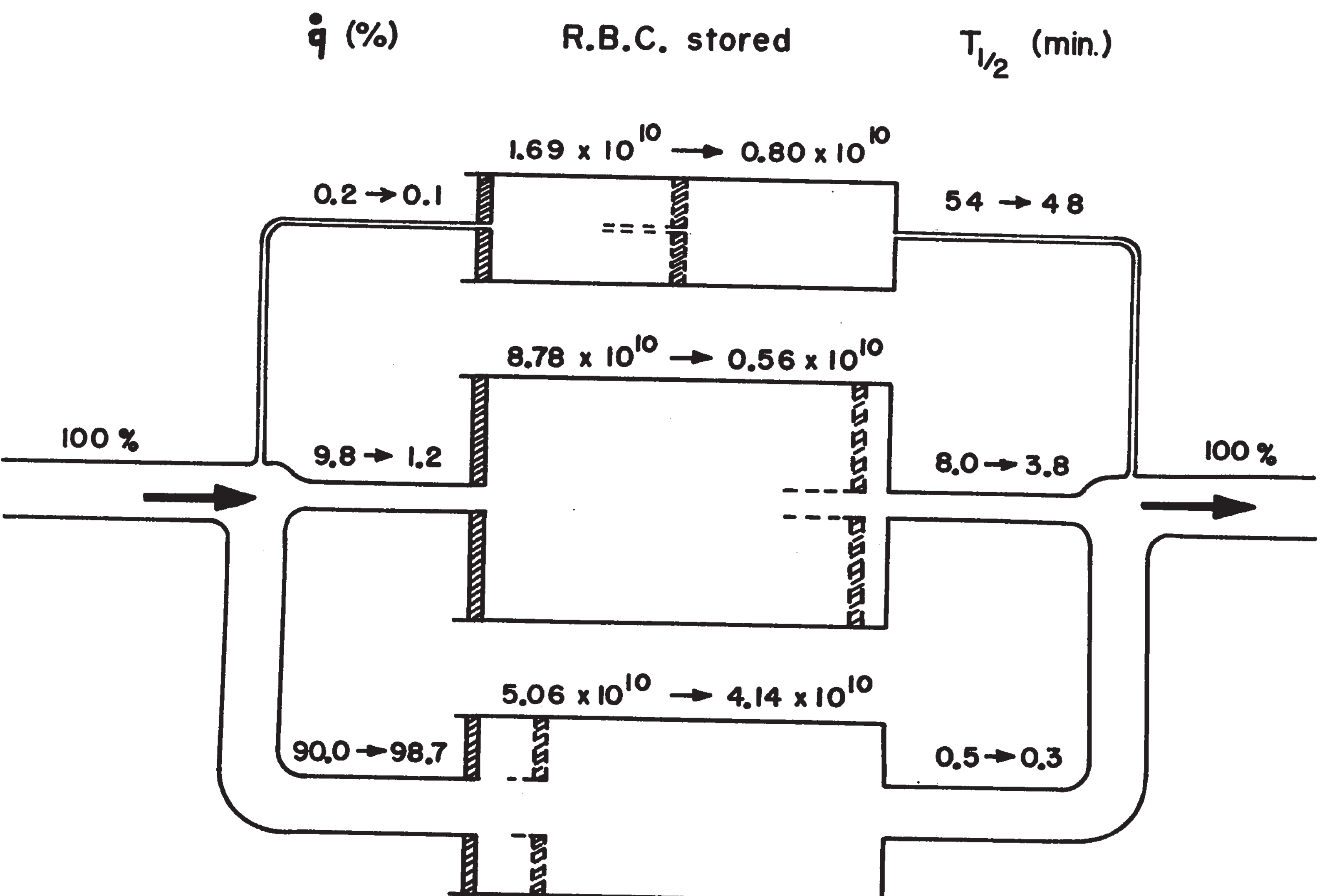
incoming cells and, at this stage of the research, we wondered whether it could be the place where aged or abnormal cells are culled and lysed.

The overall results of our experiments are summarized in Fig. 10, in which the equivalent models for cell washout from the normal and the contracted spleen are compared. The figure may appear to be complicated but it illustrates simply what happens to the three compartments when the spleen contracts. Although little change occurs in the cell stores of both the <u>fast</u> and the <u>slow</u> compartments there is a big reduction (by a factor of sixteen) in the case of the <u>intermediate</u> compartment. In fact 82% of the cells discharged into the circulation come from this <u>intermediate</u> compartment and these cells amount to 10% of the total red cell mass of the animal.

It is also apparent from Fig. 10 that the intermediate compartment undergoes, on contraction of the spleen, an eight-fold reduction in blood flow. This reduction is statistically highly significant ($p<.005$). At the same time the proportion of the total flow received by the <u>fast</u> compartment increases from 90% to 98.7% and the washout half-time remains on the order of 20-30 sec. Whether or not the circulation through the splenic pulp is to be regarded as "closed" or "open", the transit time through the spleen for 90-98.7% of the red cells entering is exactly comparable to that found in vasodilated skeletal muscle (Groom, 1968). This is highly suggestive that the fast compartment represents

FIGURE 10

Three-compartment model for the splenic microcirculation, showing what happens when the spleen contracts. $\dot{q}$ = flow to compartment (% of total inflow); RBC stored = actual number of red cells in compartment; $T_{1/2}$ = washout half-time. Numerical values to the left of each arrow refer to the normal spleen and on contraction these change to the values on the right. The relative numbers of cells stored are represented by the volumes of the cylinders (the diameters shown are proportional to the square root of the cell store in the normal spleen). On contraction the pistons move to the right to take up positions shown by the dotted lines.



q̇ (%)
R.B.C. stored
T1/2 (min.)
1.69 x 10^10 → 0.80 x 10^10
0.2 → 0.1
54 → 48
8.78 x 10^10 → 0.56 x 10^10
100 %
9.8 → 1.2
8.0 → 3.8
100 %
5.06 x 10^10 → 4.14 x 10^10
90.0 → 98.7
0.5 → 0.3

a route consisting of ordinary vascular lumens. However, the morphological nature of the three compartments remains to be investigated, since the research thus far has been confined to the "black box" approach which can elucidate only functional aspects of the splenic microcirculation.

(D) Validity of a perfusion method with Ringer's solution

In order to eliminate the influence of the autonomic nervous system and any effects of humoral substances, an isolated, perfused preparation was used. It was therefore necessary to ensure that the spleen really was in a physiological condition during each experiment. The infusate was Ringer's solution containing the proper amount of glucose. The pH of the solution was 7.8 to 8.0 without CO_2 equilibration and was 7.4 to 7.2 with 5% CO_2 in O_2. An adequate O_2 supply to the spleen could be maintained during three hours of perfusion with oxygenated Ringer's solution, presumably because of the low O_2 consumption rate (0.5-0.6 ml/min/100g) of this tissue (Boxall, 1966; Cherniack, Edelman & Fishman, 1970). Confirmation that the tissue was well oxygenated at all times came from the color of the organ, for the hemoglobin contained in the residual red cells acted as an indicator of pO_2 in the splenic microcirculation. In every experiment the spleen remained a healthy pink color, but when the perfusate was changed for Ringer's solution equilibrated with 5% CO_2 in N_2 the color changed to the dark blue of reduced hemoglobin.

The circulation to the dog spleen can be arrested for at least ten minutes without a change in the sensitivity of the splenic capsular and venous smooth muscle to a standard stimulus. This was true with cat spleens when norepinephrine was introduced into the infusate after three hours perfusion. If the circulation is restored for five minutes between the periods of occlusion, observations can be continued for three to four hours (Webb-Peploe, 1969). In the present experiments the spleen responded well to norepinephrine (0.5γ) even after three hours of perfusion with oxygenated Ringer's solution.

The perfusion pressures ranged from 50-250 mm Hg depending on the flow rate and the size of the spleen. These pressures are comparable to those reported (Frohlich and Gillenwater, 1963) for the isolated dog spleen perfused with blood (67-270 mm Hg) and for the in situ, non-isolated dog spleen (75-315 mm Hg). Splenic venous pressures ranged from -3.5 to + 25 mm Hg (mean value 18 mm Hg), similar to those reported for the dog (Frohlich and Gillenwater, 1963).

(2) Distribution of red cells in the spleen

Our model proposed in the previous section for the splenic microcirculation was derived entirely from the kinetics of cell washout and should be considered simply as a biophysical model. Whether or not there exist morphological counterparts to these three compartments in the spleen remains to be demonstrated, and this is the subject of this section. We have examined microscopic sections of cat spleens prepared at different time intervals after the start of perfusion with Ringer's solution. In this way we have compared the location of red cells in the spleen before perfusion and after cells from the fast and intermediate compartments respectively have been almost completely washed out.

The red cells observed in the spleen can be divided into three groups:

(i) free cells in vascular channels and sinuses,

(ii) cells adhering to reticulum cells or sinus endothelium,

(iii) red cells in the cytoplasm of macrophages.

(A) Free red cells

Most of the red cells in the spleen fall into this group. In histological sections the free cells are so highly concentrated that they tend to form clusters or aggregates, and their presence makes it difficult to observe cells from groups (ii) and (iii). After perfusion of the spleen by 600 ml of Ringer's solution, most of the red cells remaining come under the last two categories and can be seen quite clearly.

Sections in which Wright stain was used showed that there were both granular leukocytes and R.E. cells in the red pulp, but the 'bound' red cells (ii) were associated only with R.E. cells.

No free cells were found in the vascular lumens of sections taken from spleens perfused by 50 ml to three liters of solution. However, free cells were seen in the sinuses until 600 ml had been perfused. A section (Plate IA) taken after perfusion by 150 ml shows the vascular lumens completely cleared of cells and the red pulp in the process of being cleared. In Plate IB, which shows a section from a spleen contracted by norepinephrine and perfused by 300 ml, no clusters of red cells are discernible in the red pulp. Instead, R.E. cells, which are seen much closer together than in the non-contracted spleen (Plate IA), dominate the red pulp. After much perfusion the red pulp can be cleared almost entirely of red cells. This is shown in Plate IC which presents a section from a non-contracted spleen perfused by three liters of Ringer's solution. Washout of red cells from the spleen seems to have occurred very uniformly, and details of the splenic structures stand out clearly. Although there appear to be no red cells in the sinuses, numerous cells can be observed when the sections are examined at higher magnification.

When the spleen is contracted by norepinephrine a 16-fold reduction occurs in the number of red cells representing the intermediate compartment (see IV-1-B) and this must mean

PLATE I

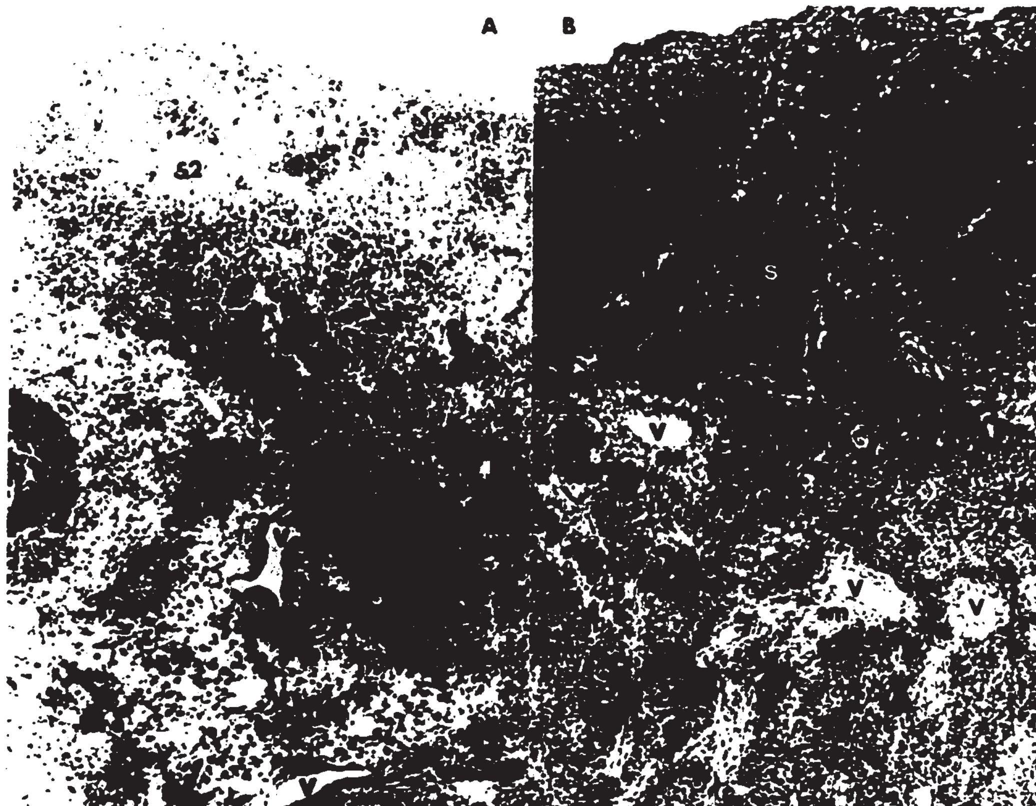
(a) - Normal spleen perfused by 150 ml. Shows empty vessels (V), sinuses filled with red cells (S1) and sinuses cleared of red cells (S2). x40.

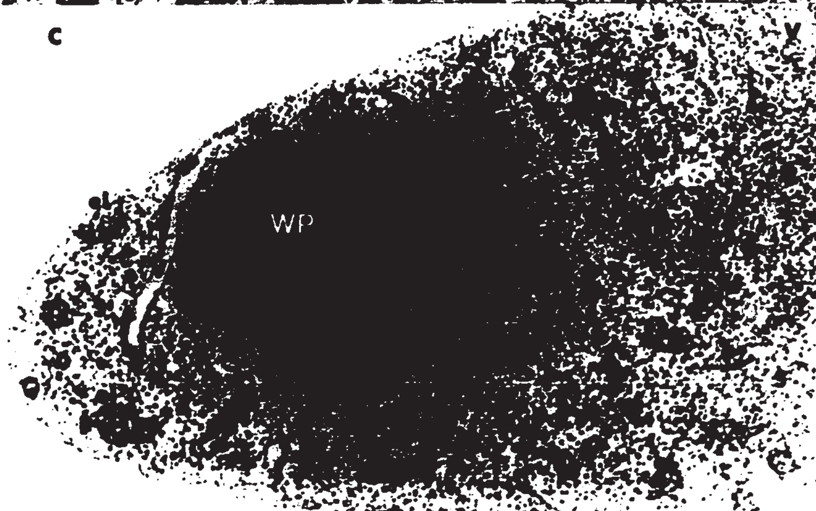
(b) - NE treated spleen perfused by 300 ml. The capsular, trabecular and vascular smooth muscles (sm) are thicker than in a and c, due to contraction. Sinuses (S) are contracted and vessels empty (V). x40.

(c) - Normal spleen perfused by 3.0 liters. Sinuses (S) are all cleared of red cells. One germinal center of the white pulp (WP) and many ellipsoids (el) are seen. x40.

PLATE I

PLATE I





that the free cells in the red pulp are reduced 16-fold. This is in agreement with the observations of Knisely (1936) on the emptying of sinusoids due to splenic contraction. It is known that the smooth muscle of the capsule and the trabeculae is responsible for the contraction. This is evident in Plate IB where an increase in thickness of both capsule and trabeculae may be noted. If the findings of Koyama _et al_. (1964) and Hirasawa and Tokuhiro (1970) are right, then cytoplasmic filaments in the sinus endothelial cells and microfibrils in reticulum fibers would cause contraction of the sinus wall. This would lead to a decrease in area available for bound cells (ii), as well as contribute to the expulsion of free cells (i) from the red pulp. However, we have not been able to show after norepinephrine a difference in size of the slow compartment that is statistically significant (see Tables V and VI). Furthermore, sections stained with Masson's trichrome showed well distributed elastic fibers in the vascular walls and the capsule but there was no evidence for elastic fibers in the sinus walls.

(B) Bound red cells; Group (ii)

Sections taken from spleens perfused by 1.3 to 2.5 liters of solution are shown in Plate II. At a magnification 40x these three sections closely resemble Plate IC, but at 400x large numbers of red cells are seen. Most of these cells are bound to reticulum cells and sinus walls but a few are within the cytoplasm of macrophages, i.e. categories (ii) and (iii) above. A comparison between A and B of Plate II

PLATE II

(a)- Normal spleen perfused by 1.3 liters. All red cells are attached to reticulum fibers or sinus walls. Some red cells show a tear-drop shape (arrows). Sinus lumens (S) are cleared of cells and an ellipsoid (el) is seen. Some free macrophages (m) are seen among the reticulum cells. x400.

(b)- Normal spleen perfused by 1.5 liters. Fewer red cells are attached to reticulum fibers than in a, and red cells are bound more to some RE cells (arrow) than to others. x400.

(c)- Normal spleen perfused by 2.5 liters. A further reduction in the number of bound red cells has occurred and tear-drop shaped cells (arrows) are present more frequently. Some free polymorpho-nuclear cells (P) are seen. x400.

PLATE II

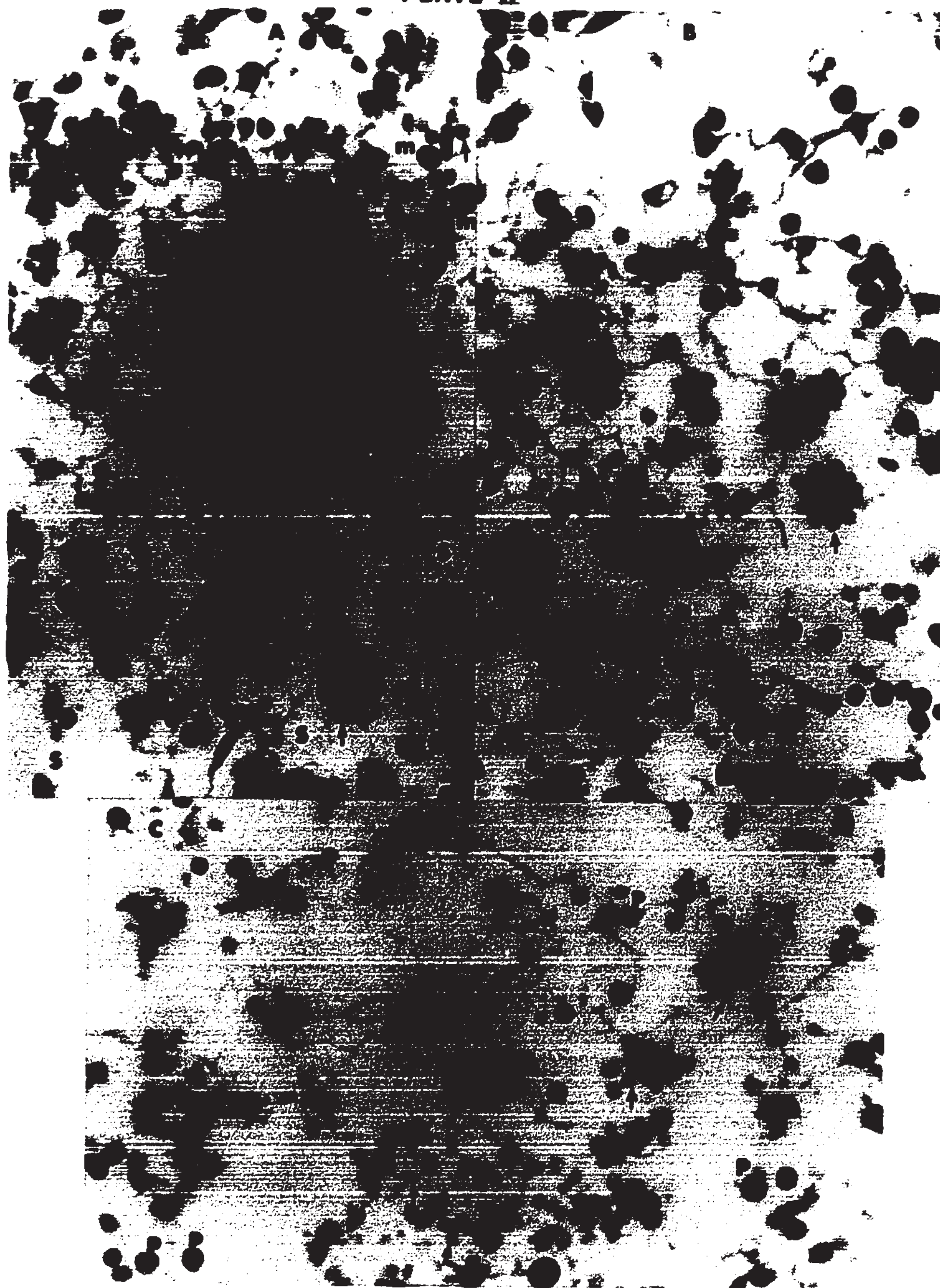
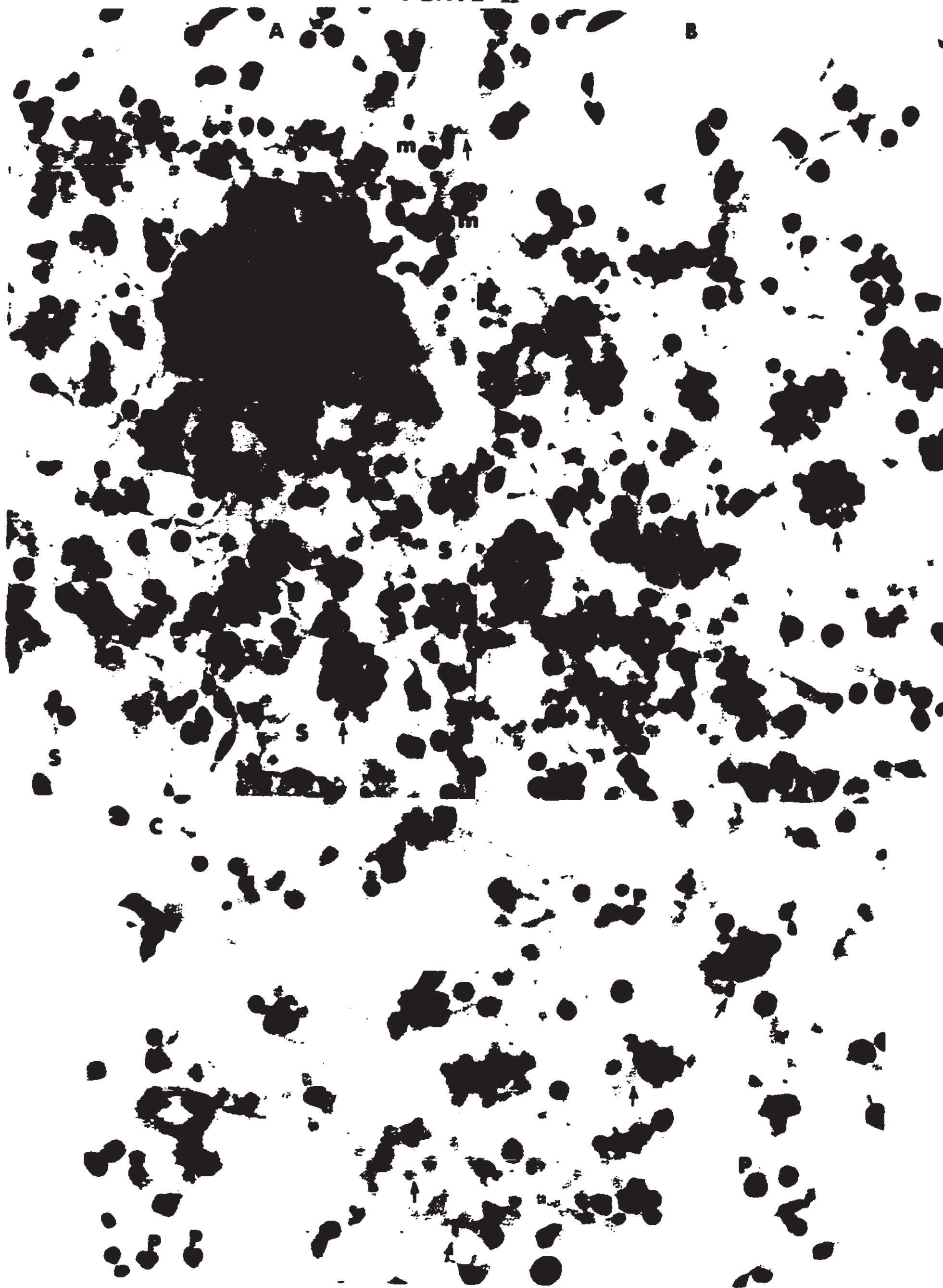


PLATE II



shows definitely that bound cells can be washed out by continued perfusion of the spleen, yet even after a total of 2.5 liters (Plate IIC) or 3.0 liters (Plate IIIC) many red cells still remain in the sinuses. In the venous outflow the concentrations of cells washed out from the spleen were not less than 10^5 per ml after perfusion by 3.0 liters of solution. Since it was surprising to find so many red cells remaining in the spleen under these conditions, the sections were examined further under oil-immersion (Plate III).

No free red cells were found in the sinuses and the framework of reticulum fibers did not appear to undergo any change during prolonged perfusion. Even though some of the red cells maintained their normal discoidal shape other forms were also found (e.g. tear-drop shape). The red cells observed were either in close contact with reticulum fibers and R.E. cells or else were already within the cytoplasm of R.E. cells, suggesting a possible process of erythro-phagocytosis. Both in Gomori trichrome and in Wright's stains, many of the bound red cells appeared to be a purplish red color or darker red color and this could imply they might be reticulocytes.

It is clear that in Plates II and III the number of red cells, as well as R.E. cells, can be counted. Using the three categories of red cells already defined, we found that free red cells (i) were too dense for accurate counting, 'bound' red cells (ii) could be counted when more than 600 ml was perfused, and phagocytosed cells (iii) were negligible in

PLATE III

(a)- Normal spleen perfused by 1.3 liters. Tear-drop shaped red cells (arrows) and a few platelets (Pt) are seen. Nucleated cells consist of monocytes (mc), small lymphocytes (lc) and reticulum cells (rc). x1000; oil immersion.

(b)- Normal spleen perfused by 1.3 liters; section shows another part of the same spleen as in a. Three tear-drop shaped red cells are seen attached to an RE cell (arrows). One of the red cells appears to contain an inclusion body (i). x1000; oil immersion.

(c)- Normal spleen perfused by 3.0 liters. A few bound red cells are seen and some cells are being phagocytosed (arrows). Different types of nucleated cells are recognizable and the framework of the sinuses appears intact. x1000; oil immersion.

PLATE III

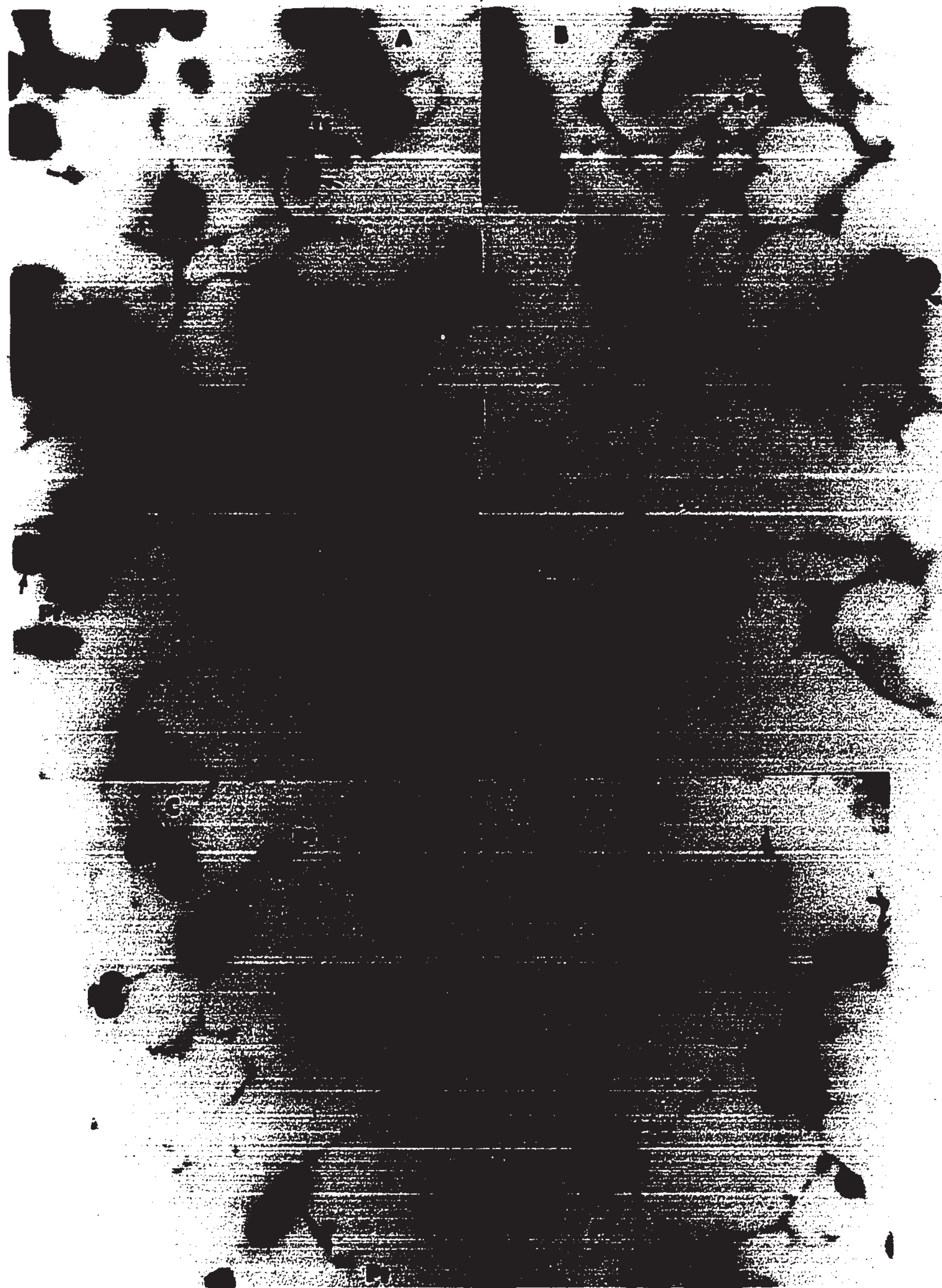


PLATE III

number compared with (ii).

The numbers of 'bound' red cells/cm^2 in the sections examined from six different spleens ranged from 2.3×10^5 to 3.2×10^3 (Table VII). From these data the total number of red cells in each spleen was calculated and the values lay between 4.9×10^9 and 2.2×10^8 cells. If all these cells are capable of being washed out eventually, then the concentration of red cells per ml of spleen is surprisingly high compared with the concentration of cells in the venous outflow. We therefore determined cellular concentrations in the fluid draining from splenic tissue. In Table VIII are given data from 10 spleens and it is clear that the cell concentrations in the intrasplenic fluid (I.S.F.) were always much higher than those in the outflow. The ratio ranged from 5.6 to 85.7 with a mean value of 40.8 ± 8.2 (S.E.).

Discussion

It would be interesting to know what is special about the red cells that are bound. Koyama *et al.* (1964) suggested, from studies on human spleens, that red cells of tear-drop shape arise only as a result of 'pitting' by sinus endothelial cells. In that case all such red cells should be attached to sinus endothelial cells. However, our observations indicate that tear-drop shaped cells can be attached to any of the R.E. cells or even to reticulum fibers in the red pulp (Plate III). Thus the tear-drop shape must result not only from pitting (Weiss, 1962; Wennberg & Weiss, 1968) but also from other processes such as cell fragmentation (Weed &

TABLE VII - Total residual red cells in six perfused spleens (determined by cell counts on microscopic sections).

Volume perfused (liters)	Red cells per cm^2 of cross-section units of 10^4	Volume of spleen (ml)	Total residual red cells in spleen: units of 10^8	
			from sections*	from washout kinetics
1.30	17.9	11	49.0	24
1.50	23.3	8	46.6	18
1.50	9.1	6	13.6	18
2.00	8.1	11	22.0	8.5
2.50	2.3	6	3.4	4.0
3.00	0.32	28	2.2	1.8

*probable limits of error ± 50% (see text)

TABLE VIII - Cell concentrations in intrasplenic fluid (C_{ISF}) and venous outflow (C_V) during slow phase of washout from 10 spleens.

Expt. No.	Volume perfused (liters)	Cell concentrations in units of 10^6/ml		$\frac{C_{ISF}}{C_V}$
		C_{ISF}	C_V	
1	0.86	251	7.84	32.0
2	1.10	88.1	5.31	16.6
3	1.20	104	4.10	25.4
4	1.30	70.7	2.98	23.8
5	1.30	89.7	1.21	74.0
6	1.40	46.1	0.538	85.7
7	1.50	16.2	2.93	5.6
8	1.80	40.1	0.652	61.5
9	2.00	165	4.50	36.7
10	2.00	256	5.54	46.2
				40.8 ± 8.2 *

*Mean value ± S.E.

Weiss, 1966; Weed & Reed, 1966). Recently Bessis and Boisfleury (1970) have obtained beautiful pictures, with the scanning electron microscope, of red cells deformed into a characteristic pear shape during phagocytosis by leukocytes. Interestingly, their studies were made on peripheral blood in vitro and they found that several red cells can adhere to one phagocyte at the same time during the process of phagocytosis. This behavior may be observed in our Plates II and III and leads us to postulate that the bound cells are in a pre-phagocytic stage. Surprisingly they are still 'exchangeable' for their number can be decreased by further perfusion.

(C) Abnormal red cells

In addition to a few red cells captured in the cytoplasm of macrophages (i.e. group iii of our earlier classification) we have found various types of abnormal red cells; (a) tear-drop shaped, (b) spiculated (Plate V-f), (c) with an inclusion body (Plate III), and (d) with a refractile body. Since the proportion of these cells to other red cells in the red pulp increased as the perfusion progressed, these cells were also considered to be a part of the slow compartment. We have not been able to identify any special structures in the spleen responsible for trapping these cells for such a long time.

<u>Discussion</u>

There is abundant evidence for erythrophagocytosis in the spleen (Isaacs, 1937; Ehrenstein & Lockner, 1958; Rifkind, 1966; Simon & Burke, 1970). In the present observations phagocytosed cells (iii) were seen even in the

spleens of normal healthy cats, indicating that this process is not restricted to overtly pathological cells (Weisman, Ham, Hing & Harris, 1955; Wennberg & Weiss, 1967; Carlson & Ham, 1968) or to abnormal spleens (Holzbach, Shipley, Clark & Chudzik, 1964; Subhiyah & Al-hindawi, 1967). The cells we have observed could well be aged cells, for which mechanisms of phagocytosis have been proposed involving either the release of a necrotoxin (Bessis, 1965) or a reduction in the density of electrical charge on the cell surface (Skutelsky & Danon, 1970a and b).

The functions of the spleen with respect to abnormal cells are not completely understood. It is still in debate whether macroreticulocytes, for example, are destroyed (Card & Valberg, 1967; Landaw, Russell & Bernstein, 1970; Sorbie & Valberg, 1970) or remodelled (Ganzoni, Hillman & Finch, 1969; Hillman, 1969) in the splenic sinuses. There is also evidence that cells containing inclusion bodies may be subjected to a "pitting" process in the spleen (Koyama *et al.*, 1964). We have observed, in the spleens of normal cats and dogs, many abnormal cells having either a spiculated or else a tear-drop shape. We had assumed, at first, that these cells were awaiting destruction (see IV-1-C). However, in view of the suggestion that certain abnormal cells can be remodelled in the spleen, one cannot exclude the possibility that these cells also may have been retained for remodelling or repairing.

(D) Rubricytes and their denucleation process in the spleen

Since I have failed to discover any reports in the literature which mention the presence of rubricytes in the spleens of healthy, adult cats and dogs, this section is the most unexpected outcome of this treatise. When the free red cells (i) in the red pulp have been washed out, all the red cells remaining in the spleen are those of the slow compartment; these cells are now relatively unobscured and may be examined easily.

Twelve cats and two dogs were used in these experiments. After the usual perfusion with Ringer's solution by volumes of 1.2 to 3.5 liters, histological sections were prepared from the perfused spleens (see III-5).

From sections prepared with blood stains it was possible to identify various stages of the erythroid series and these are shown in Plates IV and V. Most of the nucleated red cells are rubricytes having round pyknotic nuclei with either basophilic or orthochromic cytoplasm. However, proerythroblasts (i.e. having nuclei with multiple nucleoli) were not seen in any of the sections examined. A high proportion of basophilic, non-nucleated red cells was evident in all sections.

It was noted that the proportion of immature red cells in the red pulp increased considerably as washout of the slow compartment progressed. The immature cells were found everywhere in the sinuses and also in the vicinity of

PLATE IV

Cells from splenic sections prepared with various blood stains, original magnification x1600.

(a) - Single rubricyte attached to macrophage. Arrow shows fragmented nuclear material near a reticulum cell.

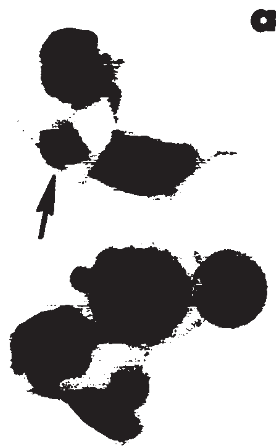
(b) - Nucleus of a rubricyte is about to be extruded

(c) - Prorubricyte contains a bi-lobed nucleus

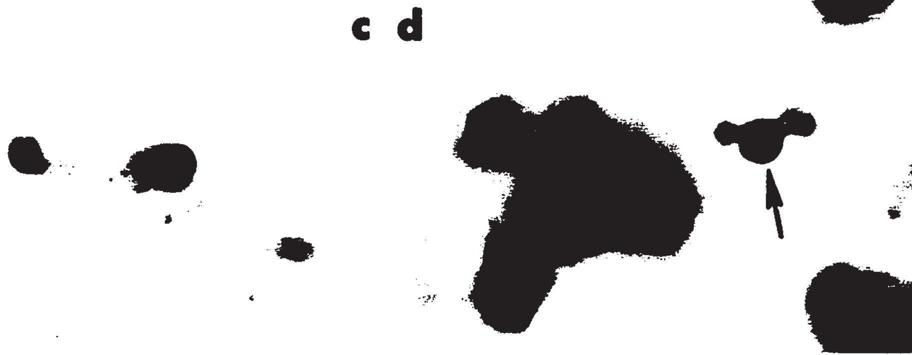
(d) - Arrow shows extruded nuclear fragments

(e) - Bi-lobed nucleus of a rubricyte being extruded

(f) - In the center a nucleus from a rubricyte shows a small protrusion


a

b


c d

e

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a
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the germinal centers. While most of the non-nucleated erythrocytes formed clusters around free macrophages or reticulum cells the nucleated cells seemed to be attached singly to macrophage (or perhaps, "nurse") cells.

The nucleated red cells appeared to have either a single nucleus (Plate IV-a, b, d) or else a double nucleus (Plate IV-c, e, f). The pictures suggest that the nucleus is distorted into the double form during the act of expulsion from the cell (Plate IV-c, e, f). However, in Plate IV-a, d, isolated nuclear masses consisting of more than two lobes may be seen (arrows). This may indicate that fragmentation of the nucleus had occurred, either after expulsion (Plate IV-d) or else, possibly, within the cell (Plate IV-a).

To determine whether the fragmentation process can proceed within the cell or can only occur after the nucleus has been expelled we searched the sections for evidence of multi-lobed nuclei. In Plate V-a, b, c, d, e, rubricytes containing three and more nuclear lobes may be seen. Furthermore, in Plate V-d the nucleus of a rubricyte is obviously undergoing fragmentation within the cell. We have, in fact, observed a similar fragmentation process occurring in rubricytes of all three stages (basophilic, polychromatophilic and orthochromatophilic).

It is possible that Plate V-e illustrates the act of expulsion, from the cell, of a fragmented nuclear mass. On the other hand, it could merely demonstrate that a nuclear mass, previously expelled from another cell, had come into close contact with an unusual red cell.

PLATE V

Cells from splenic sections prepared with various blood stains, original magnification x1600.

(a) - Quadri-lobed nucleus from a rubricyte

(b) - Rubricyte attached to macrophage shows a multi-lobed nucleus

(c) - Similar to (b), but showing tri-lobed nucleus

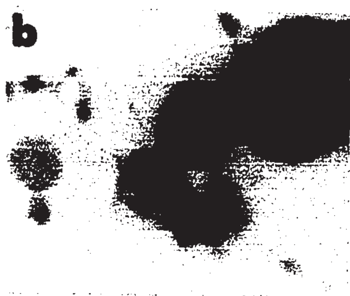
(d) - Meta-rubricyte contains fragmented nucleus

(e) - Fragmented nucleus appears to have been extruded from the crenated cell

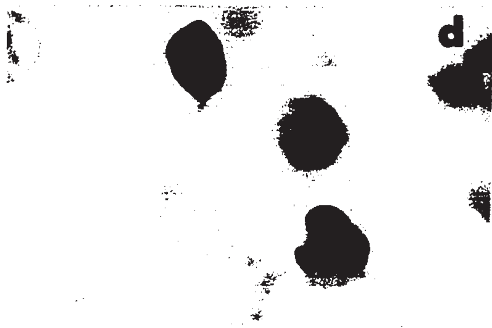
(f) - Typical spiculated red cell near a macrophage

(g) - Two free nuclei attached to a macrophage

a

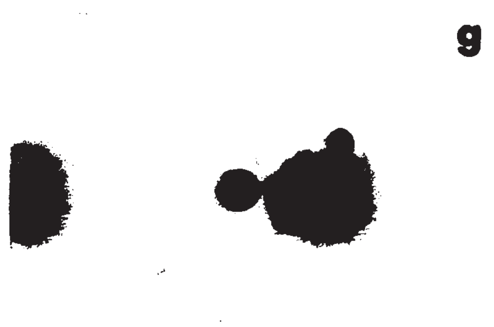

b

c


d

e

f


g

a

b

c

d

e

f

g

Discussion

There are abundant references to the sequestration of immature erythrocytes within the spleen (Berendes, 1959; Jandl, 1960; Card & Valberg, 1967; Ganzoni *et al.*, 1969; Hillman, 1969; Landaw *et al.*, 1970). Nevertheless, these studies were all carried out in either abnormal (after bleeding or phenylhydrazine) or pathological conditions. It has been suggested that immature cells could be sequestered in the spleen because of their increased stickiness (Jandl, 1960) or reduced deformability (Leblond, LaCelle & Weed, 1971). It is also possible that the immature cells could have been produced in the red pulp itself. However, erythropoiesis in the spleen has been reported only in the embryo (Thiel & Downey, 1921) or else in adult mice treated with erythropoietin (Orlic, Gordon & Rhodin, 1965; Gurney & Rosett, 1968). Our finding that there are no proerythroblasts and yet many rubricytes within the spleen suggests that immature cells, released from the bone marrow, are taken up by the spleen and there complete the maturation process.

There has been general agreement among cell biologists that, under normal conditions, the sole mechanism of denucleation in rubricytes is extrusion (Mollier, 1909; Orlic *et al.*, 1965; Skutelsky & Danon, 1967; Simpson & Kling, 1967; Campbell, 1968). Although what appeared to be fragmentation of the nucleus within the cell could also be observed, this was thought to represent cell division (Mollier, 1909).

More recently, however, Schalm (1965) has attributed this fragmentation to karyolysis. Our findings of rubricytes containing multi-lobed nuclei (Plate V) suggest that karyolysis, rather than cell division, is occurring. Moreover, our studies were restricted to normal spleens in adult animals free from any hematological disorders, so that karyolysis, followed by extrusion (Plate V-e), must also be a normal mechanism of denucleation.

It was observed by Orlic et al. (1965), in a study of erythropoietin-induced red cell formation in mouse spleen, that in all stages of development the erythroid cells formed clusters around a macrophagocytic "nurse" cell. This situation is exactly similar to what we have observed in normal feline spleens during washout of the slow compartment (Plates II & III). However, the numbers of erythroid cells forming clusters decreased gradually as the washout progressed and it was found that the proportion of rubricytes among the cells remaining in the spleen increased steadily. Presumably the macrophages, such as that shown in Plate V-c with a single rubricyte attached, may have been surrounded by a whole cluster of red cells at an earlier stage of the perfusion. It may well be that after completion of the maturation process red cells can easily be detached and thus released from the spleen.

Our findings have convinced us that the splenic sinuses in normal, healthy, adult cats and dogs contain both immature and abnormal erythrocytes. Furthermore, the

denucleation of erythroblasts indicates that maturation of the erythroid series, beginning after the prorubricyte stage, is occurring in the spleen.

(E) White blood cells in the red pulp

We have observed various types of nucleated cells other than rubricytes in the red pulp. Atypical macrophages (Plate II-a), polymorpho-nuclear leukocytes (Plate II-c), monocytes (Plate III-a) and lymphocytes (Plate III-a) were commonly found. It was of course impossible, from "static" pictures alone, to elucidate their functional relationships with other cells of the structural component in the spleen.

If bound red cells (ii and iii) were released, during perfusion, still attached to their captor phagocytes then a decrease in the number of nucleated cells in the red pulp might also be observed. However, we have counted the latter cells in sections from seven spleens perfused by 150 to 3000 ml of solution and found the range to be (1.0 to 10.2) x 10^9 cells per whole spleen (mean $\pm$ S.E.; 6.1 $\pm$ 1.2 x 10^9). The number of cells bore no relation to the volume perfused and we conclude that nucleated cells in the red pulp are not washed out.

(F) The morphological confirmation of the three compartments

It is well known that the normal spleen contains abundant red cells, in both the red pulp and the vascular lumens. Our observations on histological sections showed that

the sequence of red cell disappearance from the spleen, during perfusion with Ringer's solution, was as follows:

(a) free cells from vascular channels (zero to 50 ml)

(b) free cells from red pulp (zero to 600 ml)

(c) bound cells from red pulp (zero to infinity).

According to our kinetic studies (see IV-1a, b, c) the volumes of perfusate necessary to reduce the numbers of cells in the three compartments to 1% of their original values (i.e seven times the volume required to reduce the concentration by one-half) are 31, 490 and 3300 ml respectively. These figures agree closely with those for (a), (b), and (c) above. This indicates that free cells in vascular channels constitute the _fast_ compartment of our biophysical model. Similarly, free cells in the red pulp comprise the _intermediate_ compartment, and bound cells in the red pulp the _slow_ compartment.

During washout experiments the cellular concentrations in the outflow may be described by a single exponential function (_slow_ compartment) when the spleen has been perfused by more than 600 ml fluid. From these kinetics the total number of exchangeable red cells in the spleen at any instant can be calculated and the values given in Table VII agree well, within the range of experimental error, with the numbers of bound cells determined from histological sections. This therefore confirms quantitatively that the bound cells (ii) correspond to the slow compartment.

We have shown previously that the slow compartment comprises 11% of the splenic red cell volume and receives

0.28% of the total flow (see IV-1, a, c). On this basis the cellular concentration C_V in the venous outflow, during the slow phase of the washout, would be 0.28% of the concentration C_S in the slow compartment. On the other hand the concentration in the intrasplenic fluid C_{ISF} would be 11% of C_S if all the bound cells were freed during collapse of the spleen. The ratio C_{ISF}/C_V would thus be equal to 39.0 approximately and this agrees well with the mean value obtained from ten experiments in which C_{ISF} and C_V were measured directly (Table VIII). The agreement confirms further the physiological reality of the three separate compartments.

We have previously suggested that the slow compartment might represent aged or abnormal cells which have been trapped with a view to eventual destruction. The mechanism of trapping has been described in terms of a filter system within the spleen (Björkman, 1947; Weisman *et al.*, 1955; Crosby, 1959). On the basis of such a filter, conceived of as a series of cylindrical tubes through which some cells may not be able to pass, Canham and Burton (1968) were able to explain the distribution of area and volume of normal red cells in human peripheral blood. In the present studies, however, no cells were found trapped in narrow orifices; the cells were either phagocytosed or else in close contact with macrophages in the red pulp. Furthermore, the cells attached to macrophages could be freed and washed out by continued perfusion.

The life-span of red cells in the cat is approximately

100 days. Thus each day 1% of the total red cell volume is removed by the R.E. system and is replaced by an equivalent amount of new cells released from the bone marrow. The spleen is not the only organ which can remove aged cells and therefore we would expect to find only a part of this 1% being destroyed in the spleen. Our measurements show that the cells in the slow compartment amount to 1.8% of the animal's total red cell volume (see IV-1-c) and this would imply that the slow compartment contains cells other than aged cells. These could be either abnormal cells containing inclusion bodies (e.g. Heinz bodies) or, possibly, reticulocytes in the process of maturation.

The detailed structure of the red pulp and the pathways of red cells through the pulp are very poorly understood (Burke & Simon, 1970). Certain structures, such as ellipsoids (sometimes called penicilli or sheathed arteries) and Billroth cords, have been described differently by different investigators (Weiss, 1957, 1962; Snook, 1958; Koyama et al., 1964; Hirasawa & Tokuhiro, 1970). The present study is not aimed at clarifying the morphology of the spleen but it does suggest a new experimental approach. Thus the subtle structures of cord and sinus in the red pulp are normally obscured by red cells, but when most of these cells have been washed out it is possible to examine the microcirculatory structures in much greater detail. This method is far superior to that of "painting out the free, wandering component from the red pulp" on photographs of splenic sections (Hirasawa & Tokuhiro, 1970).

(3) <u>Nature of red cells in the slow compartment collected from the venous outflow</u>

One of the advantages of using a perfusion method to study the kinetics of red cell washout is that in this way we may collect separately the cells from each compartment (see III-5). The characteristics of red cells obtained from a particular compartment may then be compared with those of red cells from other compartments or those of normal circulating red cells.

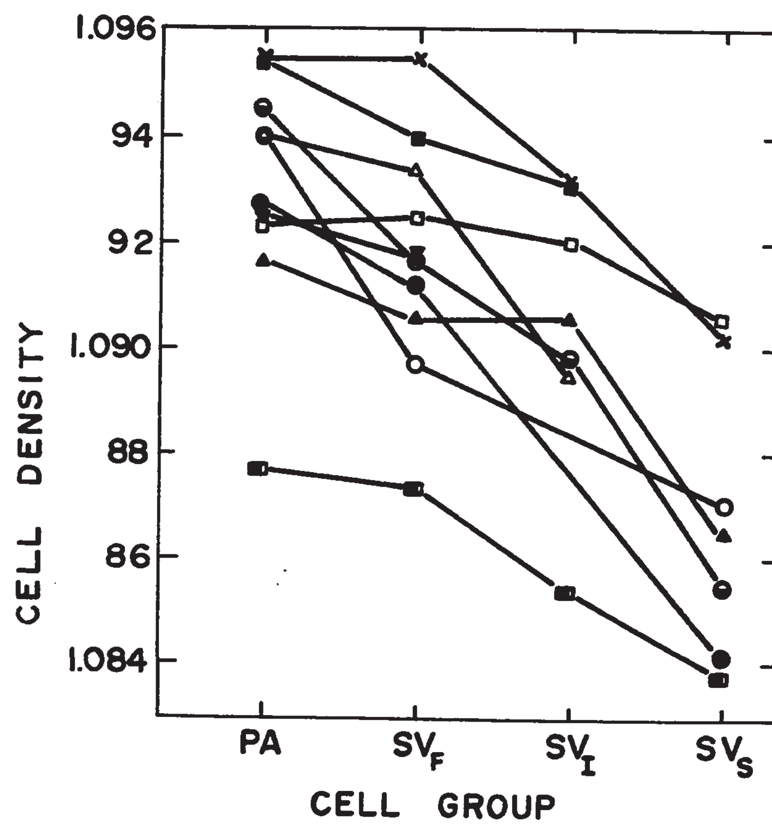
If the cells from the *slow* compartment be aged or abnormal (see IV-1) we might well expect their density to be greater and their volume smaller than average (Crosby, 1959; Danon, 1955; Bishop, 1971). This is a generalization, widely accepted, which has been confirmed particularly in man, rabbit and rat (Danon & Marikovsky, 1964; Leif & Vinograd, 1964; Piomelli, Lurinsky & Wasserman, 1967; Ganzoni *et al.*, 1971). We have therefore measured both density and volume distributions of cells from each compartment in order to determine whether or not those from the *slow* compartment are different from the rest. In addition to these physical measurements we have also studied the morphological pictures of the cells from smears treated with supravital stains.

(A) Density distribution of red cells

Ten healthy cats weighing 1.7 to 2.5 Kg were used in these experiments. During perfusion of the spleens samples containing cells from SV_F, SV_I and SV_S were collected and cellular densities were measured as described earlier (III-7).

FIGURE 11

Cell densities of the arterial (PA) and splenic venous samples. SV_F (fast compartment), SV_I (intermediate compartment), and SV_S (slow compartment): 300 mOsmol/L, 10 experiments.


1.096
94
92
1.090
88
86
1.084
CELL DENSITY
PA
SV$_F$
SV$_I$
SV$_S$
CELL GROUP

For each sample the percentage of the red cell column above the oil was plotted, on probability paper, against the density of the oil. In every case a straight line could be fitted to the data, showing that the cell density distribution was Gaussian. The mean cell density was obtained by interpolation from this graph, as equal to the density of the oil when 50 percent of the cells lay above the oil level. This mean value will be referred to as the cell density of the sample.

Values of cell density for arterial blood (PA) from 13 cats ranged from 1.0877 to 1.0965. The overall mean value for this group was 1.0932 $\pm$ .0006 (S.E.) and this is presented in Table IX together with the corresponding values for groups SV_F, SV_I and SV_S. We shall refer to these overall mean values simply as the mean densities of the various groups.

The mean densities varied from 1.0932 (PA) to 1.0868 (SV_S) and although the density tended to be less for splenic venous cells than for PA the difference was statistically significant only in the case of SV_S ($p<.01$). In Fig. 11 cell densities of the individual samples obtained in ten experiments are shown and in each case there is a tendency for the density to decrease in the order PA $\rightarrow SV_F \rightarrow SV_I \rightarrow SV_S$. When these data were treated as paired observations the differences between all three SV groups and PA were significant ($p<.02$).

Discussion

The range of densities encountered in cells from arterial blood of the cat was 1.082 to 1.105. This range is

TABLE IX - Mean densities and volumes of cells in Ringer's solution of 300 mOsm/L.

	PA	SV_F	SV_I	SV_S
Mean density	1.0932	1.0918	1.0905	1.0868*
S.E.	± 0.0006	± 0.0007	± 0.0010	± 0.0010
No. of observations	13	10	7	7
Mean Volume	42.18	41.35	42.92	44.32*
S.E. (Channel No.)	± 0.78	± 1.05	± 0.99	± 0.79
No. of observations	8	8	8	8

* $p<0.01$ compared with PA sample

comparable to those of man (1.094-1.130) and rabbit (1.090-1.118) determined by Danon _et al_. (1966). In these last two species it has been shown that the density distribution curve can be considered as representing the distribution of cell age in normal blood, density increasing with age (Danon & Marikovsky, 1964; Leif & Vinograd, 1964; Piomelli, _et al_., 1967). If this be true in general then, in our sample PA, cells with densities close to 1.082 would be the youngest whilst those near to 1.105 would be the oldest.

In Table IX the overall mean densities for samples SV_F and SV_I are not significantly different from that of PA, whereas the difference between SV_S and PA is highly significant. Despite the foregoing, when the individual data from ten experiments (Fig. 11) were compared by the method of paired observations, significant differences between each of the SV groups and PA were found. The difference between SV_I and PA can be explained quantitatively in terms of contamination of the SV_I samples with SV_S. This is clearly shown in Fig. 3 (see III-5). Samples collected between 30 and 200 ml perfused consist of not less than 85% cells from the _intermediate_ compartment and up to 15% cells from the _slow_ compartment. The latter, which can be collected as almost a pure sample after perfusion of the spleen by more than one liter of solution, have a mean density near to the bottom of the normal range for PA. This suggests that only SV_S is in fact different from PA in density.

It has been suggested that changes in the physico-chemical properties of red cells stored in the splenic sinuses may occur simply as a result of packing and storage such as could happen in vitro (Benbassat, 1969). Now Danon and Marikovsky (1964) have shown that if blood is left to stand for some time the cells become heavier, the density increasing by 0.008 approximately in five hours. In the present experiments, however, the mean density of SV_S cells, which are coming out of the spleen very slowly (washout half-time approximately 1 hr.), was <u>less</u> than that of PA by 0.0064 units. We conclude, therefore, that the difference in density between SV_S and PA cannot be explained simply in terms of the effect of storage but must be due to something else associated with the function of the <u>slow</u> compartment.

We had thought originally that the slow compartment might represent old or abnormal cells sequestered in the spleen. (See IV-1-C). However, the fact that the density of SV_S was <u>lower</u> than that of PA suggests that this is not so, but that, on the contrary, the slow compartment consists primarily of younger cells. On the basis of experiments using animals bled or treated with phenylhydrazine, it has been suggested that abnormal red cells and macro-reticulocytes are sequestered within the spleen, being remodelled (Ganzoni <u>et al</u>., 1969) or destroyed (Sorbie & Valberg, 1970). In the present experiments we have used only normal, healthy, mature animals free of any hematological disorders, and it is

therefore unlikely that the cells in SV_S were abnormal cells.

(B) Size distribution of red cells

The overall mean volume of cells from each group, in 300 mOsm/L Ringer's solution, was determined from experiments on eight cats. These values are presented in Table IX. Only in the case of SV_S was the cell volume significantly different from that of PA ($p<.01$). Moreover, even when the volumes for samples SV_F, SV_I, and SV_S were compared with the values for PA (Fig. 12), treated as paired observations, no significant difference was found except in the case of SV_S ($p<.01$).

The volumes of cells from the same samples were also determined at 200 and 400 mOsm/L and these data, together with those at 300 mOsm/L, are shown in Fig. 13. At any given osmolarity the cell volumes from all experiments covered a similar range, no matter which group of cells was considered. The mean volumes, at 200 and 400 mOsm/L, are presented in Table X, and clearly there is no significant difference between the means for the four groups. This is in contrast to what was found at 300 mOsm/L (Table IX) where the volume of SV_S was significantly larger than that of PA. This discrepancy means that the osmotic swelling at 200 mOsm/L is smaller for SV_S than for PA. We have therefore determined the osmotic volume changes (200 to 300 mOsm/L, and 300 to 400 mOsm/L) for all four groups of cells and have examined the differences between the values for the splenic venous groups and those of

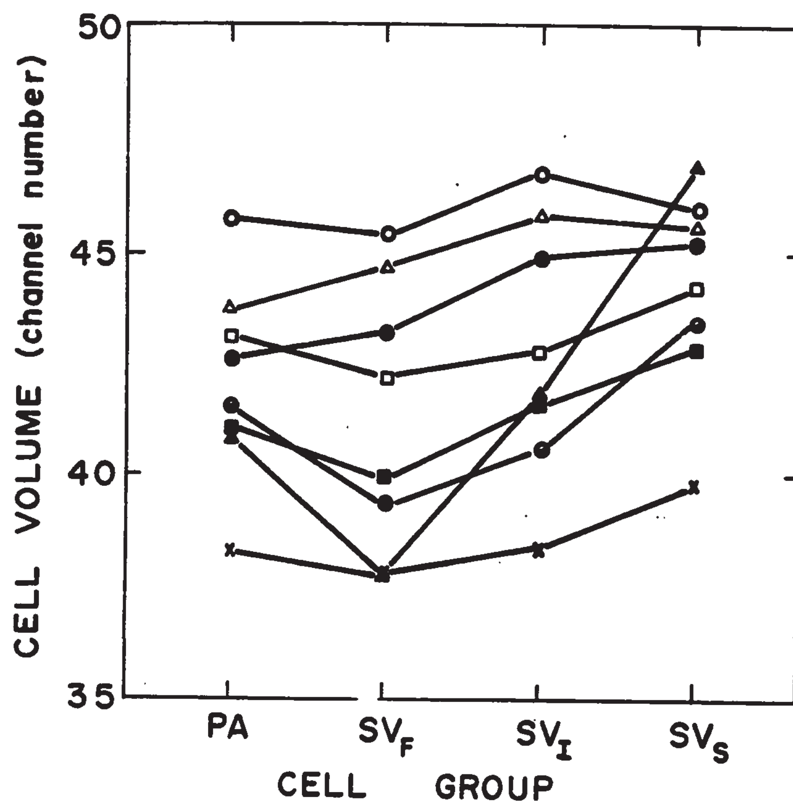
TABLE X - Mean volumes (Channel No.) of cells in Ringer's solution of 200 and 400 mOsm/L.

Osmolarity mOsm/L	Cell Sample			
	PA	SV_F	SV_I	SV_S
200	63.04*	62.51	61.62	62.81
	± 1.42	± 2.13	± 1.66	± 1.90
400	38.22	37.80	39.11	38.75
	± 1.19	± 1.54	± 1.30	± 1.01

*Values are means ± S.E.

FIGURE 12

Cell volumes for the same groups of cells as in Fig. 11: 300 mOsmol/L, eight experiments.



CELL VOLUME (channel number)
50
45
40
35
PA
SV_F
SV_I
SV_S
CELL GROUP

FIGURE 13

Cell volumes for PA, SV_F, SV_I, and SV_S in Ringer's solution at 200, 300, and 400 mOsmol/L. At each osmolarity the volumes of cells from the four different groups are drawn slightly displaced from each other (horizontally) to facilitate examination of the data.

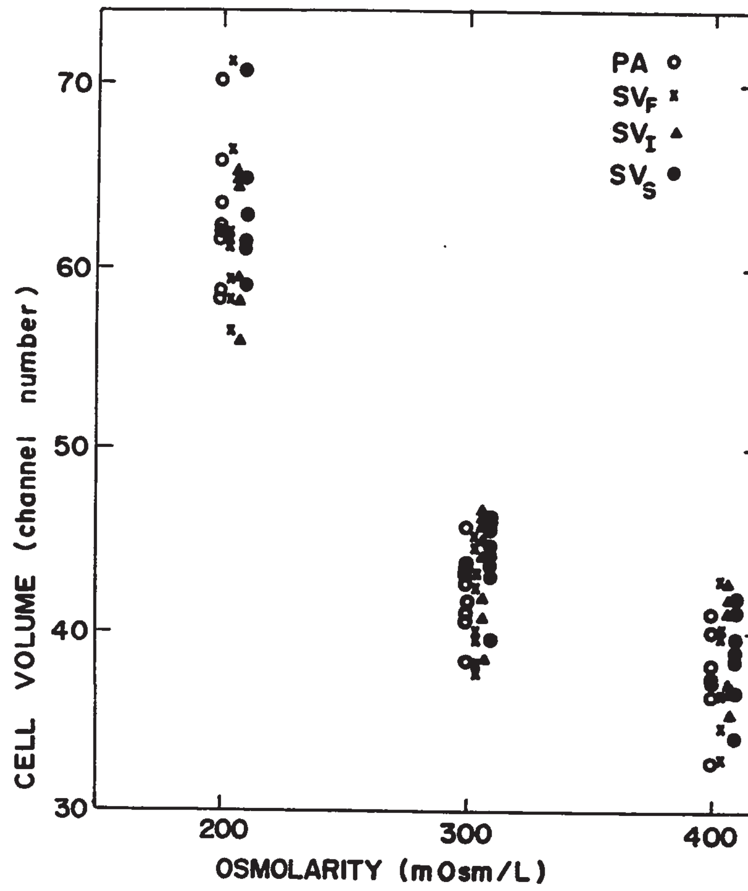

PA
SV_F
SV_I
SV_S
70
60
50
40
30
CELL VOLUME (channel number)
200
300
400
OSMOLARITY (mOsm/L)

TABLE XI - Differences in osmotic volume changes (ΔV) of red cells from the splenic vein compared with those of cells from arterial blood*.

Osmolarity (mOsm/L) changed from	SV_F	SV_I	SV_S
200 → 300			
$(\Delta V_{SV} - \Delta V_{PA})^{\dagger}$	+ 0.45	- 0.93	- 2.81#
± S.E.	± 1.40	± 1.73	± 0.68
300 → 400			
$(\Delta V_{SV} - \Delta V_{PA})^{\dagger}$	+ 0.07	+ 0.27	+ 0.84
± S.E.	± 0.45	± 0.67	± 0.64

* paired observations; six experiments

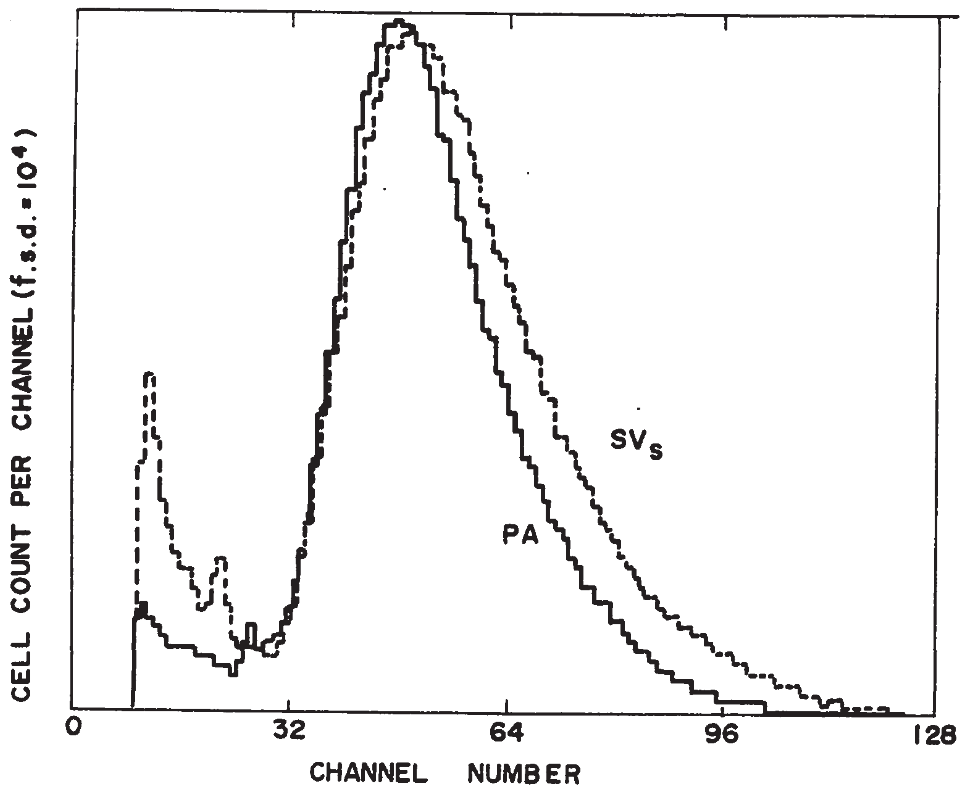
† in units of 'channel number'; a negative value means that SV cells swelled less than PA cells, whereas a positive value means the opposite

# $p<0.01$ compared with SV_F sample

arterial blood using paired observations. These data, which are presented in Table XI, show that a significant difference in the osmotic behavior of splenic venous versus arterial cells occurs only in the case of osmotic swelling of SV_S ($p<.01$).

FIGURE 14

Volume distribution curves (Celloscope) for red cells from arterial blood (PA) and the splenic slow compartment (SV_S), obtained during the same experiment.


CELL COUNT PER CHANNEL (f.s.d.=10⁴)
SVs
PA
0
32
64
96
128
CHANNEL NUMBER

Discussion

The determinations of mean red cell volume were made from volume distributions obtained with a Celloscope Counter. These distributions are often bimodal, in the case of human cells, and the secondary maximum probably represents an artefact inherent in the whole procedure (Bull, 1968; Harvey, 1969; Wechselberger, 1970). We also have encountered this situation in the case of human cells but never when using feline cells. A typical example of the volume distribution curves obtained for PA and SV_S from the same cat is shown in Fig. 14. Although both curves are skewed in the direction of increasing volume their shapes are very similar and it is quite obvious that the mean volume of SV_S is greater than that of PA.

In Fig. 12 it may be seen that the range of mean cell volumes for PA in the different experiments was $\pm$ 10%. An even larger range ($\pm$ 15%) has been found for human red cells (Canham & Burton, 1968). In addition to the inherent differences of mean cell volume between individuals a variation of $\pm$ 1.7% will be contributed by differences, between experiments, in osmolarity of the Ringer's solution (300 to 310 mOsm/L). If, therefore, the possibility of small differences in cell volume between PA, SV_F, SV_I and SV_S is to be examined (see Fig. 14) it is important that all samples from a given experiment be suspended at the same tonicity (i.e. in the same Ringer's solution) and that the data be analyzed using paired observations.

It is interesting that even when the cell volume data

for the different groups were examined in this way no significant differences between PA, SV_F and SV_I were found. This is in contrast to the analysis of cell densities where all three groups of splenic cells were significantly different from PA when analyzed by paired observations. This dissimilarity is probably due to the fact that much smaller samples are needed for determination of cell volume than for cell density. It is clear, from Fig. 3, that collection of very small samples at the appropriate time can minimize contamination from cells of other compartments.

It has been shown that in man, rabbit and rat the volume of younger cells is greater than that of older cells (Leif & Vinograd, 1964; Danon, 1966; Ganzoni _et al._, 1971). Thus the fact that the cell volume of SV_S is larger than that of PA again suggests the possibility that cells from the slow compartment might be predominantly younger cells. It may be seen from Table IX that the mean cell volume for SV_S is approximately 5% greater than that of PA. This difference agrees with that obtained for human cells from the upper and lower extremes of the density distribution (Leif & Vinograd, 1964).

Since the cells of SV_S were larger than those of PA we thought it desirable to compare the osmotic properties of splenic venous cells with those of arterial blood. The fact that SV_S cells swelled less than PA when placed in Ringer's solution of 200 mOsm/L (Table XI) must mean that SV_S cells have a smaller osmotically sensitive water volume than that of PA (Kwant & Seeman, 1970). In other words, it is likely that

the volume of solids was greater for SV_S cells than for PA. Taken in conjunction with the results for volume and density this suggests that the lipid content of SV_S cells may exceed that of PA, again supporting the idea that SV_S cells might consist of younger cells (Westerman, Pierce & Jenson, 1963).

(C) Microscopical observations on red cells treated with supravital stains

Since the foregoing results suggested that the cells from the slow compartment were not aged but, rather, younger cells there was the possibility that SV_S might contain more than the usual proportion of reticulocytes. We therefore prepared smears from samples PA, SV_F, SV_I and SV_S in six cats, using supravital staining (Brilliant Cresyl blue) and for each smear counted the number of reticulocytes per 1000 erythrocytes. The results (Table XII) show that in fact more than one-half of the SV_S cells were reticulocytes. This confirmed the validity, in the cat, of the generalization that younger cells are larger and lighter than older cells. The animals used were fairly mature (average weight 2.3 Kg) and it was not likely, therefore, that erythropoiesis was being carried on in the spleen. This raised the interesting possibility that the spleen could be the place where immature red cells, released from the bone marrow, are sequestered and matured. Because of the importance of the discovery of a high proportion of reticulocytes in the SV_S sample it was considered necessary to undertake further experiments to examine this in greater detail.

TABLE XII - Reticulocyte counts in the splenic venous outflow (SV_F, SV_I, SV_S).

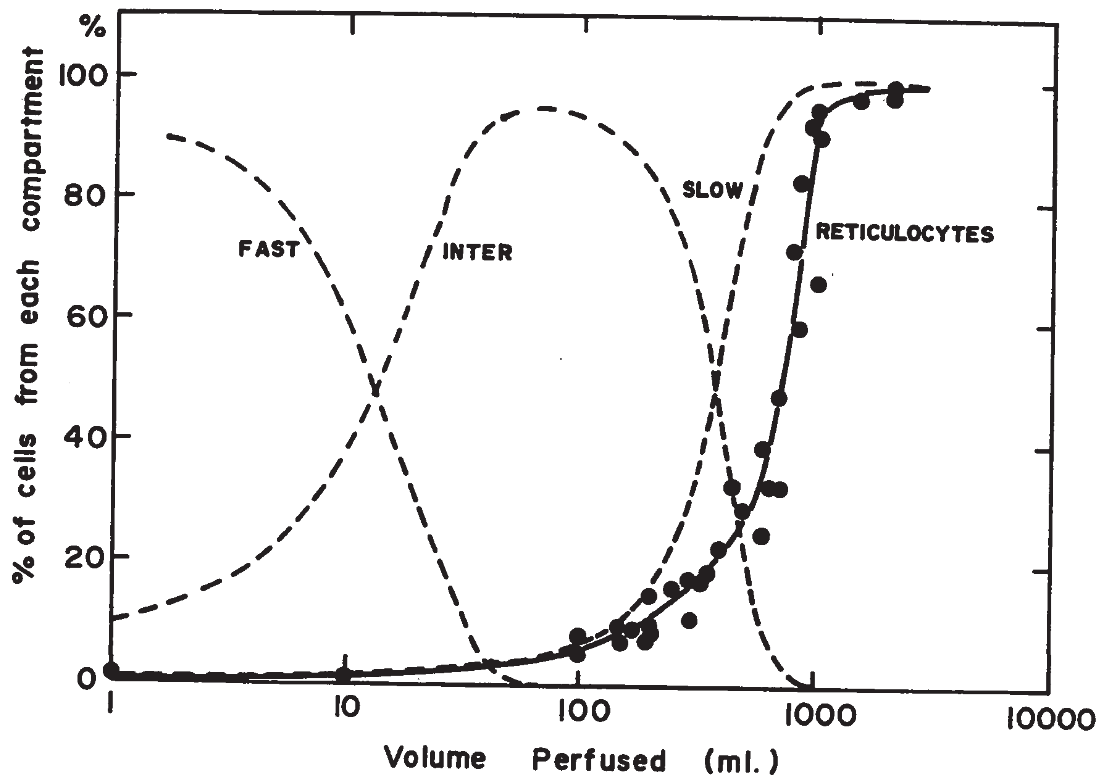
	SV_F	SV_I	SV_S
% Reticulocytes	0.41 ± 0.11*	10.1 ± 1.0†	58.0 ± 3.8†

* Values are means ± S.E. Data from five animals

† $p<0.001$ compared with SV_F sample.

FIGURE 15

Percentage reticulocytes in the outflow, during washout of feline spleens with Ringer's solution, versus the volume of solution perfused (experimental points with solid line). A logarithmic scale has been used for the abscissa because the volumes cover a range of more than three orders of magnitude. For comparison, the percentages of the total cells which come from each of the three splenic 'compartments' are shown (fast, intermediate and slow: dotted lines).


%
100
80
60
40
20
0
% of cells from each compartment
FAST
INTER
SLOW
RETICULOCYTES
1
10
100
1000
10000
Volume Perfused (ml.)

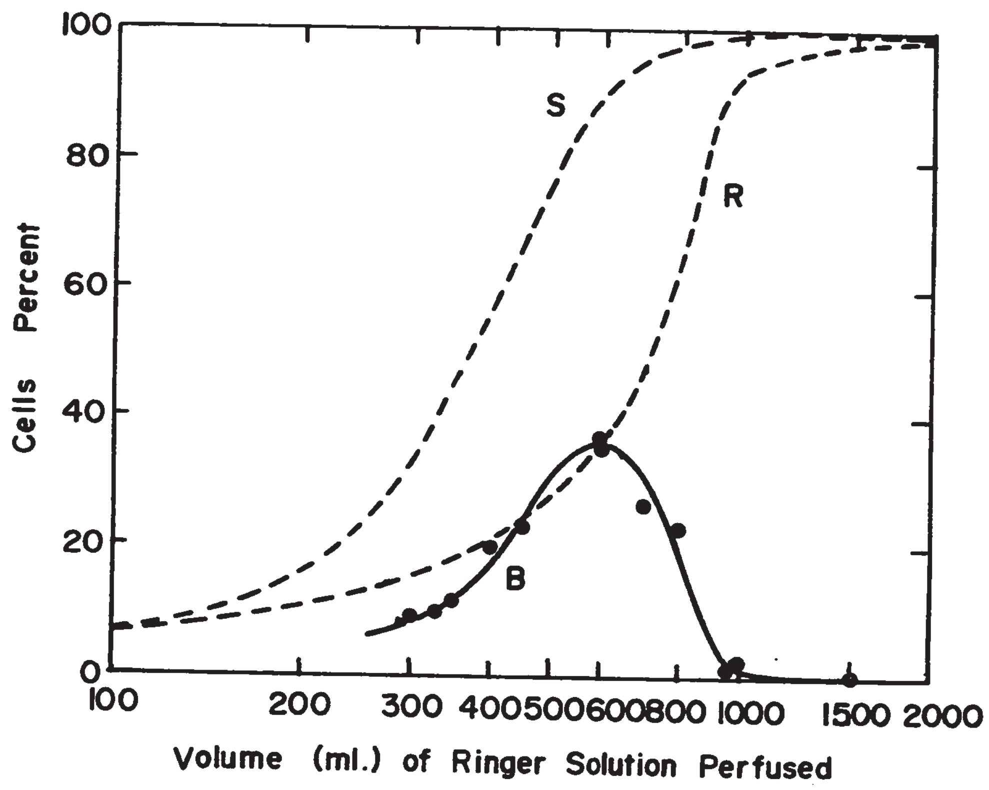
Fifteen cats weighing 1.7 to 3.5 Kg and two dogs weighing 8.2 and 9.5 Kg respectively, were used in these experiments. No significant differences in reticulocyte count (%) were found between samples collected directly and those in which the red cells were concentrated by centrifugation as described above (see III). Values of the percentage reticulocytes in the outflow from feline spleens are plotted in Fig. 15 against the corresponding volumes of Ringer's solution perfused. Each point represents the mean value determined for a given sample from six smears obtained by the two different methods of collection and treated with various stains. The percentage of reticulocytes increased from an initial value of less than 1% up to 99% as the washout proceeded. This general pattern (solid line) was found consistently in each of the spleens examined.

Between 100 ml and two liters perfused we have observed many bluish-stained (basophilic) red cells in the outflow and in 12 samples we counted those separately from the reticulocytes. The percentage of basophilic cells vs volume perfused is shown in Fig. 16 (solid line). It is apparent that the count rises to a maximum value of approximately 36% at 600 ml perfused and thereafter declines to only 2% at one liter perfused. In other words, the basophilic cells are washed out more quickly than reticulocytes.

Our previous studies (see IV-1-A) showed that the red cell washout curve consisted of the sum of three exponential

FIGURE 16

Percentage basophilic red cells in the outflow from feline spleens versus the volume of solution perfused (experimental points with solid line: curve B). Dotted lines S (slow compartment) and R (reticulocytes) are redrawn from Fig. 15.


100
80
60
40
20
0
Cells Percent
S
R
B
100
200
300
400500600800
1000
1500
2000
Volume (ml.) of Ringer Solution Perfused

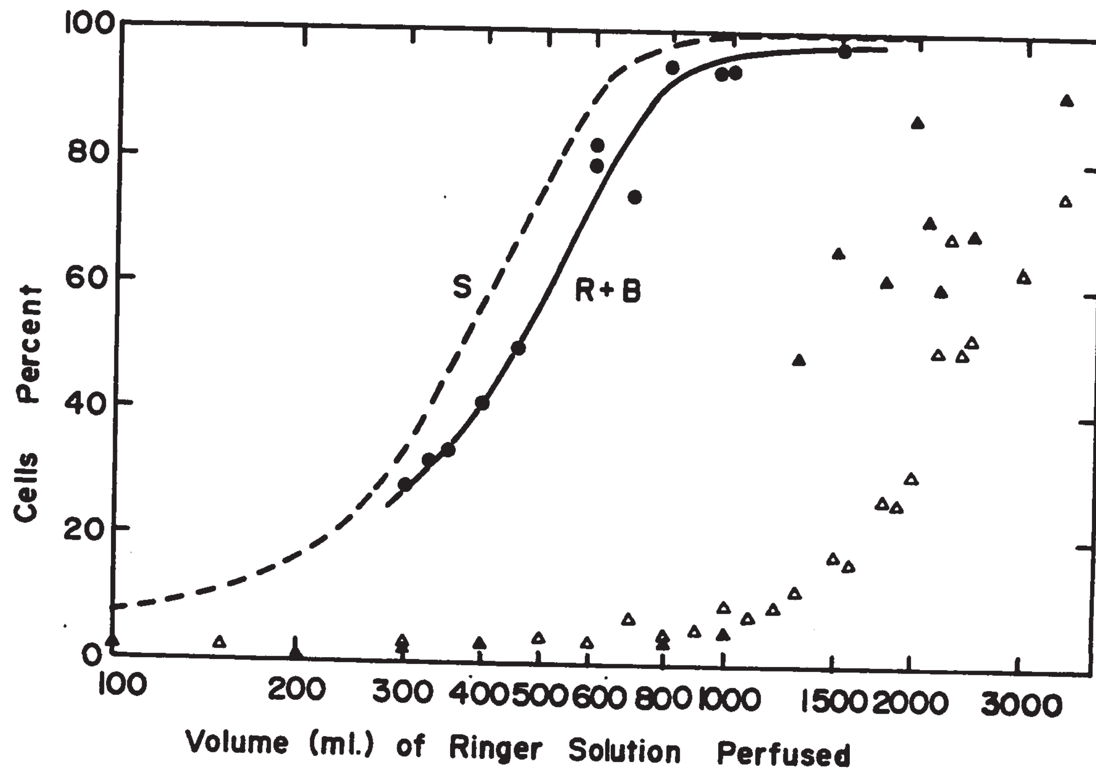
terms. From these terms we may calculate the fractions contributed by the three compartments to the cell concentration in the outflow at any stage of the perfusion. These contributions, expressed as percentages, are drawn as dotted lines in Fig. 15. A logarithmic scale has been used for the abscissa because of the large range of volumes involved. It is quite clear that the reticulocyte counts follow the same general shape as the slow compartment curve. The two curves in fact overlap below 100 ml and above two liters perfused, but in between these limits the percentage of reticulocytes is always lower than that of the slow compartment cells.

Since the onset of release of basophilic cells into the outflow is similar to that of reticulocytes, it would appear that the reticulocytes plus basophilic red cells must together constitute the slow compartment. In Fig. 17, therefore, we have plotted the sum of reticulocytes and basophilic cells obtained from the 12 samples in which both were counted. The scales of Figs. 16 and 17 are identical and it may be seen that the solid line of Fig. 17, drawn by eye through the experimental points, is much closer to the curve (S) than is the curve representing reticulocytes alone (R: Fig. 16).

The discovery of such a high percentage of reticulocytes in the slow compartment of the feline spleen raised the question whether this could be a species-dependent characteristic or not. We therefore carried out similar studies in spleens of two dogs and these results are shown

FIGURE 17

The sum (percentage) of reticulocytes and basophilic red cells in the outflow from feline spleens versus the volume of solution perfused (solid circles with solid line: R + B). Dotted line S (slow compartment) is redrawn from Fig. 1 for comparison. Also shown are reticulocytes counts (%) in the outflow from spleens of two dogs (open and solid triangles respectively).



100
80
60
40
20
0
Cells Percent
S
R+B
100
200
300
400
500
600
800
1000
1500
2000
3000
Volume (ml.) of Ringer Solution Perfused

as triangles in Fig. 17. Up to one liter perfusion the reticulocyte count was less than 10% but thereafter it increased rapidly. After perfusion by 2.5 liters the reticulocytes amounted to more than 50%. In one spleen contraction and relaxation occurred several times during the course of washout; this was accompanied by fluctuations in both the total red cell concentration and the percentage reticulocytes (solid triangles). In the other spleen this problem was not encountered (open triangles). Nevertheless, in both experiments, the washout of reticulocytes followed a similar pattern to that observed in feline spleens.

A significant difference between the two species was apparent in the case of basophilic red cells. Thus, in the cat, up to 37% of these cells were found in the outflow, whereas in the dog the maximum number was less than one-tenth of this. The general shape of the reticulocyte washout curve for the canine spleen is most clearly seen in the experiment shown by open triangles in Fig. 17. This is very similar to the curve (R + B) for the cat, except that all points on the curve are displaced laterally, on a logarithmic scale, by a constant amount. This shift is equivalent to a factor of six and corresponds to the different size of the organ in the two species.

In five cats the proportion of rubricytes among the nucleated cells of the red pulp was determined from histological sections. Three to five sections from each spleen were examined

and a total of 500 to 1000 nucleated cells in each section were counted. The percentage of rubricytes ranged from 1.5 to 4.0, with a mean value of 2.60 ± 0.46 (S.E.).

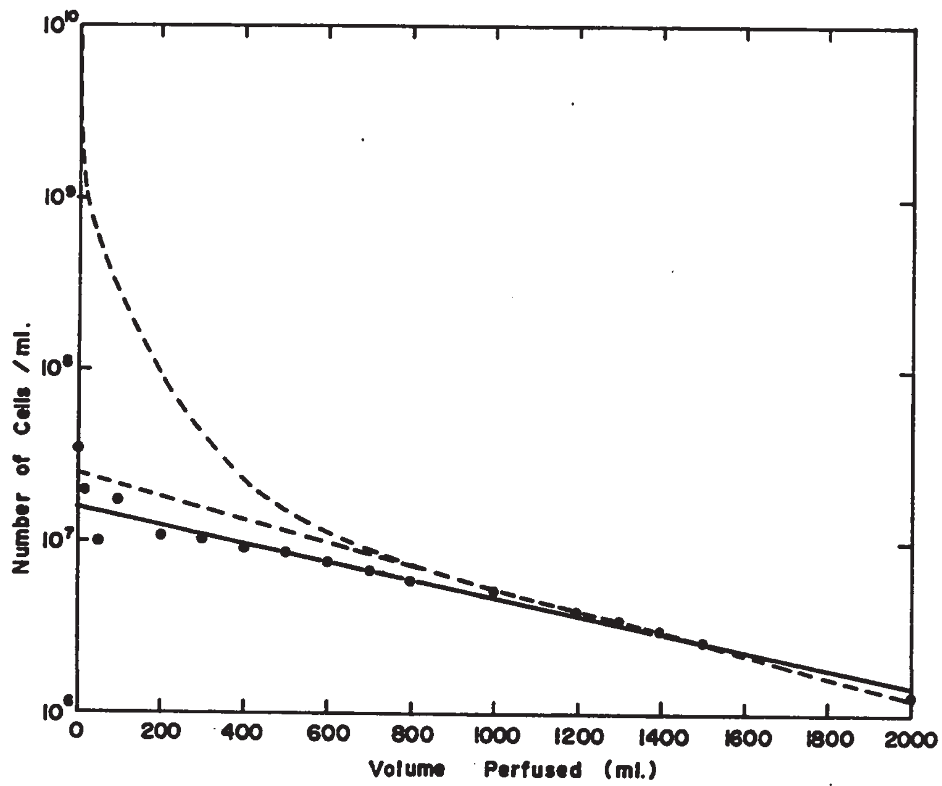
Discussion

An increased proportion of reticulocytes in the spleen of normal dogs was reported by Berendes (1959). She demonstrated reticulocyte levels as high as 10% in smears prepared by the splenic imprint method and we have obtained a similar value (10.6%) from imprints of one feline spleen which had not been perfused with Ringer's solution. The cell washout data (Figs. 15 & 18) show that, under physiological conditions, reticulocytes are retained within the spleen and released slowly. In addition, the sum of reticulocytes and basophilic red cells in the outflow at any stage of washout is almost equal to the predicted number of cells from the slow compartment (Fig. 17). In fact, the difference between the two straight lines, solid and dotted, shown in Fig. 18, is not statistically significant because of the magnitude of the standard errors of the intercept ($2.5 \pm 1.1 \times 10^7$ cells.ml^{-1}) of the slow compartment line (see IV-1-A, Table V).

We have also found a few nucleated cells and even free nuclei (expelled from rubricytes) in the outflow during the later stage of the perfusion. Although these cells and nuclei were present only in small amounts (<3%) they would account for a part of the difference between (R + B) and (S) shown in Fig. 17. The nucleated cells consisted mostly of

FIGURE 18

Cellular concentrations, in the outflow from perfused feline spleens, of reticulocytes plus basophilic red cells versus the volume of solution perfused (experimental points with solid line). Also shown for comparison are the total red cell concentrations in the outflow during washout (dotted curve), together with the slow compartment of the washout (dotted line) derived by backward extrapolation from the final linear portion of the washout curve.



Number of Cells /ml.
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Volume Perfused (ml.)

the lymphocyte series but a few rubricytes were found towards the end of the perfusion.

The washout curve of reticulocytes is of interest for two reasons. Firstly, although it is derived from measurements of total cell concentrations which extend over three orders of magnitude, the washout pattern is strictly linear on a semi-logarithmic scale. We can now present experimental data covering the range which was formerly inaccessible to direct measurement (0-600 ml) being derived only on the basis of a backward extrapolation from the later part of the washout curve. This strengthens the validity of the washout analysis. Secondly, we may calculate, from the washout curve the total store of reticulocytes in the spleen. This amounts to 1.2×10^{10} cells and agrees well with the value $(1.7 \pm 0.9) \times 10^{10}$ cells for the slow compartment (Table V); it corresponds to approximately 8% of the total red cells in the spleen.

It is of course possible that the reticulocytes could have been produced in the splenic red pulp, for erythropoiesis in the spleen has been reported in the embryo (Thiel & Downey, 1921) and in adult mice treated with erythropoietin (Orlic *et al.*, 1965; Gurney & Rosett, 1968). Although we have found many rubricytes within the normal feline spleen, we have never found proerythroblasts (see IV-2-d). The reticulocytes found in the spleen could therefore have originated from these rubricytes although the rubricytes themselves must have come from somewhere else. If this is true, then assuming a

maturation sequence similar to that occurring in bone marrow, i.e. four days in the rubricyte stage and two days as reticulocytes (Harris & Kellermeyer, 1970), we would expect the ratio of rubricytes to reticulocytes in the spleen to be approximately 1:1 as in bone marrow.

We have determined the percentage of rubricytes among the nucleated cells of the red pulp and found this to be 2.60 ± 0.46 (S.E.) %. Now the total number of nucleated cells in the red pulp is 6.1 ± 1.2 (S.E.) $\times 10^9$ per spleen (see IV-2-e) and therefore the total number of rubricytes in the red pulp must be 1.6×10^8 cells. Thus the ratio of reticulocytes to rubricytes in the spleen is $1.2 \times 10^{10}/1.6 \times 10^8$, which equals 75:1. This ratio is almost two orders of magnitude higher than that expected if the reticulocytes were derived from the rubricytes in the spleen. It therefore excludes the possibility that the high percentage of reticulocytes in the spleen might have resulted from erythropoiesis within the organ itself.

It has been suggested that immature red cells could be sequestered in the spleen because of their increased "stickiness" (Berendes, 1959; Jandl, 1960) or decreased deformability (Leblond *et al.*, 1971). If the latter be true we would expect to find evidence, from histological sections of the spleen taken during the slow compartment of washout, of red cells trapped in orifices or channels too small for them to pass through. However, our earlier morphological studies showed only red cells adhering to fine structures of the red pulp (see IV-2-b,f). These structures form a network whose

effective surface area is so large that it would be almost impossible for cells having increased adhesiveness to pass through the red pulp without being retained. This system constitutes a very efficient filter for immature cells and, we believe, is responsible for the accumulation, from the circulating blood, of such a high proportion of reticulocytes within the spleen. If we assume that the difference between the slow and intermediate compartments is primarily a function of cell adherence to splenic structures then the basophilic red cells, which are washed out sooner, must be less sticky than reticulocytes. Further, if stickiness decreases with cell maturity (Jandl, 1960) then the youngest reticulocytes would be washed out last of all. On this view the basophilic cells would be considered as an intermediate stage of development between reticulocytes and mature red cells.

The question then arises: what is the purpose under physiological conditions of such a large-scale sequestration of reticulocytes within the spleen? In the case of macrocytic reticulocytes, produced under erythropoietic stress, sequestration could indicate either destruction (Card & Valberg, 1967; Landaw *et al.*, 1970; Sorbie & Valberg, 1970), or maturation (Crosby, 1963) and remodelling (Ganzoni *et al.*, 1969; Hillman, 1969). In the present study the normocytic reticulocytes stored in the spleen comprise 1.3% of the total red cell mass of the animal. This is equivalent to 1.5 times the daily production of reticulocytes; it could be explained if all the reticulocytes released from the bone marrow were

retained in the spleen for a one to two-day period of maturation and then returned to the circulation. It is hardly conceivable that in normal animals such a large number of reticulocytes could be undergoing destruction in the spleen at any time. We therefore suggest that under physiological conditions the sequestration of reticulocytes allows maturation to proceed in the spleen.

Sequestration in the spleen has been demonstrated for macrocytic reticulocytes in rabbits (Sorbie & Valberg, 1970) and for both macrocytic and normocytic reticulocytes in rats (Dornfest _et al_., 1971). We have examined histological sections obtained from spleens of guinea pig, rat, mouse and rabbit (unpublished observations) and have found, in all these species, specialized structures in the red pulp similar to those seen in cat and dog. It is likely, therefore, that sequestration and maturation of reticulocytes is a general role of mammalian spleens. Moreover, this role may not be restricted to reticulocytes, for Spry (1971) has shown that immature eosinophils leave the bone marrow of normal rats and develop in the spleen, before being released to circulate in the blood. In summary, it is conceivable that sequestration and maturation in the spleen may well be a general phenomenon involving all the formed elements of the blood.

(4) Summary of results and discussion

We have studied the kinetics of red cell washout by perfusing isolated feline spleens and concluded that the kinetics were equivalent to those from a simple three-compartment model. The compartments, representing 11, 56 and 33% of the red cell content in the spleen (1.55 x 10^{11} cells), gave desaturation half-times of 54 min, 8 min, and 30 sec, respectively. These were equivalent to perfusion by 0.2, 9.8 and 90% of the total splenic blood flow and were referred to as the slow, intermediate, and fast compartments.

The kinetics of red cell washout from contracted spleens were studied and again the result corresponded to a three-compartment model. The fast and slow compartments did not differ from those in non-contracted spleens, but the cell content in the intermediate compartment decreased by a factor of 16. This reduction is equivalent to 10% of the total red cell mass of the animal, and the intermediate compartment may therefore be identified as the reservoir.

Morphological counterparts to these compartments were examined from observations, on microscopic sections, of spleens perfused by different volumes of Ringer's solution. We concluded that the compartments of our model (fast, intermediate and slow) represent respectively free cells in vascular channels, free cells within the red pulp, and cells adhering to sinus walls and R.E. cells.

By collecting the venous outflow at three particular stages of the washout, fairly pure samples (>85%) of the cells

from each compartment have been obtained. We have measured cellular density, volume and osmotic changes and have compared these properties for the three groups of cells. Cells from the fast and intermediate compartments were not significantly different from those of arterial blood, but cells from the slow compartment were lighter, larger in volume and swelled less in 200 mOsm/L Ringer's solution. These differences indicated that cells from the slow compartment might be predominantly younger cells.

Using supravital stains, we have determined the percentage of reticulocytes in the outflow from spleens of cats and dogs. Reticulocyte counts increased from 0.4 to 99% as the perfusion progressed. The results show that the slow compartment consists entirely of reticulocytes.

While studying the morphological characteristics of the cells in the slow compartment, we found many rubricytes, but no proerythroblasts, in the spleen. Histological sections showed immature red cells in various stages of maturation, and denucleation evidently occurred by extrusion of either an intact or a fragmented nucleus. We concluded that in normal healthy animals, cells released from the bone marrow after the proerythroblast stage undergo maturation in the spleen.

The ratio of reticulocytes to rubricytes in the spleen was found to be 75:1. The reticulocytes could not therefore have been produced in the spleen but must have been accumulated from the circulating blood. The mean total number

of reticulocytes so stored was 1.2 x 10^{10} cells, equivalent to 1.5 x the daily production in the whole animal. From these data we concluded that reticulocytes released from the bone marrow are sequestered and matured in the spleen.

V. GENERAL DISCUSSION

Any discussion of morphology and function of the spleen is bound to be difficult. At least two factors may confuse the investigators; one is that the spleen contains both white and red pulp, and the other is that the microcirculatory system in the red pulp has been described by anatomists as open, closed or a combination of both. Our experimental approach in this thesis has been restricted to some aspects of splenic function with respect to red blood cells. These studies indicate certain relationships, previously unrecognized, between the red pulp and red blood cells.

The question whether the circulation in the red pulp is open or closed is still in debate. Most anatomists have regarded the system as a combination of 'open' and 'closed', but there is no conclusive evidence for this and we certainly do not know which part is open or closed. Our kinetic studies showed that the red cell washout contained three different components, of which the fast and intermediate resulted from the washout of free red cells from vascular channels and sinuses respectively. These different cellular locations and the very different transit times involved ($T_{1/2}$; 20 - 30 sec vs 8.4 min) are strongly suggestive that the fast compartment

could result from the washout of a closed system while the intermediate might be due to the washout from an open system. This suggestion is also supported by the results from experiments where we failed to perfuse the spleen homogeneously. When the spleen is perfused through only one branch of the splenic artery, the pattern of red cell washout from the red pulp can be seen by the naked eye from the changing color of the splenic capsule. The red cells are cleared first from the well perfused lobe and then very gradually the boundary between the pale part and the dark red part is pushed further and further into the formerly non-perfused lobe of the spleen. Such a result would certainly be expected if the whole red pulp were in communication via an open system. Although this subject is not one of our present goals, it provides an important basis for the design of future experiments.

Recently immunologists have been investigating the relationship between lymphocytes and plasma cells in the spleen (Roseman, 1969; Cosenza, Leserman & Rowley, 1971). It has also been established that circulating hematopoietic stem cells (HSC) may be converted into various blood components (Fried, Knospe, Gregory & Trobaugh, 1971; Grigoriu, Antonescu & Iercan, 1971; Hellman & Grate, 1971), but the organ or tissue where these stem cells are differentiated is not yet known. In view of the present results and those of Spry (1971), it is plausible that the site of differentiation could be the spleen. Since the spleen has the dual pulp system - white and red, it seems to me possible that the spleen might guide the

HSC to differentiate into one particular type of blood cells or another. Certainly, the HSC entering the spleen could pass through either the white pulp or the red pulp. The mechanism governing the differentiation of the HSC into a particular type of blood cell might well be dependent upon the pulp factor where the HSC were held, e.g. the HSC might develop into plasma cells in the white pulp, but into the erythron in the red pulp. At present we do not yet know what mechanisms are responsible for holding these cells or initiating their differentiation. The only factors recognized to date as being important are erythropoietin and the antigen-antibody relationship. However, the site of action of erythropoietin is not yet known.

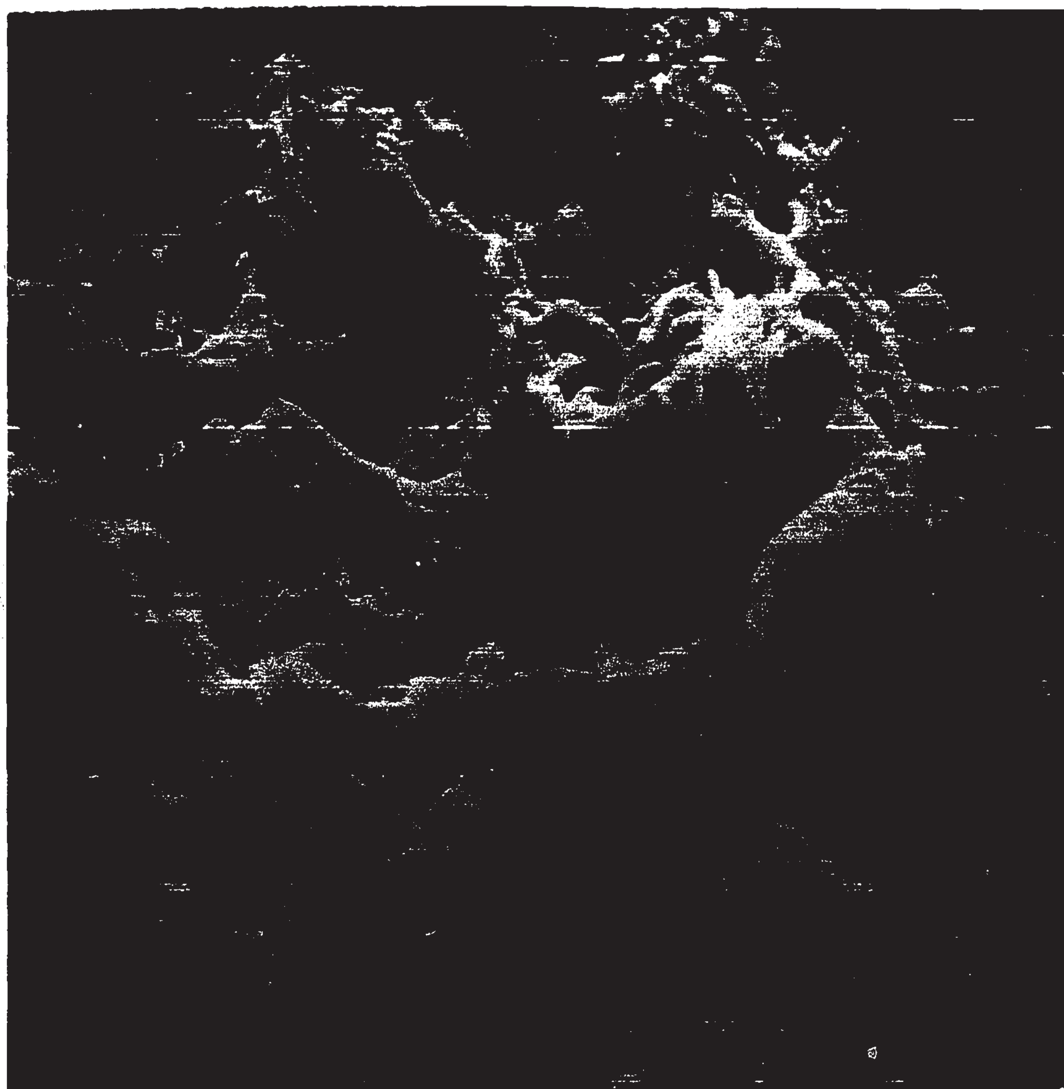
In our histological studies of the spleen we have observed many free nuclei expelled from rubricytes. It is interesting to observe these nuclei, which are surrounded by a small amount of cytoplasm and are confined within a cell membrane. If these nuclei were destined simply to be eaten up by phagocytes, the cytoplasm (which appeared to contain hemoglobin and to be enveloped by an intact membrane) would be completely wasted. The fact that in the denucleation process the expulsion of both fragmented and intact nuclei occurred raises the question whether or not the intact nuclei might be re-utilized. Indeed, if the erythron has to be replaced every day by as much as 0.8% of the total body red cell mass, the system must be operated with a minimum energy requirement. Therefore, it may not be too fanciful to

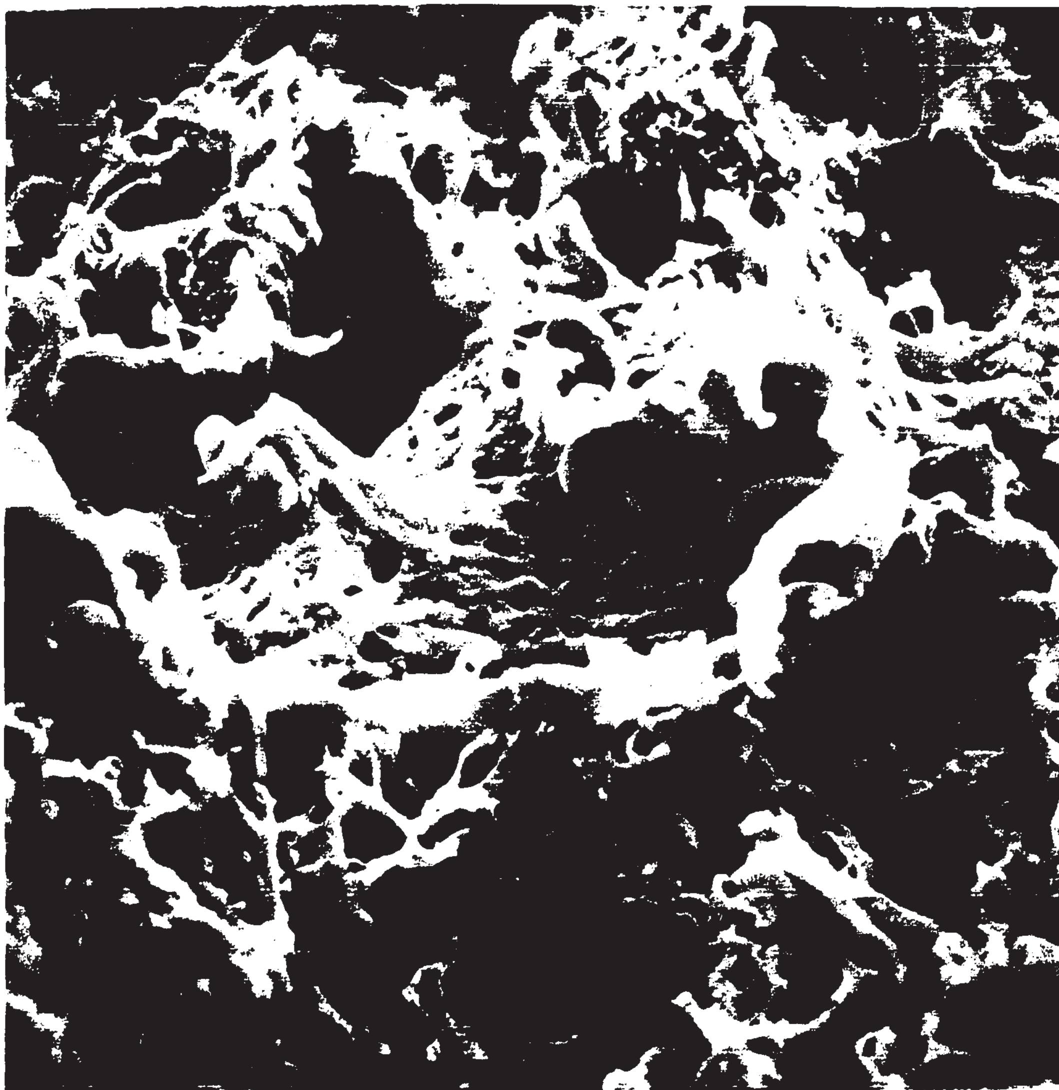
conjecture the re-use of an intact, expelled nucleus until it eventually becomes fragmented. If the macrophages were the 'nurse' cells shown in Plate V-g (see IV-2-d), the free nuclei attached to such a nurse cell could be nourished to increase the amount of the cytoplasm and would be detached as soon as the cell had grown enough to function as a matured red cell. If this conjecture were valid, the spleen could be an organ in which the HSC are differentiated and matured. During fetal life, hematopoiesis is an important function of the spleen; it seems possible that during adulthood the spleen may provide differentiation and maturation of the HSC.

The question of how the spleen holds so many red cells at hematocrit ratios up to 90% may be discussed in terms of the fine structure of the red pulp as seen under the scanning electron microscope. We have recently examined the spleens of dog, cat, rabbit, rat and guinea pig in this way, using the camphene method for preparation of specimens (Watters & Buck, 1971) and the Cambridge Stereoscan instrument, made available through the courtesy of Dr. L. N. Johnson. Plate VI shows highly developed reticular structures with fenestrated endothelium. Before the perfusion, the hollow space between reticulum cells was previously occupied by red cells. If we compare what could be seen when red cells fill this space, it is clear that two different relationships of red cells to the splenic structure exist. Red cells located far away from the reticulum fibers are unattached whereas other red cells,

PLATE VI

Scanning electron micrograph of a dog's spleen after perfusion with Ringer's solution. Note the fenestrated endothelium and tear-drop shaped red cells. The hollow spaces between reticular fibers were filled with red cells before perfusion began. Original magnification x2000.





initially near to the fibers, are attracted by the fibers. We called the latter type "bound" cells (see IV-2-b). It is plausible to assume that some physico-chemical interaction could occur between the molecules on the surfaces of red cells and those on the reticulum fibers. It is generally accepted that red cells have a negative surface charge and it is therefore conceivable that an electrostatic force might well exist between cells of different types. If Robinson's results (1928) were true (see II-1-b), and the younger red cells had the denser negative charges, then the slow compartment representing reticulocytes in our experiments might be due to the electrostatic force between red cells and the framework of the splenic red pulp. We do not yet have evidence to confirm or refute this suggestion.

As a summary of the ideas which this work has generaged in his mind, the author is proposing the following hypotheses:

(i) The microcirculatory system of the splenic red pulp is oriented in both morphology and function by a combination of closed and open types of circulation.

(ii) The splenic red pulp is the tissue where the erythron is differentiated and matured, while the white pulp is responsible for the development of immune response cells, both of which may possibly be originated from the HSC.

(iii) Therefore, erythropoietin may be used up in the splenic red pulp as well as in the bone marrow.

(iv) During the developing stage of immature red cells, the intact nucleus expelled from the rubricyte seems to continue hemoglobin synthesis until it becomes fragmented. Thus from a single nucleus many non-nucleated red cells can be generated until the DNA in the nucleus becomes deteriorated.

(v) The red pulp may also play a role in the repair of damaged red cells, or if they are beyond repair, remove them by phagocytosis.

These are of course merely hypotheses and it remains to design critical experiments to test them.

VI. FUTURE PROPOSALS

The following are titles of interesting future studies arising from the results of this work:

(1) Analysis of the red cell washout from the spleen of the animal which has high reticulocyte counts (reticulocytosis) due to either bleeding or phenylhydrazine intoxication.

(2) Analysis of the red cell washout, after the spleen is perfused with mismatched red cells from different species.

(3) Analysis of the red cell washout, after perfusing the normal spleen with autologous red cells, treated to give altered membrane properties, e.g. by heat, N-ethyl-maleimide, Neuraminidase or Glutaraldehyde.

(4) Further investigations with the scanning electron microscope to show more details of the structure in the red pulp.

(5) The contractility of the reticulum fibers in the splenic red pulp.

(6) Comparison of the fenestrated endothelium between normal and contracted spleens.

(7) The nature of the erythroid development and correlation with the splenic red pulp.

(8) The difference between phagocytes and "nurse" cells in the splenic red pulp.

(9) Analysis of the red cell washout from different organs.

(10) The kinetics of platelets washout from the spleen.

(11) Immunological studies on the function of the splenic white pulp.

This work is not finished of course; it has only just begun!

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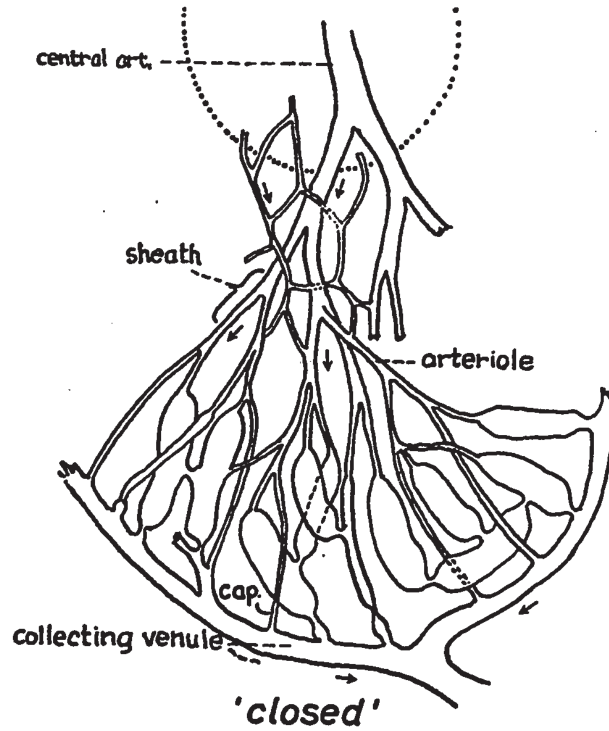
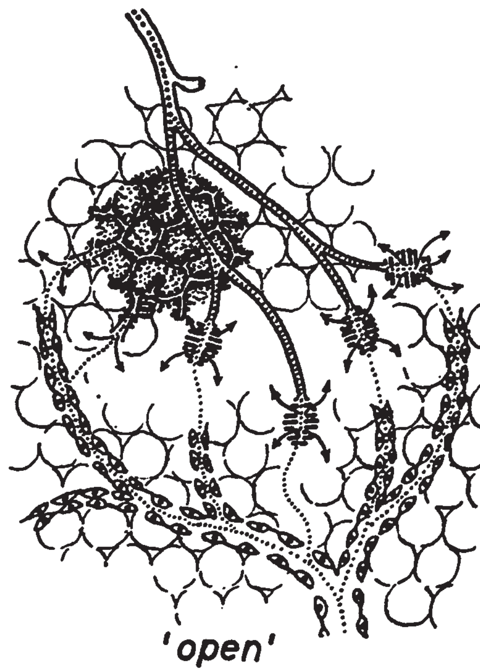
Arrows in both upper and lower graphs show the directions of blood flow. The "closed" circulation theory states that red cells pass through the splenic red pulp via vascular channels that are all covered by endothelial cells. The "open" circulation theory suggests that red cells pass from the arteries to veins, not via discrete vessels, but via a set of diffuse pathways through the meshes of the red pulp. The red cells finally drain into the veins via fenestrated vessels.

APPENDIX

'closed' vs. 'open' circulation



'closed'



'open'