I N T R A V I T A M

AND

S U P R A V I T A L

S T A I N I N G.

AN EXPERIMENTAL STUDY.

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INTRAVITAM and SUPRAVITAL STAINING.

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I. The principles and general results of intravitam and supravital staining.

Introduction.

Agents used as vital stains.

I. soluble dyes.

II. suspensoid preparations.

Dosage and mode of administration of vital stains.

I. soluble dyes of the acid dis-azo group, e.g. trypan blue.

II. suspensoid preparations, e.g. india ink.

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Distribution of vital staining in the body.

I. soluble dyes.

II. suspensoid preparations.

Intra-vitam staining may be defined as the process of staining the elements of the body in the living state. It is to be noted that the term implies a selective demarcation of certain cells or tissues, while other elements remain unstained. This end may be attained by the use of a large number of agents differing widely in their physical and chemical properties; by selecting the appropriate agent and by varying the conditions of the experiment it is possible to obtain a strikingly selective staining of particular structures. The term "intra-vitam" or "vital" staining appears to be justified, since similar results cannot be obtained with dead cells or tissues, though certain of the phenomena of vital staining may be observed in the tissues of an animal treated immediately after somatic death, and before molecular death of the cells and tissue elements has occurred. That is to say, certain of the phenomena can be observed by applying the reagents to the cellular elements removed from an animal during life or immediately after death, and to such measures the term "supra-vital" staining has been applied. As a special variety of vital and supra-vital staining, we have the addition of vital staining agents to the cells of tissue cultures, and here histological pictures closely resembling those seen in the cells of the living animal are said to be obtained. The essential difference between vital and supra-vital methods of staining is that the/

the latter require agents which, while not toxic to the cells, are yet capable of producing their maximum effect within a few minutes, whereas most of the agents used as vital stains require many hours to produce a visible staining of the cells. Certain vital stains can also be used to stain selectively non-cellular elements of the body, e.g., trypan blue stains the elastic tissue of blood vessels, and this dye has also the property of diffusely staining damaged or dead tissue, even if such tissue does not take up the stain when healthy. This property which will be dealt with more fully later, has obvious uses in pathological work for mapping out areas of damage in a tissue which normally remains free from the stain (McClellan and Goodpasture, 1923).

Hitherto, however, attention has been chiefly devoted to the study of the granules which become visible in certain cells of the body when suitable vital stains are administered to the living animal. There has been much controversy as to the nature of these granules, whether they represent preformed structures in the cells rendered visible by the selective action of the vital stain, or whether they are elements newly formed as a reaction to the stimulus of the stain. It cannot yet be regarded as definitely settled whether the accumulation of the vitally stained granules within the cytoplasm of the cells is dependent on the physical or on the chemical nature of the stain. The/

The evidence concerning the different views will be discussed below. We shall deal first with the phenomena of vital staining in the normal animal, and later take up the application of these results to the study of pathological problems. It appears that too often little attention has been paid to the normal phenomena in interpreting the results of vital staining in pathological experiments, and that many sources of error have thus not been recognised.

While there are numerous earlier instances on record in which substances of various kinds have been administered to living animals with a view to demarcating certain cells and tissues, the study of intra-vitam staining may be said to date from the work of Bouffard (1906) and Goldmann (1909). Previously experiments in vital staining had been carried on chiefly with basic dyes such as neutral red and methylene blue, though, in some experiments (Ribbert, 1904) lithium carmine, indigo carmine and various suspensoids had been used. The staining of the osseous system by feeding madder to growing animals has also been known for many years, and has long been used to demonstrate areas of new osseous deposition.

Goldmann studied in detail intra-vitam staining with acid colloidal dyes. Working under Ehrlich's guidance, he was the first to describe the distribution of vital stains in the animal body after repeated injections, and he elaborated methods which secured extremely high degrees of staining. In these/

these experiments pyrrhol blue was mainly used, as after extensive observations this was found the most satisfactory dye. In his later work Goldmann used also isamine blue - a dye of similar constitution - but he frequently stated that no dye was as good as his original pyrrhol blue. In our own experiments, this difficulty has been experienced and none of the samples of isamine blue or pyrrhol blue used have given such intense and selective results as in Goldmann's earlier work. It seemed to Goldmann that vital staining offered the prospect of a further insight into cell physiology. He regarded the stained granules as evidence of secretory activity on the part of the cells in which they appeared and sought to identify them with other known cell constituents. He recognised that while many cells of widely differing types were capable of exhibiting vitally stained granules in varying degree, yet the dye appeared predominately in certain elements which he named "pyrrhol" cells. Many of these are wandering mononuclear cells of the tissues, and Goldmann investigated the reaction of such pyrrhol cells in disease conditions. At all times, however, he retained the view that capacity for vital staining was intimately connected with cellular secretory activity. While most recent authors differ from Goldmann in the interpretation of the phenomena, the accuracy of his description of the appearances is unquestioned. Shortly after Goldmann's work there appeared/

appeared that of Kiyono (1914), on the results of an extensive series of experiments with lithium carmine and certain other vital stains.

Since 1914, there have been many papers dealing with different aspects of the problems. We shall deal here only with the most important publications concerning each branch of the subject. Evans and Schulemann (1914) claim that the essential phenomenon of vital staining, i.e., generalised staining of the tissues together with the appearance within the cells of granules stained by the dye, can be explained in terms of physical chemistry, and that the effectiveness of a dye as a vital stain, depends on the colloidal state of its solution, and is independent of its chemical constitution. According to this view, when the solution is in a suitable colloidal state it accumulates as granules within the cytoplasm of the cells. These authors have dealt at great length with this aspect of the problem and their work will be referred to more fully when the nature of vital staining is considered.

The study of vital staining in tissue cultures especially by the Lewises (1915, 1924) has yielded information concerning chiefly the nature of the vacuoles and granules which become visible after application of the stains and their relation to other cell constituents. Mitochondria have/

have also been studied by supravital staining and this method is stated to be the most satisfactory for demonstrating these elements (Michaelis, 1899; Bensley, 1911; and Cowdry, 1918).

The knowledge obtained from observations on normal vitally stained animals raised great hope that by means of the criteria which demarcated certain very clearly defined groups of cells, one could follow the activity of these cells in inflammatory and other reactive processes. Thus in recent years a great mass of literature has appeared dealing with the study of problems of pathology by methods of intra-vitam staining. Maximow (1902, 1927) and his co-workers have studied the relation of the "wandering connective tissue cells" of the body to other cell types; McJunkin (1919, 1920) and Foot (1920) have investigated the relations of the vascular "endothelial cell" to the elements of the blood and to the wandering cells of connective tissue; the researches of Sabin (1923, 1925) and her co-workers have dealt with the specificity of the cellular elements of the blood, lymph and serous sacs.

In the field of experimental embryology vital staining has been used to demonstrate the origin and mode of growth of the lymphatics in amphibian larvae (Wislocki, 1917). Goldmann observed that in rats and mice vital staining of the mother was not transferred to the foetus, although the membranes and placenta/

placenta became deeply stained. His observations have been generally confirmed by Wislocki (1920, 1921) in guinea-pigs, rabbits and cats, and by Schmidzu (1922) in rats and mice, but in the rodents Wislocki found that a very small amount of dye might be transmitted to the amniotic fluid and foetus producing a faint staining; no trace of such staining ever appeared in cats. Wislocki has further shown (1921) that soluble dyes injected directly into the amniotic sac are absorbed into the foetal circulation and bring about vital staining of the foetus, but no evidence of absorption in the maternal circulation could be found.

AGENTS USED AS VITAL STAINS.

I. Soluble Dyes.

There are many dyes which are capable of staining the tissues of the living animal but comparatively few are suitable for use as intra-vitam stains. Some dyes are absorbed so rapidly that they literally flash through the animal, which becomes stained within a few minutes; but excretion is equally rapid, and within a few hours decolorisation has followed. Certain dyes stain the tissues locally at the site of injection but are practically not absorbed or diffused at all, while others owing to intense local action produce necrosis and sloughing. A suitable dye for intra-vitam staining must be sufficiently harmless to enable an amount to be administered which will produce visible staining in the tissues, the staining must persist for a relatively long period and the dye must be segregated within the cytoplasm of the cells. For histological purposes the stain must also be capable of fixation in the tissues by suitable reagents so that it will withstand paraffin embedding, for frozen sections are rarely adapted to the fine histological methods necessary.

Generally speaking vital stains are divided into two main groups:-

(a)/

(a) The rapidly absorbed dyes which produce generalised vital staining within a few hours or days, e.g., trypan blue and vital new red;

(b) Dyes absorbed only slowly, producing intense local staining but requiring repeated injections over several weeks to bring about generalised staining. Isamine blue (pyrrhol blue), and diamine fast scarlet are examples of this type.

For the chemical constitution of the dyes mentioned in this paper, reference may be made to the Colour Index of the Society of Dyers and Colourists (1923) in which nearly all the dyes used are fully described. Each dye in this Index is designated by a number (abbreviated to C.I.No.-) which I have quoted after each dye mentioned.

The following are all sulphonated dis-azo dyes.

Trypan blue (C.I. No.477).

Vital new red is not mentioned in the Index, but it corresponds closely to C.I.No.353, from which it differs only in the absence of the OH groups and in the substitution of $NHCH_3$ in place of each of the two primary NH_2 groups.

Diamine fast scarlet 10 B F (C.I. No.321).

Benzopurpurin 4 B (C.I. No.448).

Isamine blue 6 B (Pyrrhol blue) (C.I. No.710) is a sulphonated triphenyl-methane dye.

While the results obtained with all these dyes are similar/

similar, great differences can be produced by varying the conditions of the experiment. Thus pyrrhol blue, as used by Goldmann, produces a very intense and lasting vital staining of the tissues, but this can only be obtained after about 6-8 injections at intervals of a week. A similar picture may be obtained with trypan blue by 3-4 injections every second day, but, whereas the staining with pyrrhol blue persists for months with scarcely diminished intensity, that obtained with trypan blue fades in the course of a few weeks. With vital new red a rapid and bright staining of the tissues occurs, which fades rapidly in intensity; yet after a series of injections a residuum of granular dye persists in the cells for many months and indeed appears to be a lifelong possession.

As an extreme variety of the class of slowly absorbed dyes (see above b) we may mention those which produce staining of the cells in the vicinity of a subcutaneous or intraperitoneal injection, but which do not become absorbed to a sufficient degree to cause generalised staining (negative dyes).

Certain dyes, e.g., benzopurpurin 4B cannot be administered intravenously in solution, as the solution flocculates on contact with the plasma and causes death of the animal by embolism, though a freshly prepared cold <u>suspension</u> of the dye can be injected intravenously without ill effects. Benzopurpurin 4B if injected intraperitoneally in true aqueous colloidal/

colloidal solution causes local staining of the tissues of the peritoneal cavity, but absorption into the general circulation appears not to occur or to be so slight as to fail to produce visible staining of the general tissues of the animal, at least after a single injection.

Evans and Schulemann (1914) stated that many of the so-called negative dyes could not be injected intravenously owing to the occurrence of embolism. Schulemann (1917) further recognised that isamine blue when injected intraperitoneally or subcutaneously was precipitated in the form of coarse flocculi from which the dye was liberated into the tissue fluids only slowly. Apparently he regarded this simply as a salting out or electrolytic precipitation of the dye. I have also found that this is a general occurrence with the "negative" dyes. The voluminous flocculi are not, however, composed merely of precipitated dye; apparently the dye becomes bound in some way to the proteins of the tissue fluids, for the flocculi thus formed are no longer soluble in water after simple drying, and they are capable of fixation by histological reagents in a way that is not applicable to dye precipitates formed by salting out, e.g., with NaCl. It would thus appear that while other physical and chemical properties may be important factors, the solubility of a dye in the body fluids plays a large part in determining its effectiveness as a vital stain. Schulemann (1912) appears to have held this view originally, but he later (1917) abandoned/

abandoned it in favour of the purely physical theory of vital staining.

With regard to the factors which determine whether a dye shall act as a vital stain, it cannot be said that any general law has been established correlating chemical constitution and physiological action. Schulemann (1917) especially has considered this question in detail; he states that the effectiveness of dyes as vital stains is independent of their chemical constitution, but is related particularly to the physical characters of their solutions. In the group of compounds studied, Schulemann has found a close parallelism between the rate at which dyes diffuse through gelatin and their rate of diffusion through the animal body after subcutaneous injection. Dyes which failed to diffuse _in vitro_ remained localised to the site of injection _in vivo_; those which diffused with moderate rapidity _in vitro_ were absorbed _in vivo_ and produced generalised vital staining with formation of pigmented intracellular deposits. On the other hand, those dyes which diffused very rapidly _in vitro_ were also rapidly absorbed _in vivo_, and stained the tissues quickly, but were excreted so rapidly that decolorisation was complete and no intracellular pigmented granules were visible after twenty-four hours.

Schulemann and Evans have sought to correlate the physical and biological properties of over 260 dyes. Schulemann (1917)/

(1917) tested among others 31 dyes related to trypan blue, 32 dyes related to benzopurpurin, 11 dyes related to vital new red, and 4 dyes resembling isamine blue. Certain members of each of the four series produced good general vital staining. The only other character common to these vital stains was a physical one, viz., the rate at which they diffused in gelatin. In a particular series this property was modified according to the number of sulphonic groups in the molecule. If there were too few of these groups the substance was relatively insoluble, non-diffusible and incapable of producing vital staining except locally; on the other hand, excess of sulphonic groups rendered the substance too highly diffusible and soluble, and vital staining was fugitive.

The general conclusion was reached that dyes with large molecules form highly colloidal solutions and therefore possess physical characters which render them ill adapted for vital staining, while those with somewhat smaller molecules are semi-colloids or crystalloids and form solutions the physical state of which renders them capable of diffusion _in vitro_ and _in vivo_. It must be emphasised, however, that the number of sulphonic groups required to produce a dye of suitable solubility and diffusibility varied greatly in the different series, and further that the number of sulphonic groups required was not directly dependent upon the size of the molecule. The influence/

influence of other groups, e.g., the substitution of methyl groups for H atoms, and alterations in the position of the sulphonic radicals in dyes otherwise identical in constitution, produced wide and quite irregular variations in physical and biological action; such structural changes exerted an influence not only on the effectiveness of the dyes as vital stains, but also on their toxicity. Great differences in physical and biological properties were found in dyes which possess molecules of approximately equal size and whose chemical constitution is somewhat similar, e.g., trypan blue and benzopurpurin 4B. Accordingly it would seem to us that no valid comprehensive generalisation can be formulated regarding the relationships between chemical constitution of dyes and their action *in vivo*. Clearly, however, the chemical structure of the dye molecule is the factor which determines whether a substance may be capable of acting as a vital stain. If this capacity exists but is masked owing to unsuitable physical characters, then those may be suitably modified, e.g., by sulphonation.

II. Suspensoid Preparations.

The use of the term "vital staining" as applied to the results obtained by the administration of suspensions of particulate matter appears to be justified in view of the definition given above. A large number of substances have been used/

used for this purpose, but two have proved specially useful, viz., carbon suspensions in the form of india ink, and saccharated oxide of iron (soluble or colloidal ferric oxide). These substances are usually administered by intravenous injection. It may be pointed out that such suspensoid preparations are actually particulate, the size of the great majority of the individual particles approaching the limit of visibility with transmitted light. By dark ground illumination, however, the particulate nature of the "solutions" is obvious. These suspensoids possess no powers of diffusion and when injected intravascularly their distribution is limited, for some time at least, to the vascular channels.

Sugar of iron suspensoids behave differently from india ink or carmine suspensions in that the former are stable in contact with the blood plasma, whereas carbon and carmine particles are rapidly agglutinated into larger aggregates and are removed from the circulation by the action of the platelets, which become greatly reduced in numbers in the peripheral blood (Simpson, 1922; Wright, 1927; Elvidge, 1926). These aggregates of particles and platelets are removed from the circulation largely by the filtering action of the pulmonary capillaries, and when the precipitation has been very marked actual emboli may form. There is also a general precipitation of the particulate matter on the capillary walls in all situations soon after injection, but within a few hours these masses are removed and/

and the injected material accumulates in the cells of the reticulo-endothelial system. Wislocki (1924) has shown that the rate of injection of india ink has a marked influence on the amount of precipitation, but found it impossible to prevent some degree of aggregation of the particles even when a dilute solution was injected very slowly. Foot (1920) has insisted that carbon suspensions must be prepared in a colloidal medium. Most of the commercial preparations of india ink contain a protective colloid and I have not observed any marked difference in the distribution of carbon when different brands of india ink were used. Samples from different makers have, however, varied in their toxicity, but it is probable that this is due to other substances present in the ink rather than to the carbon particles. Müller (1926-7) has shown that certain of the toxic effects of specimens of india ink are due to shellac used in their manufacture. To avoid this danger lamp-black may be used, as a suspension in a gum arabic base or in gelatin solution (McJunkin, 1919), but it is difficult to prepare a suspension of sufficiently fine particles. This difficulty has been encountered also in attempting to prepare suspensoids of carmine, and I have never succeeded in producing carmine suspensions in such a fine state of subdivision as is the carbon in commercial inks. Even when carmine was freshly precipitated from solution in the presence of a protective colloid/

colloid (gelatin or gum arabic), aggregation into larger particles always occurred, and differential centrifuging to remove the coarser particles left the suspensions too weak for my purpose. Other substances of a particulate nature have been employed by various writers, e.g., collargol (Oppenheimer, 1908; Tschaschin, 1914), and the so-called colloidal metals, especially silver and iron (Voigt, 1914, 1925). The latter preparations are stated to be suspensions of ultramicroscopic particles of the actual metals; they do not appear to offer any advantage over these which have been described above.

The immediate and remote effects of intravenous injection of saccharated oxide of iron have formed the subject of a special investigation which will be reported separately. This substance differs from carbon suspensions in its stability in contact with the blood plasma, and also in its capacity for undergoing certain transformations as a result of the action of the living cells. Sugar of iron also offers the advantage that it is relatively inconspicuous in the cells and tissues until brought into prominence by the prussian blue reaction, and thus the details of cell structure are not so obscured as with ink. A difficulty which has been encountered with this substance is a lack of uniformity in the state of the solutions formed by different samples. The preparation with which the most of my experiments have been carried out formed an opalescent brown "solution/

"solution" which proved on microscopic examination to be very finely particulate, but the solution could be filtered without appreciable loss through two thicknesses of fairly fine filter paper. Other samples of the preparation have formed clear transparent brown solutions of colloidal (non-dialysable) iron oxide, which could be made opalescent by the addition of traces of acetic acid. The process once begun, however, appears to progress to a complete precipitation of the iron; suspensions of this kind are found to precipitate rapidly in contact with the plasma and thus produce massive capillary emboli in the pulmonary circulation. The clear solutions of saccharated iron oxide appear to possess similar properties to those of the opalescent type first used, but there are minor differences in the distribution of the iron which have not been fully worked out. It is apparent, however, that such solutions do not behave like solutions of soluble dyes, and no immediate diffusion from the blood vessels into the tissues occurs.

DOSAGE AND MODE OF ADMINISTRATION OF VITAL STAINS.

I. Soluble dyes of the acid dis-azo group, e.g., trypan blue.

There are certain general principles to be observed in the administration of dyes to the living animal, and the technique must be adapted to the particular needs of the experiment, and to the species of animal employed. It is well known that/

that the smaller laboratory animals, e.g., mice and rats, tolerate much larger doses in proportion to their body weight than do the larger animals such as rabbits, guinea pigs and fowls. Solutions of dyes for intra-vitam staining are best prepared in distilled water, as saline precipitates certain dyes. Boiling is generally sufficient to ensure sterility if the distilled water and vessels for preparing solutions have previously been autoclaved. It is not advisable to autoclave solutions of dyes as some are not stable under such conditions.

If the subcutaneous route be chosen the needle puncture should be closed with a small clip to prevent leakage of the solution and the site of injection should be gently massaged to spread the dye through the tissues and thus facilitate absorption. This is especially important when a long series of injections has to be given, as it tends to diminish induration and prevents necrosis at the site of injection. After intraperitoneal injection absorption is rather more rapid than after subcutaneous inoculation. Intramuscular injection has only occasionally been employed for special purposes; it possesses no advantage over the subcutaneous route for the purpose of producing general vital staining. Intravenous injection of blue dyes is, in mice, limited to the first dose, as the resulting blue staining of the tail renders the veins almost invisible. With red dyes the veins can usually be distinguished and repeated injections can be given if desired. Intravenous/

Intravenous administration leads to the most rapid and even distribution of the vital staining but it possesses no other advantage, and with certain dyes it is definitely undesirable.

Trypan blue is employed in the form of a 0·5 per cent solution in distilled water. The solution is boiled and filtered when cool. (With certain samples of this dye, solutions in 0.75 per cent. saline form a flocculent precipitate when boiled or autoclaved.) Subcutaneously or intraperitoneally the dosage for mice is generally 1·0 c.c. of 0·5 per cent. solution per 20 grms. body weight; this produces a generalised vital staining in 24 hours. Intravenously only half the above quantity must be given. If it is desired to produce very intense staining, the above dose may be repeated subcutaneously or intraperitoneally after 24 or 48 hours, but in such cases it is better to use a smaller initial dose, e.g., 0·5 or 0·75 c.c. and to repeat it every second day until four doses have been given. This more gradual introduction of the dye seems to lead to a greater proportionate retention, and so to a deeper staining of the cells. Very young mice, under 15 grms., tolerate less in proportion, and very large mice, 30-40 grms., are sometimes hypersensitive to doses calculated on a weight basis.

A corresponding dose of stain for a 2 kilogram rabbit would be 50 c.c., but such doses are rarely given as they do not appear to be well tolerated. Rabbits may be stained with/

with trypan blue by repeated administration of 5 c.c. of a 1·0 per cent. solution of the dye per kilogram body weight. I have generally used the intraperitoneal route or where that is undesirable the intravenous route, the ear veins can generally be distinguished in spite of the dyeing of the tissues so that repeated injections can be given. Guinea pigs have always been stained by intraperitoneal injections repeated every day or every second day; 1 c.c. of a 1·0 per cent. solution per 100 grms. weight has been found satisfactory.

Trypan blue is readily fixed in the tissues by most of the ordinary fixatives, but formalin and corrosive sublimate are most suitable. Zenker's fluid - and other chromic fixatives - tend to change the blue dye to a dirty bluish gray colour, while Bouin's fluid and other picric acid solutions tend to bleach trypan blue so that fine granules may be destroyed. Sections can be prepared by the paraffin method, as the dye once fixed is relatively resistant to decolorisation by alcohol. Most of the usual staining methods can be employed, but it must be noted that the dye is rapidly bleached by potassium permanganate and oxalic acid, and I have observed that in sections treated with picric acid solutions, e.g., picrocarmine or van Gieson's stain, the vital staining is definitely reduced in intensity. Trypan blue is also readily bleached by chlorine, and by hypochlorite solutions; sometimes after/

after bleaching a colourless compound seems to be left in the cells and this can be restained with certain basic dyes such as thionin or fuchsin. The results, however, are inconstant. (See p.108).

Vital new red is less toxic than trypan blue, and mice will tolerate as much as 1·0 c.c. of a 2·5 per cent. solution per 20 grms. weight subcutaneously. It is unnecessary to use such large amounts and I have generally employed 1 c.c. of a 1·0 per cent. solution for a 20 grm. mouse. Subcutaneous, intraperitoneal or intravenous routes may be used, and three to four injections at intervals of 24 or 48 hours will produce a very bright red vital staining. This dye is more difficult to fix in the tissues than trypan blue, and formalin is unsuitable, as the dye dissolves rapidly in the weak alcohols during embedding. Vital new red is insoluble in absolute alcohol, and this may be used for very small pieces or for thin spreads of tissue on coverglasses. Saturated solution of corrosive sublimate is the best fixative in my experience, but Zenker's or Bouin's fluids may be used.

As the dye is very soluble in distilled water, concentrated solutions may be prepared in order to stain larger animals. Rabbits and guinea pigs can be stained by repeated injections of appropriate amounts of a 2·5 per cent. or 5 per cent. solution, e.g., 5 c.c. of a 5 per cent. solution per kilo. for rabbits, and 2 c.c. of a 2.5 per cent. solution per 100 grms. for/

for guinea pigs. With these concentrated solutions intravenous administration is probably best; but the solution should be given slowly to allow gradual admixture with the circulating blood.

Lithium carmine may be conveniently considered here. Though not an acid azo dye it is similar in its behaviour to vital stains of group (a), and it forms a useful contrast to the blue dyes commonly used. Lithium carmine is prepared by adding 5 per cent. by weight of pure carmine to a saturated aqueous solution of lithium carbonate. The carmine dissolves rapidly, forming a deep red solution, which can be sterilised by autoclaving. The solution should be filtered when cool to remove any insoluble impurities. The stain is best administered intravenously; the maximum tolerated dose for mice is 0·2 c.c. of the 5 per cent. solution per 20 grms. body weight. Subcutaneously or intraperitoneally it is more toxic. Rabbits and guinea-pigs may be stained by appropriate doses; the former have generally received 10 c.c. of the 5 per cent. solution intravenously, the dose being repeated several times at daily intervals or longer. Guinea pigs have received 0.5 to 1 c.c. per 100 grms. weight, and repeated injections were always given. Lithium carmine is very rapidly excreted by the kidneys and only a relatively small portion of the carmine is retained within cells in the form of granules. For this reason it is necessary to resort to repeated injections in/

in order to attain high degrees of generalised staining.

The dye is readily fixed in the tissues by formalin which appears to be the best fixative for general use. For films and spreads of tissue Bouin's fluid is excellent. The dye may be removed from the tissues by alternate treatment with acid and alkali, but the intracellular granules if once well formed are relatively resistant to decolorisation and in this respect they differ markedly from carmine itself.

Isamine blue - (Pyrrhol blue) is employed subcutaneously or intraperitoneally in the form of a 1 per cent. solution in distilled water. In this concentration it is flocculated by 0.75 per cent. NaCl, and by blood plasma or serum, and if injected intravenously tends to produce death from embolism. Solutions of 0.4 per cent. or less can be injected intravenously without serious ill effects, but I consider even this undesirable as small flocculi may be produced which are filtered out by the lung capillaries; these may be mistaken for true vital staining within cells. When this dye is injected subcutaneously, it is essential to massage the site of injection for a few moments. Each injection results in intense local staining, but in order to induce marked general staining, a series of injections at weekly intervals is necessary. After 8 to 12 doses each of 1 c.c. of a 1 per cent. solution per 20 grms. weight for mice, a very beautiful vital staining/

staining is produced, which persists without noteworthy diminution in intensity for several months. Excretion of isamine blue is very slow, and the areas of intense local staining probably act as depôts from which the loss by excretion is made good, and the intensity of staining in the tissues is thus maintained.

Isamine blue is difficult to fix in the tissues so that the intracellular granules will resist decolorisation in the ascending alcohols during paraffin embedding. After formalin fixation, the dye is rapidly extracted by weak alcohol; corrosive sublimate gives rather better results, but Zenker's fluid is the most consistently successful for all tissues. Even after Zenker's fluid dehydration must be rapid and the weaker alcohols must be avoided as much as possible. With every precaution, however, it is almost impossible to obtain the granules in sections as sharply defined as they appear in the living cells or in thin spreads or smears. Bouin's fluid is good for spreads of thin tissue on coverglasses, and for cellular exudates, but is less satisfactory as a fixative for tissues to be embedded in paraffin. Isamine blue is not appreciably affected by picric acid in staining fluids for sections, e.g., van Gieson or picrocarmine. When the dye has been fixed in the tissues by Zenker's fluid it is much more resistant to bleaching by permanganate of potash and oxalic acid or by the hypochlorite/

hypochlorite preparations than is trypan blue, but it can be removed by prolonged treatment by these reagents or by weak alcohol. Diamine fast scarlet 10 B.F. is very useful in double staining experiments as in colour it contrasts well with trypan blue or pyrrhol blue in sections. It is generally employed as a 1 per cent. solution in distilled water and is administered in doses of 1 c.c. per 20 grms. weight by subcutaneous or intraperitoneal injection, though very much larger doses may be given without harm. It is unsuitable for intravenous injection owing to its tendency to flocculate in contact with the plasma, but it rarely gives rise to death from embolism, as the flocculi are of small size. I have, however, often observed numerous small masses of precipitated dye in the lung capillaries after intravenous injection of solutions of 1 per cent. or less, and I therefore consider that this mode of administration is undesirable.

Corresponding doses may be administered to rabbits and guinea pigs. Since the dye is very slowly absorbed no toxic effects are generally observed even with enormous doses, e.g., repeated doses of 20 c.c. of 5 per cent. solution per kilo. have been administered to rabbits without ill effect. Guinea pigs may receive correspondingly 2 c.c. of 5 per cent. solution per 100 grms. weight.

This dye is readily fixed in the tissues by Bouin's fluid/

fluid, corrosive sublimate, Zenker's fluid and formalin, but the first two are best. After formalin fixation the dye granules are not so sharply defined, while after Zenker the colour of the dye is altered. When removing the picric acid of the fixative from sections of Bouin-fixed material, all contact with alkali must be avoided, as this dye is then rapidly dissolved out of the cells by weak solutions of sodium bicarbonate, ammonia, lithium carbonate, &c. It may also be removed by potassium permanganate and oxalic acid.

II. Suspensoid preparations, e.g., india ink.

Intra-vitam staining with suspensoid preparations such as india ink or saccharated oxide of iron must be performed by intravenous injection (see page 72). In mice the tail veins are used, and as the number of veins is limited care must be taken to avoid extravasation into the subcutaneous tissues during injection. This is especially important with carbon suspensions, for the slightest extravasation into the perivascular tissues permanently obscures the veins and renders another injection almost impossible. Sugar of iron suspensions present less danger, as the solution is of pale brownish colour, and even if a little is injected along the perivascular tissues the veins are not obscured to any serious extent after the fluid is absorbed. Injection into the perivascular tissues is undesirable/

undesirable since the fluid may be forced directly into the lymphatics and transported to the lymphatic glands along the aorta. A marked deposition of the injected suspensoid in the reticulo-endothelial cells of the glands follows, and this may be a source of error in estimating the distribution of the injected material by the intravenous route.

India ink has usually been employed in the form of Higgins' water proof drawing ink, but other brands have also been used. The ink is diluted with distilled water (see below) and is filtered through two thicknesses of filter paper to remove all coarser particles. The mixture is then boiled or autoclaved and injected when cool. A certain amount of carbon is removed by the infiltration, but the filtrate is quite strong enough for use. I have generally employed for mice a 10 per cent. or a 33 per cent. dilution: 50 per cent. ink, used by some writers, is not suitable for mice as the dose has to be so small that it is difficult to control: 0.2 c.c. of 33 per cent. ink is the maximum safely tolerated dose per 20 grms. weight, but I have found that while not immediately fatal even this dose sometimes leads to gradual loss of weight, progressive emaciation, and ultimately death of a proportion of the treated animals. It also produces rather heavy precipitates in the lungs and for most purposes I have therefore used the higher dilution, i.e., 10 per cent. giving 0.4 c.c. per 20 grms. weight. This amount is quite sufficient/

sufficient to produce a well marked vital staining of the reticulo-endothelial cells which are in contact with the circulating blood. The dilution in which india ink is used is an important factor in determining whether or not death will ensue within 24 hours: 0.2 c.c. of 50 per cent. ink is almost invariably fatal, often within a few minutes, but 0.5 c.c. of 20 per cent. ink is survived by a large proportion of the mice, at least for several days. This variation is probably due to the rate at which the ink particles are introduced into the blood stream, the more rapid the injection the more gross the precipitation and the more severe the blocking of the pulmonary circulation by capillary emboli.

I have not used vital staining with india ink in guinea-pigs, but rabbits may be conveniently treated with appropriate doses. Owing to the greater volume of the circulating blood more concentrated ink mixtures may be injected provided the injection is given slowly. I have used 2 c.c. of a 50 per cent. mixture of Higgins' ink per kilo, for single injections, but if it is desired to repeat the dose a smaller quantity should be given and the mixture should be diluted.

India ink is of course insoluble and cannot be removed from the tissues by any subsequent treatment. Any fixative may be used; I have generally employed formalin or Zenker's fluid, and for the study of rabbit's marrow Maximow's Zenker/

Zenker-formol mixture.

Saccharated oxide of iron is the most useful of all the suspensoids for vital staining and is generally used as a 10 per cent. solution by weight in distilled water. It mixes readily and requires no protective colloid to prevent sedimentation. The mixture may be boiled but must not be autoclaved, as the sugar reduces the ferric oxide and a black precipitate appears. Prolonged boiling should be avoided; if absolute sterility must be guaranteed fractional sterilisation may be utilised.

For mice I have found that the maximum dose safely tolerated is 0.3 c.c. of a 10 per cent. solution per 20 grms. weight. Individual susceptibility varies and some animals survive 0.5 c.c. of 10 per cent. or 0.3 c.c. of 20 per cent. solution, but these doses are lethal to a proportion of the animals. After repeated intravenous injections of sugar of iron, the animals appear to acquire an increased tolerance, and will withstand considerably larger doses without ill effects. I have only occasionally used this substance in rabbits, and employed a dose of 5 c.c. of a 25 per cent. solution by weight in distilled water per kilo., taking care to administer the solution very slowly as this concentration of sugar of iron is rather thick. This amount gave excellent demonstration of the reticulo-endothelial system, but/

but on the whole rabbits are less suitable than mice for a study of the ultimate distribution of the iron owing to the great natural variation in the amount of iron in the organs of individual rabbits.

Formalin fixation gives excellent results and is preferable to corrosive sublimate or Zenker's fluid for the preservation of iron. In some cases tissues have also been fixed in alcohol, but no noteworthy difference was observed in the amount of iron subsequently demonstrable by histological means. After sectioning in paraffin the prussian blue reaction was developed by means of a warm solution of equal parts of 4 per cent. potassium ferrocyanide and 4 per cent. hydrochloric acid. In some cases sections were first treated with ammonium sulphide and then with potassium ferricyanide, but this procedure did not demonstrate any more iron than did the simple prussian blue test. The reagents were generally heated to about 50°-55° C., but in the examination of spread preparations this was found to cause the tissues to curl and leave the coverglass, so that a lower temperature was necessary, 37°-40°C. being generally used. The use of warm prussian blue reagents results in a much sharper picture than if the treatment is carried out at room temperature.

Benzopurpurin 4 B. This dye is only very slowly soluble in cold distilled water, and a fairly fine suspension can/

can be prepared by rapid trituration in a mortar. A 1 per cent. suspension by weight is suitable for intravenous injection and may be administered to mice in doses of 0.5 c.c. per 20 grms. weight. It is advisable to allow the suspension to stand for about five minutes before use in order to get rid of coarser particles which would give rise to capillary embolism. No ill effects have been observed after intravenous injection of this substance in the suspensoid form, and repeated injections may be given. Larger doses may also be administered and I have given as much as 0.8 c.c. of a 1 per cent. solution without ill effect. The contrast is very striking between the behaviour of the suspensoid dye and the same substance in solution in water obtained by boiling the suspensoid and allowing it to cool. The solution on standing passes into a gel state, which later retracts and separates off a clear fluid which is much less deeply coloured than the gel. (This phenomenon is not uncommon with dyes). The colloidal solution of benzopurpurin is very readily flocculated by saline, serum or blood plasma, and when injected intravenously it produces immediate death from embolism of the lungs. If, however, repeated injections of the suspensoid form are given intravenously, the dye is deposited first in the reticulo-endothelial system like any other suspensoid, but later some of the dye is liberated into the plasma in a/

a soluble form and gradually brings about a striking vital staining of the general tissues of the animal. The dye is presumably altered in some way not at present understood, and circulates in the blood and tissue fluids. Its behaviour in this way is of great interest as it resembles that of the previously described sugar of iron.

True aqueous solutions of benzopurpurin when injected subcutaneously or intraperitoneally produce intense and persisting local staining of the tissues with which the solution comes into contact, but no generalised vital staining has been observed by this means, even after repeated injections (Schulemann, 1917). The solution is apparently not absorbed to a sufficient extent.

Benzopurpurin may be fixed fairly well in the tissues by saturated solution of corrosive sublimate; none of the other fixatives employed has given good results. Dehydration must be rapid or the dye will be partially extracted by the weaker alcohols. If desired it may be removed in this way from sections.

SUPRAVITAL STAINING WITH BASIC DYES.

As a special variety of vital staining we have the application of basic dyes to portions of tissue removed during life or immediately after somatic death. This process is known as supravital staining, and it has been extensively used in the study of living blood cells (Sabin, 1923) and the cells of serous exudates. Evans and Scott (1921) have utilised this method to assist in the differentiation of the cells of connective tissue, and the Lewises (1924) have studied the cells of tissue cultures by its aid. The dyes used as supravital stains are all of basic nature, and have smaller molecules than the vital stains previously considered, i.e., the acid dis-azo dyes. When applied to living cells removed from the body these dyes produce staining within a few minutes, whereas the acid dis-azo dyes require to circulate in the body fluids for several hours to produce their effect. Both classes of dyes, however, are concentrated within the cells in a similar manner. The basic dyes used as supravital stains when given to the limit of tolerance produce only transient vital staining; it is not certain whether their toxicity is the only factor in limiting their action as vital stains.

Many basic dyes have been used in the study of living cells/

cells, e.g., neutral red (C.I.No.825) (Israel and Pappenheim, 1896; Rosin and Bibergeil, 1904); methylene blue (C.I.No.922) (Horseley, 1897); brilliant cresyl blue (C.I.No.877) (Cesaris-Demel, 1907); bismarck brown (C.I.No.331); janus green (C.I. No.133); (Michaelis, 1899); but neutral red and janus green have been found especially useful. When suitably applied neutral red stains the cytoplasmic vacuoles ("grains de ségrégation," Rénaut, 1907; segregation-apparatus, Evans and Scott, 1921) and janus green the mitochondria. According to Michaelis (1899) and Cowdry (1918) only one brand of janus green is satisfactory, janus green B. (Hoechst), stated to be chemically diethylsafranin-azo-dimethylanilinechloride,* and it is further stated that the action appears to depend on the presence of the diethyl group. Janus green B in a dilution of 1-20,000 to 1-500,000 in saline is said to stain specifically the mitochondrial elements of the protoplasm, and this is the most satisfactory method of demonstrating them. Unfortunately no method is known whereby the stain may be fixed in the cells, and thus permanent preparations cannot be obtained, but Lewis (1924) has found that fixation of the stained mitochondria in iodine vapour preserves their appearance for a/

* I have not been able to trace a diethylsafranin or a janus green of this constitution in any of the Dye Tables. Presumably this is a special product not now used commercially.

a considerable time. When used in higher concentrations -- 1-10,000 to 1-1,000 -- the stain tinges various other cellular constituents, e.g., the segregation apparatus, and may prove toxic to the cells.

Janus green may be applied either by immersing the tissue in the dye solution, by injecting saline solution containing the dye into the tissues locally, or by perfusion through the blood vessels immediately after death. Also living cells or small portions of moist tissues may be mounted on a slide on which a thin film of solution of janus green in absolute alcohol has been previously allowed to dry (dry film method). The dye dissolves in the tissue juice and stains the mitochondria. This method is particularly serviceable in the study of fresh blood or the cells of serous exudates (Simpson, 1922; Sabin, 1923), but for spreads of subcutaneous tissue it is better to use the dye dissolved in saline solution. Brilliant cresyl blue is used by the dry film method in studying the reticulocytes or immature red cells (Cesaris-Demel, 1907; Key, 1921). The technique of supravital staining with neutral red is similar to that with janus green, and the two dyes may be advantageously combined, either in the dry film method (Sabin, 1923) or in saline solution.

The method of preparing the dry dye films is as follows:- Saturated solutions of neutral red (preferably that/

that of Grübler for vital injection) and of janus green B. (Hoechst) are prepared in absolute alcohol; these form the stock solutions which will keep indefinitely. To 10 c.c. absolute alcohol add 20-30 drops of the stock solution of neutral red or janus green. This constitutes the dilute solution used to prepare the slides when either dye is used separately. For the combined method, 2-3 drops of the stock solution of janus green are added to 1 c.c. of the dilute alcoholic neutral red solution; this mixture must be prepared freshly before use as a precipitate forms on standing. The alcoholic dye solution is poured over the slides and the excess is drained off; the slides are then allowed to dry in the upright position. Slides must be absolutely clean in order to obtain a film of dry dye of even thickness. Dye coated slides thus prepared may be stored indefinitely without deterioration, and it is thus convenient to prepare a considerable number at one time. When supravital studies are being made on leukaemic bloods, it may be necessary to increase the amount of dye on the films on account of the large number of cells present.

In general it may be stated that neutral red in appropriate concentrations brings into prominence intracellular granules closely similar in morphology to those which appear during vital staining with the acid dis-azo dyes such as trypan blue. Nevertheless the action of the former is much less selective than that of the latter group. When neutral red is applied/

applied in vivo or supravitally, red-staining granules appear in many different types of cells which never exhibit vital staining with the trypan blue class of dyes. The term "granules" is used here for convenience of description and does not imply any theory as to the nature of the structures demonstrated by this method of staining. Thus, while the behaviour of cells to neutral red has much biological interest and varies greatly in different cells, it can be used only as an aid to the differentiation of cell types and there is no sharp line demarcating cells which exhibit neutral red "granules" from those which do not.

It must be emphasized that neutral red demonstrates "granules" in many cells which do not stain vitally with the acid dis-azo dyes, e.g., the lymphocytes of circulating blood, epithelial cells such as those covering the cornea (Matsumoto, 1918) and the epidermal cells in amphibian larvae (Clark and Clark, 1919). In such cells, however, the neutral red "granules" are always small and uniform in size and bear little or no resemblance, other than in colour, to the structures which appear in the true vitally staining cells such as the connective tissue histiocytes. It is probable that the granules and other structures stained by neutral red in different varieties of cells are not all of the same nature, e.g., the reticulum of living immature red blood corpuscles may be demonstrated by neutral red, but/

but this structure is in no way comparable to the segregation apparatus.

For a detailed study of the action of these supra-vital stains on individual cells, the following method has been found most suitable. A small amount, e.g., 0.5 c.c. of normal saline containing the dye in very weak concentration (prepared from a fresh 1 per cent. solution of the dye in distilled water) is injected subcutaneously into the tissues of the back or inguinal region. Janus green should be used in a dilution of 1-50,000 or more in this method, as I have found that even 1-20,000 causes staining of the segregation apparatus in some of the cells. Neutral red is also best employed in very high dilution, 1-50,000 to 1-250,000. The two dyes may be advantageously combined in solution, but the mixture must always be prepared freshly before use, as it rapidly deteriorates on standing, owing to the formation of a precipitate. After the injection of the dye solution, the animal is allowed to live for 5-10 minutes, and is then rapidly killed by a blow on the head or by coal gas; chloro-form should not be used as it is apt to damage the mitochondria. The site of injection is markedly oedematous from the injected saline; a small portion of this swollen tissue is snipped out with a fine pair of pointed scissors and forceps, and is spread out as rapidly as possible on a perfectly clean slide. It is then/

then immediately covered with a thin coverslip, and ringed with melted paraffin. Owing to the presence of abundant fluid in the tissues the connective tissue fibres are separated and the cells are readily distinguished; also there is no danger of damage to the cells from desiccation during manipulation. By this procedure more even staining of the cells is produced than in the method of Evans and Scott (1921) in which small fragments of subcutaneous tissue are spread on dry slides and mounted in saline with or without the addition of supravital stain. In the case of tissues already vitally stained with trypan blue, isamine blue, &c., saline solution alone may be injected into the subcutaneous tissue, and parallel pieces may then be mounted on clean slides and on others coated with a film of dry supravital stain as previously described. The dye dissolves rapidly in the abundant fluid in the tissue fragments, but the supravital staining is not so uniform as when the dyes are injected into the tissues.

With a little practice one can pick out the cells in the saline preparation quite readily under an oil immersion lens, while in supravitally stained preparations they are even more easily distinguished. The cells of the subcutaneous tissue of a healthy mouse fall mainly into three groups: (1) histiocytes, (2) fibrocytes, and (3) mast cells. The last mentioned cells are very prominent in the mouse; they are/

are large, about 15 to 20 μ in diameter, with a small round centrally placed nucleus and an enormous number of uniform small highly refractile granules in the cytoplasm, which are clearly visible when the tissue is mounted in isotonic saline. Mast cells are relatively fragile and they frequently rupture during the preparation of the specimen, giving rise to long strings of separate granules. They tend to occur in clusters of four or more cells. In the neutral red preparations the granules stain rapidly and uniformly of an orange yellow colour except in very high dilutions, i.e., 1-50,000 or more. It must be emphasised that this staining appears to result from specific affinity of the granules for the dye, since in fixed preparations the granules of the killed cells are similarly stained; accordingly we need not concern ourselves further with them.

In normal animals it is relatively easy to distinguish fibrocytes from histiocytes in satisfactory spread preparations of subcutaneous tissue rendered oedematous by local injection of saline containing neutral red and janus green. The fibrocytes are distinguished by their elongated shape and their long very fine branching tapering processes; their cytoplasm is relatively homogeneous, of clear glassy transparency, and it contains numerous rod-like and fine granular mitochondria, which are scattered through the cytoplasm, and extend even into the tips of the fine processes (fig./

(Figs.1,b). Neutral red granules are small and are generally rather scanty (fig. 1b); they are often grouped together towards one pole of the nucleus, and only rarely are widely scattered in the cytoplasm. The fibrocytes vary considerably in size, and in the normal resting state practically all such cells exhibit the fine branching tapering processes described above. They possess a relatively large nucleus in which two nucleoli can usually be seen in the living state, and in fixed preparations the nuclear texture is much less dense than that of the histiocytes. Occasionally one finds a cell otherwise resembling a fibrocyte in which there are one or two larger neutral red "vacuoles" in addition to the smaller granules usually found. If the dye be used in too high a concentration the vacuoles and granules of the segregation apparatus of the fibrocytes appear to become larger and more numerous, and thus the sharp distinction which can usually be made between fibrocytes and histiocytes may no longer be so clearly defined, and difficulty may be experienced in deciding to which group a given cell belongs.

The histiocytes are of more compact structure than the fibrocytes, and their processes are blunt and few in number. Some exhibit long slender processes, but at the ends of these there are small rounded or club shaped masses of protoplasm (fig.1a). The cytoplasm of the histiocytes is/

is opaque like heavily ground glass (fig.1 a), thus differing sharply from that of the fibrocytes, and contains numerous small and large "vacuoles" which rapidly become tinged with neutral red. The latter constitute the "segregation apparatus," and in different cells they vary greatly in number and in the intensity with which they stain; frequently they appear to contain smaller more deeply stained granules adhering to the walls of the "vacuoles." (fig. 1 a). In the normal resting state of the histiocytes these structures are smaller and more uniform in size than those which appear after administration of true vital stains; and if neutral red is used in the high dilution specified, e.g., 1-100,000, the dye seems to stain only structures preformed within the cell. These structures, designated by Evans (1921) and his co-workers the "segregation apparatus," have been thought to be intimately concerned with the nutritional activities of the protoplasm (Shipley, 1919). An increased number of granules and vacuoles appear within the cells when stronger concentrations of neutral red are applied, e.g., 1-10,000 or more; the vacuoles are also larger and of deeper tint. Evans (1921) believes that the structures brought to light by vital staining with the acid dis-azo dyes and other agents are merely a hypertrophied form of segregation apparatus which has increased in response to the presence of the large amounts of/

of vital dye in the environment of the cells.

In unstained histiocytes there can be seen in the cytoplasm small granules which possess a refractile character intermediate between the segregation apparatus and the fat granules; after staining with janus green these become deeply coloured, and appear to be in part at least the mitochondria. They are distinctly plumper and more granular than those of the fibrocytes, and often resemble diplococci (fig.1a); I have not observed frankly rod-shaped mitochondira in typical histiocytes. The mitochondria of the histiocytes are generally more numerous in the perinuclear zone of the cytoplasm, and are only occasionally present in the outer zone or in the blunt processes. From their shape alone it is impossible to distinguish them from the smallest granules of the "segregation apparatus." If the janus green be used in a higher concentration which is sufficient to produce staining of the "segregation" granules and vacuoles, it is quite impossible to separate the latter from the stained mitochondria of the histiocytes, although those of the fibrocytes may still be distinguishable. When the "segregation apparatus" is stained with neutral red and the mitochondria with janus green the latter can be seen to lie between the neutral red granules (fig.1a), and it seems justifiable to conclude that these structures are separate entities. It is not clear, however, that all the minute granules which stain even with the highest dilutions/

dilutions of janus green are actually mitochondria, and one must be critical in interpreting the results of supravital staining in this respect.

In the larger and presumably older histiocytes, mitochondria are more difficult to see owing to the overcrowding of the cytoplasm with neutral red granules, and some writers (Cunningham, Sabin and Doan, 1925) state that they have been unable to find mitochondria in fully developed histiocytes. In the younger type of cells the segregation apparatus is smaller and so one can more easily distinguish the mitochondria, which are always of the granular type (fig.1,c). Probably owing to unsuitable optical conditions, I have never been able to observe the very fine streaming processes around the periphery of the histiocytes which Carrel and Ebeling (1926) describe as the "undulating membrane."

In addition to the typical histiocytes, one finds in normal subcutaneous tissue many cells of smaller size, whose general character points to their close relation to the histiocytes. The reduction is chiefly in the cytoplasm; the nucleus is generally of about the same size, but may be slightly indented, or actually reniform. These cells contain fewer neutral red vacuoles than the typical histiocytes and those present are generally of smaller size. They possess fairly numerous plump granular mitochondria, and all intermediate stages are met with between small cells resembling lymphocytes/

lymphocytes and the fully formed histiocytes (figs. 1, 2). These are probably the cells which Maximow (1902) designated polyblasts; he regarded them as derived from lymphocytes at least in part, and for this view we think there is much to be said. From their presence at all times in the subcutaneous areolar tissue, it is clear that there is a continuous new formation of histiocytes by the gradual growth and development of small lymphocyte-like cells through all stages up to the fully formed histiocytes. The disposition of the mitochondria in these cells is not quite identical with that of blood lymphocytes. We are inclined to regard them as young undifferentiated forms, analogous to the small round cells of the tâches laiteuses and perivascular sheaths of the omental and mesenteric vessels, - the adventitial cells of Marchand. It might be suggested that these smaller cells arose by division of the pre-existing histiocytes, but, in the examination of a large number of preparations of living and fixed subcutaneous tissue, mitotic figures have been encountered only among the smaller cells around vessels and nerves. Mitosis of mature histiocytes has not been observed in the normal loose areolar tissue. Mallory (1898) regards such small mononuclear cells as migrated endothelial elements from the vascular capillaries; while Foot (1927) and Masugi (1927) suggest that they are emigrated monocytes. Their exact origin is not settled but it is necessary to emphasise their presence, as we believe that they/

they are of considerable importance as a source of error in interpreting the results of experimental investigations conducted by intra-vitam staining with the acid dis-azo dyes.

While we have described typical examples of each variety of cell, it must be pointed out that frequently it is a matter of considerable difficulty to determine to which group a particular cell belongs even in the normal resting state. When the cells have been stimulated by the injection of vital stains, this difficulty is greatly increased, and cells of apparently intermediate character are frequently met with.

In healthy living cells the nucleus never becomes even faintly tinged with the supravital stain. If, however, the stain be too concentrated and be toxic to the cells, or if the preparation be watched over a long period until cell death occurs, one can observe the staining process taking place in the nuclei. Occasionally the neutral red staining in the cytoplasmic granules fades, though this is not so common in subcutaneous tissue histiocytes as in those from other situations, e.g., spleen. The nucleus becomes granular and then tinged with the dye, the nucleoli first showing staining, and in a short time the whole nuclear structure is displayed. If preparations of subcutaneous tissue be made by the method Evans and Scott (1921) and stained supravitally by/

by mounting in neutral red saline, it can be seen that while the cells in the centre of the fragment stain normally, those around the margin, where the tissue has dried during manipulation, fail to show staining of the segregation apparatus, but on the other hand exhibit nuclear staining in varying degrees. In the intermediate zone between killed and healthy living cells, one finds nuclear staining and granule staining in inverse proportion due to the different degree of injury inflicted on the cells during the preparation of the slide. From this it is clear that the appearance of supravital staining of the segregation apparatus is an indication of the vitality of the cell; dead or severely injured cells fail to concentrate the dye within their cytoplasm, on the other hand their nuclei become stained. The tint of the nuclear staining is, however, decidedly more reddish than that of the granules.

It may be added that, if janus green is injected into the subcutaneous tissues in a high concentration, e.g., 1-5,000, it produces rapid staining of the cell nuclei and cytoplasm, but owing to its toxic action in this strength the mitochondria are not stained. The staining of the cell bodies, however, brings into view the fine processes of the fibrocytes which are usually difficult to see in living cells in the optical conditions which obtain in such preparations. These/

These processes are much more delicate and branched than is commonly supposed, and remind one of neuroglial cells demonstrated by silver impregnation (fig. 2 b). This method is one which might well be utilised to demonstrate the morphology of these cells for teaching purposes.

DISTRIBUTION OF VITAL STAINING IN THE BODY.

I. Distribution of Soluble Dyes.

If an animal be subjected to intra-vitam staining by the administration of several suitable doses of trypan blue, it is found that the tissues of the body everywhere become of a blue tint the depth of which varies widely in different organs. This blue colouration is due to two factors, (a) a diffuse staining of certain structures, e.g., the epidermis, bone (especially the growing ends), elastic and connective tissues; and (b) the accumulation of dye within certain cells in the form of granules and "vacuoles." It is chiefly on this second factor that the intensity of colouration of the organs depends; those which contain a large number of vitally stained cells appear to the naked eye deeply coloured, e.g., liver, and those which contain few or no vitally stained cells, e.g., brain, appear uncoloured. It must be noted that a varying number of these cells are present in the connective tissue/

tissue stroma of all organs except the brain. For this reason, organs such as pancreas, thyroid gland and skeletal muscle, the specific parenchyma cells of which do not stain vitally, appear on naked eye examination coloured in varying degree. In general it may be stated that granular staining is found chiefly in the cells of the reticulo-endothelial system, and the distribution of these cells will be considered in detail later (See p. 81).

Diffuse Staining.

This occurs very soon after intravenous injection of trypan blue, and in a few minutes the animal becomes of a definite pale blue colour. Thorough microscopic examination of the tissues at this time shows no evidence of the dye, which is located in the body fluids. Diffuse staining of the epidermis occurs rapidly and persists for many days after a single injection; the skin on reflection may appear quite blue while the subcutaneous tissues are much paler. Rous (1925) has noted that after vital staining with litmus the epidermis stains of quite a deep diffuse blue colour while the subcutaneous tissues become red owing to the accumulation of granules of litmus in the vacuoles of the connective tissue histiocytes, this indicating that the contents of the vacuoles are definitely acid. Rous (1925) also notes the diffuse staining of bone, cartilage and connective tissue with indicators such/

such as litmus or the phthaleins, and finds that those tissues have all a definitely alkaline reaction whereas the macrophages both in the connective tissues and in the organs exhibit granules and vacuoles with a markedly acid reaction.

The intima of blood vessels and the elastic tissue in their wall, particularly the internal elastic laminae of the arteries, become deeply and diffusely stained with trypan blue, but this staining fades after a few days. Petroff (1922) has used this method to study the mode of nourishment of the different layers of the arterial wall, and has also applied the results of his observations to the study of experimental arterial disease.

This diffuse staining is held by most writers to be a simple absorption which is determined by the physical characters of the structure stained. It is to be noted that it has a selective character which remains only as long as the tissue is healthy and molecular life persists.

As has been mentioned previously death of individual cells is followed by loss of power to concentrate the vital stains within their cytoplasm and the nucleus which in the healthy state remains unstained then becomes progressively coloured. Similarly, seriously injured or necrosed areas in an organ or tissue become diffusely stained by the dyes, e.g., trypan blue, even if such tissue is normally free from the stain.

Recently/

Recently McClellan and Goodpasture (1923) have utilised this method to assist in the localisation of the lesions of herpetic encephalitis in the rabbit's brain, the injured areas presenting a striking colour against the quite unstained healthy brain tissue. Trypan blue has also been utilised by Ramsdell (1928) to demonstrate the immediate skin reaction in rabbits and guinea-pigs sensitized to horse serum. The site of injection of the test dose rapidly becomes of a much deeper blue colour than the surrounding skin and it is thus possible to detect the reaction earlier and with higher dilutions of antigen than in unstained animals. This is a valuable property and one which should be of considerable value in pathological investigations.

If we now consider in detail the appearances in a mouse which has received several doses of trypan blue over a period of say a week, the general features of vital staining will be readily appreciated.

The animal is coloured a bright blue generally. The skin and mucous membranes are of bright blue colour, but the fur is not affected. The subcutaneous tissues are of paler blue colour; in female animals the mammary glands are more deeply coloured than the areolar and fatty tissue in which they lie. This is especially marked in pregnant and lactating animals. The skeletal muscles appear greenish, chiefly owing to/

to the presence of vitally stained cells in the interstitial tissue, and the tongue and heart are often more deeply coloured than other muscles. Tendons are of shining blue silky appearance and their insertions into bones are often more deeply coloured. In young animals the growing ends of the bones are coloured deep blue owing to intense deposit of the dye along the lines of ossification. This fact has been used extensively by Shipley and Macklin (1917) in studying the distribution of osteogenic centres in the bones. Thus in young growing animals the costo-chondral junctions appear much more deeply stained than the rib or cartilage on either side. The lymphatic glands are of very deep blue colour and the fat in which they lie is of a pale greenish hue.

In the internal organs the peritoneal and pleural tissues appear of a pale blue hue similar to that of connective tissue, but it must be emphasised that where injection is made hypodermically or into a serous sac the connective tissues at the site of injection or around the serous sac are very deeply stained while the more distant organs and tissues are relatively less intensely coloured.

The liver is of very deep blue colour, and is usually the most intensely stained organ in the body. The colour is uniform throughout but the gall-bladder is relatively pale and the bile does not appear to be coloured by the dye.

The spleen is also deeply coloured to naked eye examination/

examination and the Malphigian bodies stand out as very pale blue dots against the more bluish pulp, but the cut surface of the organ is less deeply stained than the capsule.

The kidneys are intensely blue stained, particularly in the cortical zone, while the medulla is relatively pale, though the tip of the renal pyramid is always more deeply coloured than the intermediate zone.

The bone marrow is of deep blue colour throughout the short bones, and in laboratory animals generally the staining is present throughout the long bones also, but is usually more intense towards their ends. Here the colour may be partially masked by the red colour of the marrow itself, while the more fatty portions toward the centre of the shaft of, say, the femur take on a slightly greenish yellow hue.

The suprarenal glands are pale blue in colour, the intensity of which is greatest in the outer cortical zone, while the inner layers of cortex are relatively unstained. The junctional zone with the medulla is usually rather more deeply coloured.

The pancreas and salivary glands are of pale blue colour but there may be areas of more intense staining owing to the presence of small islets of lymphoid tissue.

The stomach shows varying degrees of staining in different sites; the cardiac end and body of the stomach are/

are of very pale blue colour, but the pyloric canal and ring are much more deeply stained, and this deeper hue is carried on over the small intestine with gradually diminishing intensity. The large bowel is more deeply coloured, especially the region of the caecum, in which the colour is especially intense. The lymphoid follicles in the bowel wall are always more deeply stained than the gut itself. With isamine blue, on the other hand, the caecum is of paler colour than the small bowel.

The omentum is pale blue generally with numerous deeply pigmented areas, some of which are in relation to blood vessels, while others constitute the so-called _tâches laiteuses_. The mesentery is of similar pale blue colour, with more intense aggregations around the vessels, while the lymphoid tissue is darker in hue, similar to that of lymphoid tissue elsewhere.

In the male the testes are coloured rather more deeply than the pancreas or salivary glands but less deeply than lymphatic glands. In the female the ovaries are generally of pale blue colour but exhibit several sharply circumscribed areas of much deeper colour which correspond to Graafian follicles. The Fallopian tubes and uterus are often quite deeply coloured, and this staining often persists with scarcely diminished intensity over a long period, after the other/

other organs and tissues have become almost completely decolourised.

The walls of the blood vessels are pale blue, but the staining of vessel walls is more pronounced within a few hours of injection of the dye, and fades rapidly to a uniform pale blue. In rodents, staining of the vessel wall is usually diffuse in character, but in dogs and cats Okuneff (1926) has shown that in the early stages vital staining of the aorta is patchy in distribution, the region just above the aortic cusps, the origin of the innominate and subclavian arteries and the insertion of the ductus arteriosus being first and most pronouncedly stained. Glasenow (1926) obtained similarly selective staining of local areas of the aortic wall by perfusing rats and other rodents with a weak solution of trypan blue. This localisation is similar to that obtained by Anitschkow (1913) and others in early lipoid infiltration of the wall in rodents. The significance of these observations is not very apparent.

The blood is tinted with the dye, the intensity depending on the length of time after injection. At first, the red corpuscles are tinged with blue so that in fresh preparations they appear greenish, but this staining disappears within a few hours. The white corpuscles remain quite unstained generally. The blood plasma carries the dye throughout/

throughout the body and, as the diffusion occurs from the vessels into the tissues, the colouration of the plasma gradually diminishes. At the same time, the dye content of the blood plasma is being lowered by excretion of the dye through the kidney and intestine, so that eventually the concentration in the plasma falls below that of the tissues which then commence to yield up their dyestuff again to the plasma.

Decolourisation of the animal goes on slowly in this way and traces of dye can be demonstrated in the plasma or serum of animals for comparatively long periods after the administration of the dye.

The urine of animals stained with trypan blue contains a considerable amount of the dye. In the early stages of excretion, i.e., within a few hours of administration, the kidney excretes chiefly a red component of the dye - probably an impurity - so that the urine is purplish in colour. After twenty-four hours, however, the red dye appears to be entirely excreted, and the urine becomes pure blue and finally, as the concentration of dye falls, greenish blue. The intestinal contents are also stained with the dye, which appears to be excreted partly by the large intestine.

The cerebro-spinal fluid, in moderate degrees of vital staining, is quite clear and dye-free but, under very heavy/

heavy dosage, a small amount of dye seems to escape into the cerebrospinal fluid, which becomes very pale blue in colour (Goldmann, 1909, 1913). It is difficult to understand why the vascular capillary endothelium, which is elsewhere freely permeable to dyes of the trypan blue class, should appear to prevent almost completely diffusion of the dye through the vessel walls, and so into the Virchow-Robin spaces and the cerebrospinal fluid. In the choroid plexus, however, the cubical lining cells become quite deeply stained with fine uniform granules. Perhaps the lack of colouration of the cerebrospinal fluid depends on the total lack of affinity of healthy nervous tissue for the vital stains, for the brain and cord always remain free from dye when in a healthy state, though areas of damage, e.g., softening, inflammation, &c., become deeply stained. Certainly it is striking that although the blood plasma is kept deeply coloured with the dye over a period of many days, the colour of the cerebrospinal fluid is very much less intense at all times than that of the plasma.

I have observed, however, that in all species of animals studied and with all the dyes which produce generalised vital staining, there is a small area around the tuber cinereum which becomes diffusely stained with the dye. In a trypan blue animal there is thus a bluish halo around the base of insertion of the hypophyseal stalk, but nothing has been found microscopically to account for this staining, which appears to be a diffuse/

diffuse colouration of the nervous tissue and is not referable to intracellular granules. I have observed this diffuse blue colouration in thick sections but am unable to offer any satisfactory explanation of its presence, unless it be that the diffuse staining of the nervous tissue in this situation is related in some way to the absorption of the secretion of the pituitary gland. It is generally supposed that the presence of colloid droplets in the hypophysis and tuber cinereum represents the absorption of some component or secretion elaborated by the pars intermedia of the pituitary, which passes outward in this way to be absorbed into the third ventricle. The pituitary gland itself is found to be of pale blue colour, the anterior lobe being more deeply pigmented than the posterior. The pineal body also is usually stained a similar blue. The soft membranes of the brain are also of pale blue colour. The dura is more deeply coloured than the pia arachnoid, and it is noticeable that the dura is generally more deeply stained where the cranial nerves pass through the membrane, especially over the olfactory lobes and around the Gasserian ganglion. The above description of the lack of affinity of the central nervous system for vital stains holds only for the healthy brain tissue, and areas of nervous tissue damaged by softening or inflammatory changes become quite definitely stained. (Macklin and Macklin, 1920, McCurdy and Evans, 1912). Recently Siengalewicz (1925) has shown that general/

general damage to the nervous tissue, e.g., in poisoning with carbon monoxide or with salvarsan leads to quite marked staining of the damaged areas by trypan blue.

The thyroid gland has generally a pale blue shade superimposed on its natural reddish translucent colour. The thymus gland is very pale blue and contrasts sharply with the much more deeply stained mediastinal lymph nodes.

Granular Vital Staining of Cells.

It is to be noted that with all true soluble vital stains, such as trypan blue or isamine blue, a considerable interval elapses between the introduction of the dye into the tissues and its appearance within cells in the form of "vacuoles" and granules. This period varies greatly with different dyes, and is shorter with the dyes of group (a) than with those of group (b) (See p. 9). The former require about three hours or more to become visible within the cells, while the latter do not appear until after about 6-8 hours. During this lag period the cells are bathed in tissue fluids impregnated with the dye, which is presumably penetrating the cell membrane and gaining entrance to the cytoplasm. The mechanism by which concentration of the dye is effected is at present unknown, but as has been previously pointed out, it/

it is definitely dependent on the vitality of the cells and cannot be reproduced after death.

Examination of the subcutaneous tissues of a mouse by the methods previously described, twenty-four hours after a single intravenous or intraperitoneal injection of trypan blue to avoid local reaction, reveals a picture similar to that which has been described above in the case of supravital staining with neutral red. In addition the elastic fibres may be seen to be stained of a pale blue colour: this phenomenon is much more marked at shorter intervals after the injection of dye, e.g., five hours, and by the end of twenty-four hours has faded considerably. Histiocytes are readily identified by their numerous large and small blue vacuoles varying in number from a dozen up to a hundred or more in each cell (fig. 3). Some of the vacuoles are of uniform pale blue colour, while others contain more clearly defined deep blue granules, which may sometimes be found in active Brownian movement within the vacuoles and sometimes appear to be adherent to their walls. The intervacuolar cytoplasm and the nucleus are quite uncoloured. The fibrocytes contain a few fine bluish granules usually in the vicinity of the nucleus. (fig.4b) Here and there smaller round or oval cells containing scanty vacuoles and granules of varying size are found; these are probably to be regarded as immature cells.

As/

As an aid to the differentiation ofcell types one may superimpose staining with neutral red and janus green upon cells already stained with the acid dis-azo dyes. The vitally stained vacuoles and granules of both histiocytes and fibrocytes become deeply tinged with the neutral red and frequently the latter obscures the blue dye (fig. 5). Since in most cases the neutral red is stored in the same granules and vacuoles as the previously administered trypan blue, it is clear that the acid dis-azo dye has been dealt with by the "segregation apparatus" which has in many cases become greatly increased in size.

In addition cells are frequently encountered which exhibit a considerable number of neutral red granules and vacuoles in only a few of which trypan blue is present. Some of these cells are clearly histiocytes whose segregation apparatus, though fully displayed by neutral red, contains practically no trypan blue; others morphologically resemble fibrocytes in which the "segregation apparatus" has become much more extensive than usual.(fig.5e).

In my experience the study of the mitochondria in vitally stained preparations has proved exceedingly difficult, especially when the cells contain a blue vital dye, and I have not been able to satisfy myself that these cytoplasmic elements can be distinguished with sufficient sharpness to justify/

justify their use as a criterion for separating the different types of cells vitally stained with the acid dis-azo dyes.

Evans and Scott (1921) state that the difficulty of demonstrating mitochondria in cells loaded with vital stains may be surmounted by using janus green in stronger concentrations, and they consider that the difficulty experienced by other workers is due to the presence of free acid dis-azo dye which, they state, combines with the basic janus green. In my experience, however, it has been impossible to obtain satisfactory demonstration of mitochondria in vitally stained histiocytes or fibrocytes. Even in cells vitally stained with vital new red which affords a sufficient contrast in colour to the janus green, the mitochondria have been inconspicuous in the high dilutions found best for normal tissues. By using stronger solutions (1-10,000 or 1-5,000) bluish green granules appear within the cells, but I have been unable to satisfy myself that their morphology justified the designation of mitochondria: they might well belong to the segregation apparatus, the smallest granules of which also become stained in these strengths of janus green. One must conclude, therefore, that even with the aid of supravital staining accurate classification of individual cells is frequently impossible and the presence in considerable number of cells of intermediate morphological characters must be recognised. Further since most research in pathological histology requires/

requires fixed and sectioned material, the criteria furnished by supravital staining cannot always be applied.

The maximum intensity of intracellular staining is reached about forty-eight hours after a single injection of trypan blue. At this time the blood plasma is of deep blue colour, but a state of equilibrium appears to have been reached and no further intensification of the intracellular staining occurs unless another dose of stain be administered. The staining persists with not appreciably diminished intensity for a further period of about forty-eight hours, after which gradual fading of the granules occurs, but decolourisation is not complete for some weeks. It is thus clear that the highest degrees of intracellular dye storage cannot be attained by a single injection even by raising the dose to the limit of tolerance.

It is possible, however, to give without ill effect a second injection of the dye in the same dose ninety-six hours after the first. This results in a considerable increase in the number and size of the dye deposits and the granules and vacuoles become of deeper hue. By frequently repeated injections over a considerable period it is possible to obtain very much larger and more intense intracellular dye deposits than after several injections. By administering a second dye of a different colour it is clearly shown that many/

many of the vitally stained cells are capable of absorbing and storing a further quantity of dye, and in addition cells containing only the second dye are found.

After repeated intraperitoneal injections of trypan blue, to avoid local reaction in the subcutaneous tissues, the number and size of the vacuoles in both types of cell are increased. The vacuoles of the histiocytes exhibit great variation in size; some may be as large as the nucleus, while others are minute. Their contents vary considerably in tint, some are of uniform blue colour, but others contain irregular and very intensely coloured granular masses of much deeper shade than the vacuolar fluid with which they are surrounded (fig. 3).

If the dye be injected into the subcutaneous tissue either in a single large amount or in repeated smaller doses, the site of injection is more deeply stained than parts to which the dye has spread slowly by diffusion. At the site of injection the histiocytes contain enormous globular vacuoles and granules of ingested dye, and the fibrocytes also come to contain a greatly increased amount of vital stain (fig. 6, 8). Sharp differentiation of histiocytes and fibrocytes becomes more difficult and the number of intermediate cell types and immature cells increases greatly. Even rapidly absorbed dyes such as trypan blue produce a well/

well marked cellular reaction at the site of injection, and many new "polyblastic" cells are formed in response to the mild aseptic inflammation induced by the presence of the dye.

When dyes of the slowly absorbed type, e.g., isamine blue or diamine fast scarlet, are introduced locally into the tissues they set up a more pronounced local irritation, with some oedema and more marked cellular reaction. With such dyes, the histiocytes at the site of inoculation present extreme degrees of dye storage (figs. 4, 9, 100), while the typical fibrocytes also contain numerous dye granules of relatively uniform small size. Smaller polyblastic cells of all gradations are very numerous, but it is noteworthy that mitotic figures are practically absent, and it must be assumed that the polyblasts develop from small unstained cells which have migrated into the part. Intermediate cell forms are often abundant, and thus accurate classification of individual cells is frequently impossible. Many of these "unclassifiable" cells are probably fibrocytes which have taken up an unusually large amount of the vital stain (fig. 9c).

When generalised vital staining of high degree is attained by repeated injections of such dyes the histiocytes of areas which have received the dye by gradual diffusion contain a very large number of rather uniform small vacuoles often/

often of almost homogeneous tint, while the fibrocytes are found to possess much smaller and less deeply stained vacuoles. (fig. 8) Differentiation of cell types is more readily accomplished in such areas, and it is probably for this reason that Goldmann (1909) obtained such strikingly selective pictures with his original "pyrrhol blue." Nevertheless intermediate and young cells are still present and the difficulties of classification are considerable.

If an animal subjected to intra-vitam staining be allowed to live for a sufficiently long time after the cessation of dye administration, decolourisation of the segregation apparatus of its cells gradually occurs and eventually the dye is almost completely discharged from the tissues. This occurs much more rapidly with dyes of group (a) than with those of group (b). Frequently, however, even when the tissues appear completely decolourised to naked eye examination, a careful search with an oil immersion lens will reveal tiny dye granules in many cells. These small granules appear to be very insoluble and highly resistant to excretion. They occur more readily with some dyes than others, and are always present after administering vital new red. Evans and Scott (1921) state that they are situated chiefly within the fibrocytes "and constitute probably a lifelong pigmentation of the cell." I have found it by no means easy to decide to which type these cells/

cells belong. In my opinion both histiocytes and fibrocytes are involved (fig. 10), but it is not always possible to differentiate between them without the addition of neutral red supravitally; even with this aid difficulties are met with.

A similar difficulty is encountered in studying spreads of subcutaneous tissue from animals which have been treated some time previously with trypan blue. While the general picture corresponds to that described above, the cytoplasmic vacuoles of the histiocytes are paler in colour and the dye appears chiefly in the form of small granules or irregular masses in the walls of the vacuoles or free in the vacuolar fluid. In some cells one finds also small very deep blue granules of rhomboidal shape said to be of crystalline nature, apparently embedded in the cytoplasm; no "vacuole" is as a rule visible around them (fig.11). After application of neutral red some of the rhomboidal granules may become tinged with the brownish colour, showing that they lie in small vacuoles so closely applied to the granules as to be invisible without the aid of supravital staining, but others fail to take on the supravital stain. These minute granules appear to lie in the cytoplasm between the "vacuoles" of the segregation apparatus, but it is not clear exactly how they are formed, i.e., whether they have arisen in the cytoplasm or whether they have been formed within the vacuoles of the segregation apparatus/

apparatus, and have been extruded later into the inter-vacuolar cytoplasm. These "crystalline" dye deposits are most easily obtained by prolonged administration of very small doses of trypan blue, but they have also been observed in the cells of animals which have been rapidly stained by large doses and allowed to live for a prolonged period without further treatment. In all such cases, the administration of a second dye of a different colour, e.g., lithium carmine, leads to colouration of the segregation apparatus and the formation of granules and vacuoles stained only by the second dye. In some cells, both "crystalline" deposits of trypan blue and carmine-stained vacuoles are present but have remained entirely separate (fig. 11b,c); in others the trypan blue deposits appear to have become dissolved in the contents of the vacuoles, and granules of mixed colour are found (fig. 11d).

It is not clear whether these rhomboidal granules of trypan blue are actually true crystals as Evans and Scott claim, but we have observed the occurrence of frankly crystalline intracellular dye deposits with certain other dyes (fig. 12). In this case, however, we cannot be sure whether the crystals are formed extracellularly by precipitation of the dye solution in contact with the tissue fluids and have been subsequently ingested by the cells, or whether they are built up actually within the cytoplasm. In many of the cells containing the largest/

largest amount of dye crystals neutral red granules are exceedingly scanty; in other places where the dye is less abundant fibrocytes and histiocytes can be distinguished by supravital staining (fig. 13), but many cells of indeterminate nature are also to be found.

From the foregoing description of the appearances produced by intra-vitam staining, it is clear that we can distinguish the different varieties of connective tissue cells from one another only up to a point. Typical histiocytes and fibrocytes present a characteristic morphology when vitally stained, but it must be noted that individual cells may present intermediate characters and cannot always be put into one or other category. We would emphasize that this difficulty of differentiating cell types is one which cannot be wholly overcome, even with the help of the supravital technique. If vital staining be carried out over a prolonged period, e.g., with isamine blue, the histiocytes and fibrocytes both come to contain a very large amount of dye and are thus difficult to distinguish from one another, while at the same time new polyblastic cells are continually being formed in the connective tissues. If, on the other hand, rapid staining with trypan blue or vital new red be employed, it is impossible to decide whether certain of the indeterminate cells are fibrocytes with well developed vacuoles or whether they are relatively inactive histiocytes/

histiocytes such as those previously described whose fully formed "segregation apparatus" could be demonstrated with neutral red.

It is not clear why certain histiocytes should remain relatively unaffected by vital stains, but it seems reasonable to suppose that the cells may be in a resting or inactive phase following previous metabolic activity, or it may be that their vacuoles (segregation apparatus) are already fully occupied with other substances which are not readily demonstrable by histological means. In this connection it may be noted here that this explanation is supported by the results of double staining experiments, in which two dyes of a different colour are administered at suitable intervals. In such cases many cells are found to contain only the substance first injected, others contain both in varying proportions, while a third group contains only the substance last injected (fig. 108). These facts will be discussed further later, but they appear to confirm the hypothesis expressed above.

II. Distribution of vitally staining agents of suspensoid (particulate) nature.

It has been previously pointed out that suspensoid preparations, e.g., india ink or saccharated oxide of iron, possess no powers of diffusion and when injected intravenously are confined at first to the blood channels. Such substances are removed from the circulating blood and are stored within the reticulo-endothelial cells which form the lining of the blood channels of the liver, spleen, bone-marrow, and to a less extent of the suprarenal and pituitary glands. Since the injected substance is confined within the blood channels there is no deposition in the reticulo-endothelial cells of lymphatic glands, or in the histiocytes or fibrocytes of the general connective tissues of the body. This difference between the distribution of suspensoids and soluble dyes is a purely mechanical one, due to the separation of the former from the cells of lymphatic glands and subcutaneous tissue by the endothelium of the capillaries. Such substances injected locally into the tissues are stored by the histiocytes and fibrocytes in a manner similar to soluble dyes, and a certain amount of absorption occurs along the lymphatics thus leading to accumulation of the suspensoid in the reticulo-endothelial cells of the lymph gland draining the injected area. For this reason extravasation/

extravasation of the suspensoid into the perivascular tissues during intravenous injection must be avoided. There are, however, differences between the reaction of certain organs to soluble vital stains and their response to those of particulate nature. This is seen especially in the spleen, which is not a very active participant in vital staining with soluble dyes, but which filters out particulate matter with great rapidity and retains it for long periods. The kidneys, which are intensely stained with some soluble dyes owing to excretions of dye in the urine, are unaffected in this manner by suspensoids, but I have observed that there is a much greater deposition of india ink, sugar of iron, and other suspensoids in certain cells lining the blood capillaries of the kidney than I have ever observed with soluble dyes.

After intravenous injections of india ink the liver, spleen and bone-marrow are almost jet black in colour, but in the various animal species investigated no other organ shows any permanent or consistent evidence of ink retention. In some animals the lungs are grey or even black, especially when examined soon after injection, and the kidneys, suprarenals and pituitary are also greyish in colour. The distribution of india ink in the marrow corresponds to the more cellular zones, and this method has been used by Wislocki (1921) to demonstrate the distribution of cellular marrow in various animals. After bleaching and clearing of the bones by Spalteholz's/

Spalteholz's method (1914), the ink-storing cellular marrow stands out with great distinctness.

When suspensoid substances are stored within cells, the appearances are similar to those produced by the dyes previously described. The process of formation of the intracellular granules is analogous to what occurs with the soluble dyes; the minute particles of the suspensoid seem to adhere to the surface of the cells and at first the entire cytoplasm appears as if stippled, but within a few minutes small granules which must consist of a large number of the original minute particles, are found within the vacuoles of the segregation apparatus. As this process continues the granules increase in size and in opacity and finally the morphology of the vital staining in individual cells is identical with that seen after the acid dis-azo dyes.

It must be emphasized, however, that the formation of intracellular granules occurs much more rapidly with suspensoids than with soluble vital stains. Whereas the latter require several hours to become visible within the cells, the former appear to gain entrance to the cytoplasm within a few minutes. Further it is noteworthy that intracellular storage of india ink occurs much more rapidly in the Kupffer cells after intravenous injection than in the histiocytes after subcutaneous/

subcutaneous injection: as we have already stated, vitally staining elements in different situations exhibit a certain specialisation for dealing with substances of different types, and further examples of this will be dealt with later.

SUMMARY AND CONCLUSIONS.

Animals may be stained intravitam by the administration of suitable dyes and by relatively inert substances of particulate nature. The term vital staining is justified in so far as it is not possible to obtain similar results with dead or fixed tissues. A dye is said to be suitable for intra-vitam staining if it produces after local injection generalised staining of the animal's tissues together with the formation of intracellular coloured granules. Vitally stained animals suffer no serious ill effects, and behave practically like normal animals. After a considerable lapse of time such animals may become completely decolourised to naked eye examination, but traces of the dye previously administered may be found on microscopic examination of the tissues.

Vital staining may be accomplished by many dyes of widely different chemical constitution, and as yet no general law can be formulated correlating chemical constitution and physiological action. It is believed, however, that the chemical structure of the dye molecule plays an important part in determining its effectiveness as a vital stain; but this property may not be demonstrable unless the physical character of the solution, e.g., its capacity for mixing with the/

the tissue fluids, is such as to permit the dye to exercise its effect. Unsuitable physical characters may be modified by appropriate means, e.g., by sulphonation, but the position and character of other radicals in the dye molecule have an important influence both on the capacity of the dye for vital staining and on its toxicity.

The majority of the dyes used as vital stains belong to the acid dis-azo group, e.g., trypan blue, but certain tri-phenylmethane dyes are also employed, e.g., isamine blue. Such dyes vary considerably in the rapidity with which they produce generalised vital staining, but in all cases a period of several hours elapses before intracellular colouration is observed. Vital staining of a more restricted type may be accomplished by means of intravenous administration of certain relatively inert finely divided particulate substances, e.g., india ink or saccharated oxide of iron.

<u>Vital staining</u> with <u>acid dis-azo dyes</u> is to be distinguished from <u>supravital staining</u> of surviving cells or portions of tissue by the use of <u>basic dyes</u> such as neutral red. A special variety of supravital staining is the strikingly selective colouration of the mitochondria of living cells by janus green.

Both intra-vitam and supravital staining of cells (apart from mitochondria) are due to the concentration of the dye/

dye within certain vacuoles and granules in the cytoplasm - the so-called segregation apparatus, - and it is believed that the appearances which result are due chiefly to actual accumulation and segregation of dye substance within the cytoplasm. The dye is, however, bound in some way within the cells, and the granules cannot be regarded as simple aggregates of precipitated dye.

The capacity for storing the acid dis-azo dyes which act as vital stains is much more restricted than is the supravital reaction with neutral red. In this communication only the appearance and distribution of vitally staining cells in the subcutaneous areolar tissue has been considered in detail; the distribution of these cells throughout the animal body and their variation under different conditions and in different species will be described later.

In the subcutaneous areolar tissue the vitally staining cells fall into two main groups, (a) the histiocytes, which exhibit this property in marked degree, and (b) the fibrocytes, in which it is much less highly developed. While typical examples of these cells can be readily distinguished, numerous intermediate cell forms are encountered which cannot be classified satisfactorily by the criteria of vital and supra-vital staining. Some evidence has been presented to show that the capacity for vital staining is not a hard and fast/

fast attribute of certain cells alone, but is rather to be regarded as an indication of the functional activity of the cells at the time of examination, which may be modified under the influence of environmental conditions. Thus, cells which apparently correspond to histiocytes in every other respect, even in their supravital staining reaction with neutral red, may fail to take up more than a trace of the vital stain. It is believed that such cells may be in a resting phase or more probably that their "vacuoles" are already fully occupied with some other less readily demonstrable substance.

The local injection of vital stains _per se_ produces a definite cellular reaction with the production of many new cells of "polyblast" type. These primitive cells are at first unstained, but they rapidly develop their latent capacity for vital staining, and in a relatively short time become almost indistinguishable from the previously fully formed histiocytes. These points will be considered more fully in a later communication, but it seems desirable to emphasise at the outset that they represent difficulties which have been largely overlooked by writers who have applied intra-vitam staining to the problems of pathological histology.

II. The distribution of vitally stained cells in the blood and organs.

Blood Plasma.

Erythrocytes : (a) intravitam staining.
(b) supravital staining.

Leucocytes : (a) intravitam staining.
(b) supravital staining.

Liver.

Spleen.

Bone Marrow.

Lymphatic Glands.

Omentum and Mesenteries.

Suprarenal Glands.

Pituitary Gland.

Thyroid and Parathyroid Glands.

Testes.

Ovaries.

Uterus.

Pancreas and Salivary Glands.

Kidneys.

Alimentary System.

Muscular Tissues.

Bones.

Nervous System.

Discussion and Summary.

The general principles of intra-vitam staining have been defined (p. 76), and the methods by which it may be accomplished have been dewcribed. Attention was drawn to the different groups of vital stains and three main classes were distinguished: (a) rapidly absorbed dyes, e.g. trypan blue, vital new red, lithium carmine; (b) slowly absorbed dyes, e.g. isamine blue, diamine fast scarlet; (c) insoluble suspensoid preparations, e.g. india ink, saccharated oxide of iron.

In this section the distribution of vitally stained cells in the organs and tissues is described and attention is drawn to variations which exist between the organs of different animals in respect of their reaction to vital staining agents. In connection with each organ, unless otherwise stated, a description is first given of the appearances found in mice deeply stained with a relatively diffusible dye such as trypan blue. Any differences observed with other vital stains and in other animals are then mentioned. In general it may be said that the cells in question belong to the reticulo-endothelial system of Aschoff and Landau. Their scheme and the relation of the various cells to it are discussed at a later period (see p. 171).

BLOOD PLASMA.

The introduction of dyestuffs into the blood plasma has been used chiefly as a means of determining blood volume. This method was originated by Keith, Rowntree and Geraghty (1915), who injected vital red into the blood stream and estimated by colorimettic methods the degree of dilution of the dye. Dawson, Evans and Whipple (1920) investigated a large number of dyes from this point of view, and also in relation to their rate and mode of elimination. With reference to the use of dyes for this purpose it is significant that a very rapid disappearance of a portion of the injected dye has been recorded by most observers. For this reason estimations of blood volume made by this method differ from those otherwise obtained, e.g. by the use of Carbon monoxide. The fact that the dyes employed, e.g. brilliant vital red (C.I.No.456) act as vital stains (Smith, 1925) affords an explanation (see below).

In the present series of experiments this aspect of the use of vital dyes has not been studied. Several facts have, however, been observed which have an important bearing on the use of dyes as an aid to histological differentiation by vital staining. According to Permar (1920), after intravenous administration of certain vital stains, e.g. isamine blue, the dye remains in circulation in the blood plasma for only a few hours and is soon removed by the tissues/

tissues in which it accumulates progressively. This statement has unfortunately been widely accepted without verification. My own observations, however, shew that after administration of soluble vital stains of both group (a) and group (b) the blood plasma remains deeply coloured for several days. Further, the blood plasma continues to shew traces of the dyes until the animal has become completely decolourised. This has been tested by withdrawing at varying intervals the heart blood of stained animals into citrate saline and centrifuging the mixture. In this way the blood plasma has been found to be appreciably tinted with the dye even up to several weeks after the last of a series of injections. So long as dye is present in any considerable concentration in the circulation it is available for the staining of cells in a receptive state. We regard this as of very great importance in view of the opinion expressed earlier that vital staining is merely an expression of the physiological activity of the cell at the time of examination. It follows that if it is desired to study the participation of vitally stained cells in pathological processes, it is essential to allow such an interval to elapse between the last administration of dye and the commencement of the experiment as will ensure that only a negligible amount of dye is present in the blood plasma, otherwise vital staining may appear in previously unstained cells which have emigrated or proliferated under the influence of the pathological stimulus./

stimulus. The relationship between the dye content of the plasma and that of the vitally staining cells is thus of great importance in the interpretation of the results of the application of vital staining to pathological histology.

As a result of examination of the supernatant plasma after centrifuging the citrate blood mixture from a large number of animals the following conclusions have been drawn. Immediately after intravenous injection of solutions of dyes of group (a) rapid dilution occurs in the blood plasma and a relatively uniform mixture is attained after about 5 minutes. The concentration then falls slightly during the first hour owing to the passage of the dye into the tissue fluids and lymph, and owing to diffuse staining of certain elements such as elastic tissue and also by reason of excretion. Extensive elimination of the dyes commences soon afterwards but the rapidity and route of excretion depend on the nature of the dye, those of group (a) being excreted chiefly in the urine and only slightly in the faeces, while those of group (b) are excreted chiefly by the latter route, the urine becoming only faintly coloured (see pp. 55-57). During the next 24-48 hours the maximum concentration of dye within the cells is gradually attained and this comes about sooner with the more rapidly diffusible and quickly staining dyes of group (a) than with those of group (b). After subcutaneous or intra-peritoneal injection, the maximum concentration in the blood is reached more/

more slowly and is not as great as after intravenous injection of a similar dose, since much of the dye is retained locally in the tissues, but since these routes establish a depôt, a more steady concentration of dye in the plasma over a more prolonged period is thus secured. This is of great importance in experimental work when it is desired to secure vital staining of the reacting cells in inflammatory lesions.

A further stage is soon reached when the amount of dye in the plasma and tissue fluids becomes lowered owing to excretion; then the vitally stained cells slowly give up their content of dye to the tissue fluids and plasma until almost complete decolorisation is attained. As has been previously noted, however, certain dyes, e.g. trypan blue and vital new red, give rise to small intensely coloured intracellular deposits which are very resistant to decolourisation; these have been interpreted by Schulemann (1917) and by Evans and Scott (1921) as actual dye crystals (see p. 68).

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BLOOD.

Erythrocytes.

Intra-vitam staining. The red corpuscles of the blood show no alteration beyond becoming faintly and diffusely tinged with dye when there is a large amount of the vital stain circulating in the plasma. This is naturally more marked with vital stains of group (a) than with those of group (b). This diffuse staining is best seen by examining a drop of fresh blood and does not show in dried and stained films. It is also seen in sections of tissues fixed while the plasma contains a large amount of dye. As the dye in the plasma decreases, the diffuse staining of the red cells disappears.

Supravital staining of the erythrocytes is of considerable importance, since by its aid one can distinguish young non-nucleated red cells or "reticulocytes". For this purpose brilliant cresyl blue, first used by Cesaris-Demel (1906), is now extensively employed in clinical work. Supravital staining of the erythrocytes may be effected by mixing the blood with isotonic saline containing the dye in a strength of 1-1000 or more, but the most satisfactory method is to add a small drop of fresh blood to a slide which has been coated with a thin film of dye as already described (see p. 37). Israel & Pappenheim (1896) using neutral red were the first to describe in red cells small "vitally staining" granules which they identified at that

time with basophilic punctation. Horseley (1897) then used methylene blue for the purpose. Cesaris-Demel (1907) investigated a number of basic dyes and found that brilliant cresyl blue produced more rapid and intense staining in the red cells than any other dye. He originally observed what are now known as "reticulocytes" and described in detail the "granulo-filamentous substance", showing clearly that reticulocytes were young red cells. It is unnecessary to describe in detail the numerous granular and other bodies which have been observed in red cells, for a full discussion of which the papers of Cesaris-Demel, Ferrata and Pappenheim (1907) should be consulted.

In the present work attention has been paid chiefly to the results of supravital staining with brilliant cresyl blue, neutral red and janus green. In peripheral blood of the normal human adult reticulocytes do not amount to more than 1 per cent. of the red cells though in various pathological conditions the proportion may be greatly increased. In mice the proportion is somewhat higher, often about 5 per cent. although in some animals even more occur. In newly born animals the proportion of reticulocytes is much greater (Seyfarth & Jurgens, 1928) and is commonly as high as 50 per cent. in mice.

The name reticulocyte is applied to red corpuscles which/

which on supravital staining exhibit what appears to be an intensely stained intracorpuscular <u>skein</u> or <u>reticulum</u>.(figs.14-16) Such corpuscles are best studied in animals in which anaemia has been produced either by simple bleeding or, better, by the use of a haemolytic serum, as in these a high proportion of the circulating erythrocytes may be reticulocytes. They are frequently somewhat larger than normal red cells and usually shew polychromasia, but polychromatophilic red cells are not invariably reticulocytes. The reticulum may occupy the centre of the corpuscle or may be at one side. Occasionally we have seen the reticulum in process of extrusion from the corpuscles and free reticula may be found when such cells are abundant in the blood. In some cells a complicated network with nodal thickenings is present; in others only a few thread-like bodies or coarse granules may be found. The reticulum within the corpuscles is faintly shown by dark ground illumination, but it becomes more distinct by this method after staining with brilliant cresyl blue. The reticulum cannot usually be demonstrated in dried smears stained by any of the Romanowsky methods, but we have occasionally observed macrocytes in which a faint suggestion of an unstained network could be seen as a paler structure within the slightly polychromatophilic cell. Morphologically, however, this rarely corresponds to the reticulum shown by the supravital method. If the reticulum be first stained with brilliant cresyl blue, and the film be then allowed to/

to dry the reticulocytes remain clearly stained after subsequent treatment with stains such as Leishman's. On the other hand, fixation of unstained red cells in the wet state by osmic acid or formaldehyde leads to complete absence of reticulum staining on subsequently applying brilliant cresyl blue.

In neutral red-janus green preparations there is rarely much staining of the reticulum with the weak concentrations of the dyes usually employed. In such preparations the reticulum may become stained with janus green some time after the mitochondria and segregation apparatus of the white corpuscles. With higher concentrations staining occurs more rapidly and both neutral red and janus green are capable of showing up the skein-like structure. When stained with janus green it appears coarser than with brilliant cresyl blue or neutral red. In some corpuscles, small granular coccus-like bodies have been observed to stain with janus green; these are similar to the structures figured by Cunningham, Doan and Sabin (1925) as mitochondria in erythrocytes: Key (1921), however, denies that mitochondria are ever found in non-nucleated erythrocytes, and it can be stated that such granular bodies may appear to become joined by thread-like structures as the staining progresses. Key (1921) has studied the nature of the reticulum and has investigated the differences in its form as/

as shewn by various basic dyes. He concludes that the skein-like structure is due to a precipitation of the polychromatophilic substance of the immature cells by the dye. While this is possible it cannot yet be regarded as definitely settled, and the nature of the reticular substance is at present unknown.

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Leucocytes.

Intra-vitam staining. In a mouse deeply stained by trypan blue the white cells of the peripheral blood show no constant changes, either quantitative or qualitative, at any stage which can be attributed to the staining. Examination of a large number of fresh and stained films fails to reveal any vitally stained cells in the peripheral blood. In the internal organs of such animals, however, one can generally find scanty mononuclear leucocytes which contain dye granules. These granules may constitute the sole colouration of the vitally stained cells or the latter may occasionally contain in addition granules of altered blood pigment. These vitally stained cells can be found fairly readily in the blood of the splenic veins and in the sinusoids of the spleen. They also occur in the branches of the portal vein, in the sinusoids of the liver and in the branches of the hepatic veins. Similar cells occur in the sinusoids of the bone marrow but are less numerous there. They can be found also in the right side of the heart, and in the pulmonary artery and capillaries. Most authors (Kiyono 1914) agree that they are almost entirely absent from the left side of the heart and from the peripheral circulation, and the general opinion is that they are removed from the circulation by the lungs. Masugi (1927) states that they are equally scanty in both sides of the heart and explains Simpson's (1922) observation of "showers" of macrophages/

macrophages in blood from the right ventricle as due to contamination by pericardial fluid of the blood taken repeatedly by heart puncture. Morphologically such scanty vitally stained cells as are met with in the blood are round or oval and of varying size, being generally 15-20 μ in diameter but may be even larger than this. The nucleus is generally excentric and is round, oval or reniform with the indentation toward the centre of the cell. The bulk of the ingested dye particles usually are aggregated in the centre of the cell though some may also be scattered throughout the cytoplasm. In some cells there are only one or two small granules in the nuclear indentation, in others they are fairly numerous and of irregular size and shape; sometimes, however, the cytoplasm may be so heavily loaded with vitally stained granules and other phagocytosed debris that the cell cytoplasm is no longer distinguishable (fig. 20). The nuclei possess a well-developed chromatic reticulum with coarse knots and a definite nuclear membrane, but stain less deeply than those of the lymphocytes. The protoplasm is generally weakly basophile, but this is difficult to confirm in blood smears from the veins of internal organs, as the presence of particles of dye in the cytoplasm appears to alter the reaction to the Romanowsky stains; intracellular granules of the acid dyes used as vital stains are themselves capable/

capable of retaining firmly the basic dye, thus giving the whole cytoplasm a dark blue colour. It is important, moreover, to observe that no matter how intense or prolonged has been the vital staining, the stained cells are never more than a small proportion of the blood mononuclears.

If a suspensoid preparation such as india ink be injected intravenously the effect on the circulating blood cells differs from that described above. Such substances are quickly taken up by the leucocytes in the blood stream and it is usual to find that a large proportion of the mononuclear cells contains granules of ink. It is also quite common to find polymorph leucocytes with ingested granules of ink, but McJunkin (1919) states that this can be avoided by the addition of a suitable quantity of sodium citrate to the carbon suspension. The proportion of vitally stained cells varies in different parts of the vascular system, and is lowest in the peripheral blood. Very soon after injection the number of leucocytes in the peripheral blood falls, but the leucopenia is later followed by a transient leucocytosis. We have observed that phagocytic mononuclears are most abundant in the blood during the first 24 hours, and Nagao (1920) states that they reach a maximum about 12 hours after injection. No exact study of the rate of appearance or disappearance of such cells in the peripheral blood has been made, but they have been observed at periods ranging from ½ hour to 48 hours after a single injection/

injection, and probably they are most numerous in the blood between 5 and 12 hours after the injection. Carbon-containing leucocytes, both polymorphs and mononuclears, are more numerous in the blood of the internal organs, especially the lungs but also in the liver and spleen and main venous channels; this is especially the case after a large injection of ink. (see p. 112). Their distribution is irregular, e.g. some radicals of the pulmonary or portal vein may show numerous vitally stained cells, while in others they are scanty, and this variation is even more pronounced in the interlobular veins and also in the main branches of the hepatic veins. In some hepatic veins every non-lymphocytic cell contains carbon granules, while in others such cells are much less numerous. This condition does not persist, however, and there is little doubt that the majority of these cells either liberate their pigment into the circulating blood, whence it is removed by the agency of the liver, spleen, marrow, etc., or they are themselves destroyed, probably in the spleen pulp. From 24 hours up to many weeks one may find in the blood of internal organs occasional ink-containing mononuclears; but these cells are larger and show numerous coarse intensely black granules of varying size which may obscure the nucleus (fig. 22). Therefore it is unlikely that they represent the blood leucocytes which took up ink in the first instance. Probably they correspond to the vitally stained mononuclears which appear in the internal veins after prolonged treatment with/

with trypan blue and other soluble dyes.

It is important to observe that the capacity to store suspensoid substances is possessed by polymorphonuclear leucocytes and the normal monocytes in conditions where a large amount of the substance is suddenly introduced. It seems that every possible cell is then called upon to remove the substance from the blood stream and that when this is accomplished they accumulate especially in the pulmonary capillaries, and to a less extent in the veins and sinusoids of the liver and other viscera. Such cells may be so numerous in the pulmonary capillaries as to suggest that they are temporarily retained in some way in this situation. Later they may pass into the air sacs and so be lost in the sputum, or along the lymphatics to accumulate in the peribronchial lymph nodes and mediastinal glands; but probably the majority subsequently pass on in the circulation to be destroyed by the spleen. A similar accumulation of leucocytes in the lungs was noted by Andrewes (1910) and Wright (1927) in the clearing of the blood stream of organisms. It is almost certain that this phenomenon is responsible for the remarkable claims of Oeller (1923,1925,) who thought that the endothelium of the pulmonary capillaries gave rise to monocytes and to granular leucocytes.

Foot (1920) formerly considered the ink-containing cells in the pulmonary capillaries as phagocytic interalveolar capillary endothelium; but he now regards them as monocytes flattened along the capillary walls. The interpretation of these appearances is intimately associated with the problem of the phagocytic

activity of non-specialised blood capillary endothelium. Eliot (1926) has recently expressed the view that the vitally staining reticulo-endothelial cells of the various organs are monocytes which have settled in these situations from the circulating blood. While this statement cannot be accepted for reasons discussed later (p. 106) the possibility must be borne in mind in considering the regeneration of reticulo-endothelial cells after vital staining.

When saccharated oxide of iron is injected intravenously, the effect on the circulating leucocytes is similar to that of india ink, but is more pronounced. Nearly all the leucocytes of every kind give a well-marked prussian blue reaction similar to that described in the Kupffer cells (see p. 114). The iron reaction disappears first from the polymorphs and lymphocytes, but persists in some of the monocytes for 24-48 hours, though such cells are then much less numerous than earlier. After 24 hours the diffuse character of the staining changes, as in phagocytic cells elsewhere, and granules giving a more intense iron reaction are found. Temporary accumulation of the iron-containing leucocytes in the lungs and other internal organs occurs as after india ink and the cells appear to undergo the subsequent changes already desdribed.

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Supravital Staining. Most recent studies on the white blood cells have included supravital staining with neutral red and janus green. For this purpose the dry dye film method (see p. 37) is especially suitable. The various types of leucocytes react to the supravital stains in a fairly characteristic manner and can be readily distinguished from one another. In the granular cell series the granules of the neutrophile and eosinophile polymorphs and myelocytes stain fairly readily with neutral red, while in myeloblasts and myelocytes granular and rod-like mitochondria can be stained with janus green. The latter are rather larger than the immature specific granules, but they become smaller and less numerous as the cells mature. In agreement with Sabin and her co-workers (1924) I have not been able to demonstrate their presence in fully differentiated polymorphs. In addition to the staining of the specific granules, neutrophile polymorphs occasionally exhibit one or two neutral red vacuoles of larger size, but these are not invariably present and no such vacuoles have been observed in eosinophiles. Most workers agree that mitochondria are less readily demonstrated in the eosinophile series than in other leucocytes (Cowdry 1914). The lymphocytes exhibit only scanty neutral red vacuoles of very small size, but contain very distinct granular and short plump rod-like mitochondria. These are larger and more numerous in the larger lymphocytes and tend to lie especially in/

in the broader cytoplasmic zone opposite the slight nuclear indentation. It cannot be stated definitely whether or not the neutral red granules in the lymphocytes bear any relation to the azurophile granulation of Michaelis and Wolff (1902). The reactions of the monocytes (large mononuclears and transitionals) have been investigated by many recent workers (Sabin, Cunningham and Doan, 1925; Masugi, 1927). These cells take up the supravital stain in a fairly characteristic fashion but considerable variations are met with. Sabin and her co-workers state that the characteristic feature of the monocyte is the appearance of small granules grouped together in the form of a rosette around the centrosphere lying in the cytoplasmic zone opposite the nuclear indentation; they have a salmon pink colour quite distinct from the usual orange red of the segregation apparatus. The majority of the mitochondria are described as lying around the rosette of neutral red granules. While monocytes presenting this type of supravital staining can be readily found in normal blood, the majority of these cells in my experience do not exhibit such a regular cytoplasmic pattern; the granules do not always form a rosette but may be scattered through the cytoplasm, and their colour does not always present any noteworthy difference from that of the granules of the lymphocytes and other cells (fig. 1 e). Masugi (1927) also has noted that the characteristic morphology described by Sabin is not always maintained.

The/

The relationship of monocytes to histiocytes has been much discussed. As mentioned in connection with intravitam staining, histiocytes are only rarely present in the peripheral blood. Though not uncommon in the vessels of internal organs these cells are relatively scanty in normal animals, but their number is increased after repeated injections of vital stains and other foreign substances. The blood histiocytes are generally larger than monocytes, but cells otherwise corresponding to typical monocytes are present which give on supravital staining the characteristic reactions of histiocytes elsewhere. Mitochondria are usually only demonstrable in these smaller cells as previously noted. In vitally stained animals such of the large mononuclear cells as contain the dye give also the typical histiocytic reaction on supra-vital staining with neutral red; it must be noted, however, that there are present in addition some mononuclear cells giving the histiocytic reaction with neutral red but without vitally stained granules. Thus it may be concluded that the large mononuclears fall into two groups. Difficulties arise in connectioh with cells which do not exhibit any characteristic cytoplasmic pattern, and whose neutral red granules are larger and more irregular in size than those of typical monocytes. It is impossible to decide whether such a cell is to be classified as a monocyte with unusually large granules - "stimulated monocyte" of Sabin - or a small histiocyte. As has been stated the grouping/

grouping and colour of the granules are not a reliable criterion for separating those cells. It is therefore difficult to understand how many writers conclude that histiocytes and monocytes can be clearly separated from one another in the blood stream, while expressing doubt as to the possibility of distinguishing them in the tissues or in serous exudates (Witts,1928). It seems that the deciding factor in classifying many such cells has been merely the fact that since they are in the blood, therefore they are presumed to be monocytes; whereas the nature of cells of identical morphology and staining reactions in the tissues or in serous exudates would be regarded with doubt. Sabin, Doan and Cunningham (1925) appear to have realised this when they state in reference to the separation of clasmatocytes and monocytes that "upon further stimulation the two classes of cells become indistinguishable." This statement in a paper attempting to prove that these cell groups are fundamentally different in origin and development seems to have been generally overlooked. As regards the origin of the blood histiocytes, my experiments support Kiyono's view that these cells are derived from the reticulo-endothelial cells of the organs and tissues particularly the liver, spleen, and bone-marrow.

In general it may be said that neither intra-vitam

nor/

nor supravital staining has yielded any decisive evidence regarding the origin of the monocytes. Cunningham, Sabin and Doan (1925) claim to have shown by means of supravital staining that the monocytes are derived from reticulum cells in the spleen and marrow, but may also be formed from similar primitive mesenchyme cells scattered throughout the connective tissues. Lang (1926) and Stilwell (1926) could find no evidence in support of the endothelial origin of the monocytes from experimental injection of india ink as claimed by McJunkin (1919,1920). Simpson (1922) showed that repeated injections of colloidal substances of different types, e.g. dyes and suspensoid preparations, led to showers of mononuclear cells, some apparently monocytes, others blood histiocytes, and concluded that while these two types of cell are not identical they appear to be closely related. Masugi (1927) and Bungeler (1927) agree that these cells are not identical but consider that both are derived from reticulo-endothelial elements; the former states further that monocytes may become transformed into histiocytes. Maximow (1928) on the other hand held that monocytes are produced by the maturation and transformation of large lymphocytes in the blood stream, though this may occur more extensively in the vessels of certain organs, e.g. marrow, spleen. Since in Maximow's opinion these cells also give rise to histiocytes, the numerous transition forms met with in conditions of clinical and experimental mononucleosis may be readily explained. My observations on vital and supravital staining strongly support Maximow's view (1928) that the monocytes are to be regarded

as a phase of the development of certain lymphoid cells which is normally passed in the blood stream, although cells of similar morphology and staining reactions are met with in the organs and tissues especially as a result of stimulation. The monocytes may in conditions of stimulation develop further, and act as macrophages; they are then indistinguishable from macrophages which have been derived from histiocytes, or have developed directly from more primitive undifferentiated cells. As will be shown in a later communication, there is clear evidence that in conditions of stimulation undifferentiated primitive cells situated in the connective tissues especially in the neighbourhood of the serous cavities (see p. 288) may develop into free macrophages directly or may first pass through a monocyte-like stage. In the end the macrophages from different sources are indistinguishable from one another.

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LIVER.

Of all the organs in the body the liver is the most effective in removing from the blood stream the various agents used as vital stains. This activity is due chiefly to the endothelium and stellate cells of the sinusoids and not to the cells of the parenchyma. In normal animals the endothelium lining the capillaries is seen as a layer of flattened cells with small darkly staining nuclei. Here and there larger stellate cells with paler nuclei are present which float freely in the slowly flowing blood of the sinusoids, attached to the walls by one or two long processes. These are the true "sternzellen" of von Kupffer (1876) and they appear to be produced by division of the endothelial cells at right angles to the capillary wall, so that one cell remains in the wall in a quiescent condition, while the other floats in the current. (Sabin,1920, Pratt, 1927). We have observed that both flattened endothelial cells and floating Kupffer cells take up vital stains, but the latter are more active in this respect. As in the histiocytes of areolar tissue, the accumulation of dye within the cytoplasm does not become visible for several hours after a single injection of trypan blue by subcutaneous, intraperitoneal or intravenous route; it is very scanty after 12 hours, but after 24 hours numerous intracellular granules and vacuoles are found to show the vital stain. The stained endothelial cells become swollen, and the stimulus of the dye appears to give rise to an increased number of true Kupffer cells which project into/

into the lumen of the sinusoids. It is important to note that many of the flattened cells also contain dye granules.

The distribution of the vital staining throughout the lobules is uniform, and it can not be said that the sinusoidal cells in any particular zone of the lobule exhibit dye granulation first. The sinusoidal cells are not, however, all equally active, and one may observe neighbouring cells which exhibit extreme variation in their dye content. That this is not attributable to the plane of section is shown by the examination of fragments of fresh teased or crushed liver, where the above appearances were first observed. In all cases one can find along the sinusoidal walls flattened endothelial cells which have failed to take up or at least to concentrate within their cytoplasm any trace of the vital stain, while neighbouring cells contain considerable amounts. In favourable cases one may observe that there are processes extending from the vitally stained endothelial cells into crevices between the hepatic cells; sometimes these appear to extend as far as the bile canaliculi, but no extrusion of dye into these channels seems to take place. After repeated injections of dye the amount taken up by the Kupffer cells and endothelial cells increases markedly and the latter become more swollen and project further into the lumen of the vessels. With very high dosage it is not uncommon to find the heavily stained cells becoming desquamated into the blood stream where they may be observed free in the branches of the hepatic vein (fig. 20). These desquamated endothelial cells have been termed "blood histiocytes" (Kiyono,

1914; Wentzlaff, 1924).

In rabbits irregularity in the degree of staining of individual cells is much more pronounced and it is less easy than in mice to obtain a relatively uniform staining of the Kupffer cells. With repeated doses of trypan blue some of the lining cells of the liver sinusoids of the rabbit become markedly swollen and small giant cells with several nuclei may be formed. The guinea-pig occupies an intermediate position between the mouse and the rabbit, the appearances resembling more closely those of the rabbit than of the mouse.

It has been pointed out that if an animal be subjected to intense vital staining, some of the stained cells finally desquamate and are carried off in the blood to the right side of the heart and lungs. Within a few days, others leave their situation in the sinusoid wall and migrate chiefly in three directions, (a) into the periportal connective tissue, (b) toward the central vein, which some of them enter, (fig. 20) or (c) to situations beneath the capsule of the liver, where they form small foci of deeply stained cells. Small groups of deeply stained cells may be found also in the sinusoid walls at various points. Probably these cells are on their way to the lymphatics, into which some of them migrate and so arrive at the regional lymph nodes. This 'cleansing' process is of very great importance and it is very striking to find how rapidly it occurs.

As a result of this redistribution, the original uniform arrangement/

arrangement of vitally stained cells in the liver lobules is lost and after a few days such cells are most numerous toward the centre and the periphery of the lobules, while the midzone appears less uniformly stained than at an earlier stage. New endothelial cells free from vital stain appear in the place of those which have migrated and the picture becomes entirely changed.

The newly developed endothelial cells are quite free from vital stain, and may be demonstrated by the injection of a small dose of a suitable contrasting dye, e.g. vital new red or lithium carmine. The origin of these new cells is uncertain. If they arose from proliferation of the pre-existing vitally stained cells, one would expect them to show evidence of the stain first administered, but in a large number of observations we have repeatedly obtained evidence that these new cells appear without any trace of vital staining in their cytoplasm and presumably they develop from unstained progenitors. It seems probable that they are derived from the unstained endothelial cells which have been previously described as remaining unaffected by the vital staining. It has been suggested by Eliot (1926) that the reticulo-endothelial cells of the various organs are mononuclear cells from the blood which adhere to the capillary walls and settle there at least temporarily. Foot (1927) has stated that he formerly mistook such flattened phagocytic monocytes for endothelial cells. The views of these writers illustrate the difficulty in/

in deciding how such cells may be formed, but in our opinion such views cannot be clearly substantiated. There is no satisfactory evidence to prove that such processes are concerned in the regeneration of the hepatic endothelial cells though the possibility cannot be denied. It seems more reasonable to suppose that regeneration occurs from the unstained cells to which we have drawn attention. The existence of such non-reacting cells and the rapidity with which regeneration occurs are factors of great importance in connection with the so-called blockade of the reticulo-endothelial system, which will be referred to more fully later. Dyes of group (b) produce essentially similar results if the vital staining be carried on for a sufficiently long period, but after a single injection the amount of dye absorbed is so small that only traces of staining are found in the Kupffer cells.

The healthy hepatic parenchyma cells of the mouse have never in any circumstances been found to exhibit staining with any of the vital dyes, and we have not observed any evidence of excretion of trypan blue in the bile of the mouse, rabbit or guinea-pig. Goldmann (1909) states that trypan blue can be readily observed in the bile of injected animals by dropping the contents of the gall-bladder on to white filter paper, but our experience has not confirmed this finding with the samples of dye used. With none of the other dyes employed has staining of the bile been observed. In the hepatic cells of the rabbit and guinea-pig, however, vitally stained granules are readily produced/

produced even with quite small doses of the dye. (fig. 20) These granules are scattered throughout the cell cytoplasm in an irregular fashion and bear no special relation to the bile canaliculi. Generally they appear first in the cells around the central vein; and in the guinea-pig remain limited to this situation; in the rabbit, however, they are later found in hepatic cells in all parts of the lobule. They are fairly uniform in size but irregular in shape. These granules become more numerous with prolonged and intense staining and appear also to increase in size. All vitally stained granules in hepatic cells do not appear to be of the same nature. Some appear to be newly formed dye granules perhaps analogous to those of the Kupffer cells, but others seem to be the result of imbibition of dye into or a deposit on the surface of granules pre-existing in the liver cells. Similar granules have been observed in other situations, e.g. in a few of the Kupffer cells or in phagocytic cells in the stroma of the portal tracts, and they must be carefully distinguished from those due to true vital staining. We believe that these granules are entirely different in character from the granules and vacuoles of the histiodytes and Kupffer cells. They appear to represent an imbibition of dyestuff into preformed granules in the cell protoplasm, which can be demonstrated by other means. They resist the action of digestive ferments and various decolorising agents in a manner quite different from the granules of the Kupffer cells and histiocytes. At one time it was thought that such granules might represent the physical basis of vital staining phenomena/

phenomena in general, and a number of experiments were carried out to determine the proper interpretation of the observations. It was found that if sections of vitally stained rabbit liver were bleached with potassium permanganate and oxalic acid, the vitally stained granules in all situations disappeared, but fine yellow granules remained in the liver cells which could be rendered prominent again by subsequent staining with thionine blue, methylene blue or carbol-fuchsin, whereas the granules of most of the Kupffer cells were not restored in this way. Occasionally Kupffer cells and phagocytes in the portal tract were observed to contain granules which restained with the basic dyes, but these were much less numerous than the previously vitally stained cells and corresponded to the pigment-cobtaining cells found in these situations in normal rabbits. These granules in the liver cells were also resistant to the digestive action of gastric and pancreatic juice, which removed completely the tigroid bodies of the liver cells. They were not affected by alcoholic solution of potassium hydroxide.

In control preparations of other vitally stained tissues restaining with basic dyes after bleaching generally failed to show any definite restoration of visible granules in cells previously vitally stained, though it must be noted that if only the minimum degree of effective bleaching were used, there appeared to be left in place of the dye granules a colourless compound which on restaining with the basic dye took on a diffuse colouration. In other organs and tissues in which similar/

similar yellow pigment granules normally occur in small numbers, e.g. in the mesenteric and retroperitoneal glands, heart muscle, spleen, etc., such granules wherever present stained sharply with the basic dyes. We are, however, satisfied that such granules have no real importance in vital staining phenomena.

With regard to the nature of the yellow pigment granules which naturally occur in the liver and other tissues of the rabbit, it was found that these could be dissolved out of the tissues neither by fat solvents, e.g. alcohol, ether and chloroform, nor by strong alkali; hence they are not lipochromes. They fail to give the prussian blue reaction, and are not completely bleached by oxidising agents such as hydrogen peroxide or potassium permanganate and oxalic acid; therefore they are not derivatives of haemoglobin. Since they stain with varying intensity with basic dyes, such as fuchsin, thionin, methylene blue, etc., it seems that these granules correspond most closely with "lipofuscin" Hueck (1912) and Lubarsch (1902, 1922) and haemofuscin (Mallory et al., 1924), which according to Connor (1928) are essentially the same, the differences being due probably to the age of the granules. The presence of these granules in the tissues of normal rabbits is worthy of attention since recently it has been claimed (Mallory, 1925; Hall and Butt, 1928) that a condition of pigmentation of the liver and other organs can be produced in animals/

animals by the administration of certain metals, especially copper, and these writers state that the lesions produced are analogous to haemochromatosis. Flynn & Von Glahn (1929), however, point out that the degree of pigmentation in rabbit's liver is much more closely related to the nature of the diet and consider that the increased pigmentation is of exogenous origin. Without going further into the question of the nature of the pigment found in various experimental conditions in the livers of rabbits, we would emphasize the value of vital staining as a method of bringing such granules into prominence, since where such pigment is present in the tissues, certain vital stains -especially those of group (a) - administered subsequently may be deposited in or on the pigment granules already present and may render clearly visible very fine granules which can scarcely be seen otherwise.

After prolonged and intense dosage the cells of the bile ducts contain minute granules of trypan blue situated in their basal portion. We have not observed blue stained material in the lumen of the bile ducts as described by Goldmann (see above). Similar granules in bile duct epithelium have not been observed with dyes of group (b). Fibrous tissue cells in the portal tract and in Glisson's capsule exhibit the small uniform granules characteristic of the fibrocytes of connective tissue elsewhere.

After intravenous injection of suspensoid preparations such as india ink, etc., the histological findings in the liver are similar to those with trypan blue and similar dyes, (fig. 21,22) but/

but certain differences are to be observed. It has already been pointed out that no intracellular deposition of dye is visible during the first few hours after administration, but in the case of colloidal suspensoids the Kupffer cells exhibit granules of the injected substance within a few minutes.

If an animal be killed immediately after an injection of india ink, the carbon particles will be found adhering to and outlining the walls of the liver sinusoids and Kupffer cells. Within 10 minutes pronounced phagocytosis occurs; (fig.21) at the end of an hour the greater part of the deposited ink is intracellular. With large doses this process naturally takes longer, but 5 or 6 hours after relatively enormous or even lethal doses of ink almost the entire carbon content of the liver is intracellular. As already mentioned (see p. 96) a considerable number of the circulating leucocytes ingest the particulate matter, but the number of these cells is greater in the capillaries of internal organs than in the peripheral blood, Such cells, comprising both polymorphs and monocytes, are present in the hepatic sinusoids. Deposition of carbon particles begins in the liver sinusoids around the portal tract, and the Kupffer cells at the periphery of the lobule are the first to become laden with ink particles. At the end of 5 or 6 hours, however, this irregular distribution has largely disappeared and the Kupffer cells and endothelium are engorged with carbon throughout the entire length of the sinusoids (fig. 23). This picture remains for about 24-48 hours without any marked alteration beyond/

beyond gradual increase in the amount of carbon in the cells, but here too inactive endothelial cells which have ingested no ink can be found in the sinusoid lining. No trace of ink is found in the connective tissue cells and macrophages of the portal tracts and Glisson's capsule during the first few days, but later, i.e. from 4 days onward, migration of ink-laden Kupffer cells occurs, as previously described in the dye experiments. Similar cells appear to be liberated from the sinusoids into the hepatic veins whence they pass through the right heart to the lungs. The hepatic parenchyma cells of the mouse have never been found to contain ink granules, but in rabbits scanty particles find their way into the epithelial cells after a few days, just as was observed with dyes. Nagao (1920) found that this occurred more readily in weakly rabbits, e.g. those affected by coccidiosis. In the hepatic perenchyma cells of the rabbit, the ink particles appear to be absorbed on the surface of pre-existing granules and are irregularly scattered throughout the cell body, not being specially related to the bile canaliculi.

Other suspensoid substances behave similarly. Oxide of iron and benzopurpurin in suspensoid form are deposited in the Kupffer cells in the same way as india ink, and the engorged endothelial cells have been observed to migrate into the circulating blood and into the portal tracts and subcapsular connective tissue just as they do after the administration of dyes. It is noteworthy, however, that after injection of oxide of iron, the entire cytoplasm, of the Kupffer cells gives at first a diffuse opaque prussian/

prussian blue reaction, like heavily ground glass; later the iron is segregated into granular masses, in which the colour is much more intense, as if the iron were becoming concentrated. Similar changes have been noted in other situations in which iron is stored. These substances, however, are not relatively inert like the carbon in india ink. They are capable of undergoing further changes in the body as a result of which a redistribution of the injected substances subsequently occurs. The later changes in the deposited iron and its subsequent redistribution have been followed over a long period, and will be dealt with in a separate section.

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SPLEEN.

The degree of participation of the spleen in vital staining processes depends on the agent used and the mode of administration. The more diffusible dyes of the trypan blue class, e.g. trypan blue, vital new red, etc. show relatively little tendency to accumulate in the spleen. Administration of vitally staining dyes by the intravenous route appears to produce a more extensive participation of the splenic tissue than does subcutaneous or intraperitoneal injection of similar doses. Suspensoids such as india ink and saccharated iron oxide on the other hand are removed from the circulation by the spleen with extreme rapidity, and substances of this class tend to accumulate in the spleen progressively after intravenous injection.

In animals stained with dyes no enlargement of the spleen is found. Vitally stained vacuoles and granules appear first in certain round or oval mononuclear cells or 'splenocytes' scattered throughout the pulp, and granules are also found in stellate cells which are almost certainly the reticular elements. The stellate reticulum cells in the spleen pulp may be recognised by their shape and by the larger size and paler staining character of the nucleus which is oval or sometimes reniform. In both stellate and free cells it is not unusual to find traces of pigment of golden-brown colour, most of which gives the iron reaction, and the mixture of the vital stain and the altered blood pigment produces dirty brownish granules in the cells. Cells heavily laden with altered blood pigment rarely ingest much of the dye,

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but varying degrees of admixture occur. It is noteworthy that the spleen pulp contains a very large number of mononuclear cells resembling the vitally stained splenocytes in every way except that they lack dye granules, and these unstained cells usually form a large majority. The endothelial cells of the venous sinusoids also exhibit scanty fine granules of the injected dye, but in my experience these are always small and much less prominent in mice than the vitally staining granules of the endothelial cells of the liver or bone marrow. I have never succeeded in obtaining a sharp demarcation of the venous sinusoids by vital staining, since the lining cells do not become prominent with dye granules as they sometimes do with iron in human acholuric jaundice (see Muir's Pathology, 1929, p.445, fig.265). The Malpighian bodies remain almost completely free from the vital stain; careful search with the oil immersion lens will, however, reveal scanty very fine granules in the reticular cells of the lymphoid follicles. Even with prolonged and intense vital staining the storage of dyes in the spleen is usually surprisingly slight in mice in comparison to that of other organs, e.g. lymphatic glands. This phenomenon strengthens the opinion that the reticulo-endothelial elements, while possessing certain properties in common which justifies their being grouped together into a "system", are yet strikingly selective and specialised in their activities in the various organs. The giant cells which are so plentiful in the spleens of rodents take no part in the storage of vital stains of any kind. In the capsule of the spleen/

spleen numerous vitally stained cells are present, these are to be regarded as histiocytes and fibrocytes. The peritoneal mesothelial cells covering the spleen are plumper and more cubical than elsewhere, especially after repeated intraperitoneal injections, and they tend to store the vital stains in rather greater quantity than do mesothelial cells generally.

In rabbits the storage of vital stains in the spleen has in my experience been exceedingly scanty, but possibly this is to be related to the presence of numerous phagocytes containing altered blood pigment. In guinea-pigs, on the other hand, more pronounced dye storage has been observed in the spleen, though in a series of animals subjected to the same dosage considerable variation was found. In a few cases dye containing cells were present in unusual numbers both in the pulp and in the Malpighian bodies (figs. 24,25). In the venous sinusoids of guinea-pigs, very little dye is generally present in the mural cells, though the floating cells (reticulum cells of Downey 1922, Maximow 1928, anchored clasmatocytes of Sabin 1920) contain quite a considerable amount (fig. 26). In nearly all deeply stained animals of every species, a few mononuclear leucocytes with vitally stained granules are found in the lumen of the splenic vein and in the sinusoids. It seems clear that these are derived from the vitally stained splenocytes and reticulum cells of the pulp and sinuses. In guinea-pigs vital staining of the specific granules of the polymorphs is frequently seen in the spleen pulp and vessels. This phenomenon is considered further in discussing

the bone marrow (see p. 124).

In marked contrast to the scanty storage of colloidal dyes are the results after intravenous administration of suspensoid preparations, e.g. india ink, and saccharated iron oxide. Deposition of ink and iron begins in the spleen within a few minutes after a small dose of a dilute suspension and is first visible around the Malpighian bodies (fig. 27). Within 10 minutes after an intravenous injection the lymphoid follicles are surrounded by a layer or layers of stellate cells heavily laden with carbon or iron. At this time there is also some deposit on the arterioles of the pulp cords but very little is found in the pulp cells which surround the arteriole like a sheath, or in the pulp spaces. The active phagocytic cells are probably the first pulp cells with which the blood passing through the capillaries of the Malpighian bodies comes into contact, since all this blood is emptied into the pulp at the periphery of the Malpighian bodies, whereas that passing through the arterioles of the pulp may go straight through the ellipsoids into the venous sinusoids and so into the splenic vein (NcNee 1929). This appearance in some animals is rapidly succeeded by a stage in which the ink particles spread into the interstices of the pulp,with subsequent phagocytosis by the pulp cells; but in others the general pulp remains almost free from carbon particles for a much longer time, even up to several hours. It is probable that deposition in the general splenic pulp occurs only when some of the ellipsoids divert the flow of blood out/

out into the pulp instead of allowing it to pass right through into the sinusoids. The particulate matter is then deposited on the reticular cells and along their long processes which form the meshes of the pulp spaces. Robinson (1928) has shown by transfusion experiments that this initial deposition is due to a <u>mechanical</u> filtering action and can be elicited even after the cells have been poisoned by cyanide. After an hour the particulate matter in the pulp is much more abundant and is nearly all intracellular, but as at earlier stages, it is generally most marked around the Malpighian bodies. The reticular cells of the lymphoid follicles have also by this time taken up a small amount of the particulate matter. During the next 24 hours the amount of ink or iron in the spleen increases; in the pulp spaces in all situations carbon or iron is taken up by many free phagocytic cells in addition to the fixed reticular cells. When very large doses are given the splenic pulp becomes greatly distended with phagocytic cells and a high proportion of the free mononuclear cells of the pulp take up the injected substance. Even in such cases, however - and this is very clear with the usual moderate doses - there are always many small mononuclear cells in the pulp which remain free from ink or iron. These are probably lymphocytes, immature monocytes and primitive undifferentiated cells, and they represent a reserve from which fresh phagocytic cells can be constantly formed as further demand arises. The giant cells (megacaryocytes) remain at all times indifferent to the presence of the particulate matter and exhibit no trace of phagocytosis.

In no case can all the elements, whose morphological characters and anatomical situation would suggest that they are stainable, be made to exercise this function at any one time. No matter how high the dosage or how prolonged the administration even to the limit of toxicity there are always to be found side by side cells laden with the injected substances and others morphologically similar which are entirely free. Moreover it must be emphasised that there are great variations in the amount of particulate matter in the spleen pulp in different animals of the same species at any given interval after injection. This variation is probably dependent on individual differences in the circulation of the blood through the spleen pulp. The endothelial cells of the venous sinusoids are even more indifferent to particulate matter than to soluble dyes, and it is rare to find more than mere traces of ink or iron in these cells, at least soon after injection.

Each repeated injection of particulate matter leads to the same cycle of changes and the spleen pulp becomes so rich in phagocytes that the original accentuation of the deposit around the Malpighian bodies is obscured. If such animals are allowed to survive for several months after cessation of treatment a certain rearrangement of the phagocytes becomes evident. This is chiefly due to accumulation of the engorged cells in little clumps throughout the pulp, especially along the margins of the trabeculae, as if the cells were endeavouring to migrate into the perivascular lymphatics in these situations; occasionally similar clumps of cells have been found around the central arterioles of the

Malpighian bodies (fig. 61). Long after the cessation of administration of saccharated iron oxide, granular iron may begin to accumulate within the sinus endothelial cells. This does not occur with india ink and it will be further considered later.

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BONE MARROW.

The bone marrow participates in storing vital stains of all kinds, though, as has been mentioned, its activity varies considerably in different animals. In general it may be stated that the elements of the marrow which are active in this respect are the reticulo-endothelial cells; but the histological picture varies with the nature of the vital stain and with the time of examination after its administration.

In mice the long bones are generally filled with cellular marrow in which there is only rarely more than a trace of fat. After rapid intense staining with trypan blue or similar vital stains, the dye is deposited in the form of fine granules in the endothelial cells of the venous sinusoids and in certain stellate cells scattered irregularly in the marrow pulp. but the lining cells of the arterial capillaries remain free from the dye. At first the cells contain only tiny blue granules, but as staining proceeds the amount of intra-cellular dye increases; the affected cells become swollen and some project into the lumen of the sinusoids. The degree of staining of individual cells varies considerably, as is well seen in sections which cut a sinusoid longitudinally. The vitally stained stellate cells generally possess fairly large rather pale staining oval nuclei and their cytoplasm becomes much increased in amount and filled with large granular masses of vital stain. These elements, which have generally been regarded as the reticulum cells of the marrow, lie among the masses of myeloid cells, and their processes can frequently be traced a considerable distance.

As vital staining increases in intensity, there appears a considerable number of large oval or rounded cells resembling histiocytes, with coarse vitally stained granules. It is probable that they are derived from the reticulum cells and from the endothelial cells of the sinusoids. The appearances suggest that some of the former draw in their processes and become rounded; some of the endothelial cells appear to leave the sinusoid wall and migrate outwards into the marrow pulp, while others desquamate into the lumen and appear as free vitally stained mononuclear cells in the blood just as in the liver and other organs. The bone marrow in mice is relatively inactive in storing vital stains and in a relatively short time after injections of the dye have ceased, the endothelial cells of the sinusoids are found to be almost free from dye granules. This change appears to be brought about at least partly by migration of the endothelial cells as above described, and for a time the free vitally stained histiocyte-like cells persist in considerable numbers in the intersinusoidal tissue. After a few weeks, however, they too become scanty and only occasional dye containing cells remain, the exact nature of which cannot be determined.

The bone marrow of rabbits is much more active in storing vital stains of all kinds than that of mice, and the effects persist longer. Essentially the same types of cells are affected, but those of the rabbit take up the dyes in larger amounts. The marrow of rabbits is less cellular than that of mice and contains a larger proportion of adipose tissue; it is thus easier to/

to observe slight hyperplastic changes following treatment. Doan, Cunningham & Sabin (1925) state that trypan blue exercises a slightly stimulating effect on the marrow of pigeons and rabbits, and Muller (1926) has found a similar effect from small doses of india ink. I have not observed any marked difference in cellularity between the marrow of most vitally stained rabbits and that of controls, the differences appearing to fall within the range of individual variations. Sienigalewicz and Clark (1925), however, points out that even isotonic trypan blue solutions are slightly haemolytic; they might thus be expected to give rise to mild erythroblastic hyperplasia.

In guinea-pigs the general results of vital staining more closely resemble those of rabbits than of mice, but the marrow usually contains less adipose tissue than that of rabbits and may be entirely cellular. In addition to vital staining of the endothelial cells, stellate cells and histiocytes of marrow, I have found that trypan blue produces in guinea-pigs a certain degree of staining of the finely granular myelocytes and polymorphs, but no such staining has been observed in the marrow of mice or rabbits. In fixed and stained sections the myelocytes frequently exhibit diffuse blue colouration of their cytoplasm, but in fresh preparations, the staining appears to be finely granular. The polymorphs in sections of the marrow and internal organs from such animals exhibit fine bright blue granulations. I have noted that this staining of the myeloid cells is observed only when the animals are killed at a time when there is abundant dye in the blood plasma and tissue fluids, and it is possible that

it represents an agonal phenomenon similar to those observed by Gross (1911). Sewell (1918) and Cunningham (1922) have also noted on occasion vital staining of the specific granules of the polymorphs (see also p. 241).

The intravenous injection of suspensoid substances such as india ink or saccharated oxide of iron leads to vital staining of the marrow similar to that obtained with soluble dyes. As in other internal organs, shortly after injection the blood in the venous sinusoids contains ink-storing mononuclear leucocytes; these disappear within a few hours, but their fate is uncertain though it is possible that some may pass into the marrow pulp where they become indistinguishable from the ink-storing histiocytes. Within a few hours the injected suspensoid is deposited in the endothelial cells of the venous sinusoids and also in certain spindle shaped or stellate cells in the inter-sinusoidal tissue. After 24 hours there are also found rounded histiocyte-like cells similar to those described in the trypan blue experiments, and after some days these become more abundant while the amount of particulate matter in the sinusoidal endothelium diminishes. After repeated injections of suspensoids the amount deposited in the bone marrow is increased; in my experience it has always been much less abundant in the mouse than in the rabbit. The bone marrow of mice also seems to free itself of the particulate matter more rapidly than that of rabbits.

It is at first difficult to reconcile some of these effects of vital staining with recent views on the structure

of the bone marrow. Drinker, Drinker and Lund (1922) and Doan (1922) have claimed that the circulation of the bone marrow in both birds and mammals is of the closed type. Van den Stricht (1892) had previously suggested that the circulation in avian marrow was of closed type, but believed that in mammals the open type was probably the rule, an opinion in which Muir and Drummond (1893) concurred. If the capillaries of the marrow everywhere possess a complete endothelial lining, it is necessary to assume that they are unusually permeable, since even particulate matter (india ink) passes readily into the stellate cells of the intersinusoidal tissue, which have generally been assumed to be the reticulum cells. It might be suggested that the stellate cells are not the reticulum cells, but are endothelial cells belonging to the collapsed intersinusoidal capillaries described by Doan (1922). This is, however, unlikely when we consider that the vitally stained stellate cells are numerous in the active haemopoietic regions and can be clearly seen to occur singly among foci of developing myelocytes with no trace of a capillary wall or developing erythroblasts in their vicinity (fig.34). Further, the stellate cells demonstrated by suspensoid preparations correspond in every way to those shown by the soluble dyes, which it is generally admitted are taken up by the reticulum cells. Cunningham (1922) has shown that apparently under the influence of respiratory movements, the mesothelial cells covering the peritoneal surface of the diaphragm take into their cytoplasm finely divided particulate matter (india ink, etc.) which is transferred to the endothelial cells of the lymphatics; finally

the granules become free in the lumen of the lymphatic channels and pass to the glands in the free state. Possibly the sinusoidal endothelium of the marrow possesses such permeability; this would explain the facts observed without the assumption that the circulation of the marrow is of open type. A similar permeability of the sinus endothelium of lymph glands to particulate matter has also been observed, but on the other hand the blood capillary endothelium of lymph nodes is quite impermeable to such substances.

Cunningham, Sabin and Doan (1925) state that red blood cells and clasmatocytes (histiocytes) are derived from endothelium and that reticulum cells give rise only to monocytes, lymphocytes and the granular cell series. My own observations show that free histiocytes are formed from endothelium, but I do not consider that it is justifiable to deny the participation of the reticulum cells in the formation of free histiocytes. This may occur through a monocyte-like stage, as in peritoneal exudates, but stellate reticulum cells may also draw in their processes after ingesting a large amount of foreign material and may thus become directly the free vitally stained histiocytes seen in the interstices of the marrow.

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LYMPHATIC GLANDS.

The effect of vital staining on the lymphatic glands depends on the nature of the agent employed and its mode of administration. These factors are of importance in that they govern the route by which the substance reaches the gland, whether by the blood stream or by the lymphatic channels. It may be stated definitely that staining of the gland substance depends on the penetration of the stain into the lymph stream, and that a substance capable of staining the gland cells by that route has no effect if it be confined to the blood stream, e.g. india ink administered intravenously to a mouse is not stored immediately in any of the lymphatic glands. In the case of the various dyestuffs used as vital stains the results depend on the above factors. Dyes of group (a) e.g. trypan blue, vital new red, etc., are rapidly absorbed into the blood stream after intra-peritoneal or subcutaneous injection and are thus distributed throughout the whole body. From the blood stream these substances pass readily into the tissue lymph, so that their administration by any route is soon followed by the most widespread staining possible. Dyestuffs of group (b) e.g. isamine blue, produce very intense staining of the tissues at the site of injection, and the lymphatic glands draining the area also become deeply stained, but since spread of the dye to distant situations occurs only slowly, glands not immediately draining the site of injection become stained more slowly

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and always remain less deeply coloured than those directly implicated. The still more highly colloidal dyes, e.g. benzopurpurin in solution, are scarcely appreciably absorbed from the site of injection; [illegible] staining is confined to the regional lymphatic glands and is slow in onset.

The elements of the lymphatic tissue which respond to intra-vitam staining are identical whatever be the agent employed. With dyestuffs such as trypam blue, the dye in the lymph entering the peripheral sinuses permeates through the lymphatic pathways and also through the substance of the cortical nodes, so that the sinus endothelium and the reticulum cells have almost equal opportunity to take it up. Granular deposits of dyestuff do not appear for about six hours, though long before this the glands to the naked eye may all appear of a deep blue colour. The microscopic accumulation of cytoplasmic dye granules increases steadily throughout the next 24 hours. The glands draining an area into which trypam blue has been injected subcutaneously or intraperitoneally are, of course, more deeply stained than those elsewhere, and uniform staining can only be secured by intravenous injection. The appearances produced in lymphatic glands after absorption of the dye into the general circulation as opposed to those in glands draining an injected area may, however, be studied in the axillary glands after intraperitoneal injection; it is found that such differences as exist are quantitative rather than qualitative and the same elements are affected in both cases. With moderate doses of trypan blue or isamine blue it/

it is possible to demonstrate a complete layer of long flattened endothelial cells which line the lymph sinuses of the gland, with their tapering processes filled with blue granules, while here and there one may find such cells rounding up and becoming desquamated into the lumen to appear as free macrophages (fig. 35). The reticular elements of the sinuses and those supporting the lymphocytes in their meshwork of fibrils are also electively stained and the cytoplasmic processes which extend along the reticulum fibrils are filled with dye particles.

After the prolonged subcutaneous administration of dyes of group (b) e.g. isamine blue, the number of free macrophages in the lumen of the sinuses may be very great, and it is probable that these cells are produced chiefly by proliferation and desquamation of the reticulo-endothelial elements of the gland, though some are probably also macrophages formed in response to the reaction at the site of injection, which have wandered in from the subcutaneous tissue. In some cases these cells may fuse to form syncytial masses of large size containing many nuclei (fig. 36). It must therefore always be kept in mind in interpreting the results of vital staining in pathological experiments that the vital stains themselves are capable of producing pronounced proliferative changes in susceptible tissue element.

Lymphocytes are never affected by any of the true vital stains; this applies both to the small lymphocytes and the larger so-called lymphoblastic elements which comprise the "germinal centres"/

centres" of the lymph nodes. Although, by the "supravital" staining technique with neutral red and janus green a small "segregation apparatus" can be demonstrated in many lymphocytic and lymphoblastic cells together with very distinct rod-like mitochondria, these cells so long as they maintain their normal situation and character seem to be quite indifferent to the presence of vital stains. It is not meant thereby to contradict the assertion made earlier that there is evidence that lymphocytes may become transformed into polyblasts: the question of the relation of the lymphocyte to the macrophage and the origin of Maximow's polyblasts will be discussed later in dealing with inflammatory changes.

It has been frequently observed in cases where the lymphatic sinusoids contain numerous free macrophages in their lumen that these do not all take up the dye with equal avidity; some cells are found to be loaded with dye granules while others are almost or even completely free from dye though their cytoplasm may exhibit granules of pigment or other inclusions (fig. 35). This observation is in accordance with what has been described earlier (see p. 71) in connection with the histiocytes, viz. that cells already engorged with one substance may be at least temporarily incapacitated from taking up other substances presented to them.

A remarkable demonstration of the different functional capacities of endothelium is afforded by the different reaction to vital/

vital stains shown by the endothelium lining the afferent and efferent lymphatic channels and that of the sinus endothelium. The transition is abrupt; the endothelium of the lymph channels remains quite free from microscopically visible dye deposits except in rare instances, while the endothelium lining the sinuses of the gland stands out prominently with more plump lining cells whose every process is filled with dye granules. Downey (1922) and Maximow (1927) have recently denied that the lining cells of the lymphatic pathways in the glands are true endothelial cells, and consider that they should be regarded as reticulum cells, both from the embryological and physiological standpoint.

It has previously been noted that the intravenous injection of suspensoid preparations such as india ink leads to no generalised vital staining of lymphatic glands, but, if leakage from the injected tail vein occur, or if the ink suspensoid be injected by mistake into the perivascular tissue, the lymphatic glands along the aorta very rapidly become intensely pigmented. Occasionally, when very large doses of ink or iron oxide have been injected intravenously, deposition of the injected substance has been observed on the walls of the blood capillaries which permeate the gland, but this phenomenon is merely part of a general process by which particulate matter tends to settle out of the blood stream and is found on the capillary walls in all parts of the body a short time after injection. This deposition does not persist, however, and the suspensoid material

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is rapidly removed and collects in the chief organs of storage, i.e. the liver, spleen and bone marrow. Occasionally, however, there is actual phagocytosis of the deposited carbon or iron particles by the capillary endothelium. When this occurs the endothelial cells do not seem to persist in the vessel wall but either desquamate into the blood stream or migrate outwards through the capillary wall into the surrounding connective tissue. Intraperitoneal injection of india ink leads to very rapid staining of the anterior mediastinal and retroperitoneal lymph glands, while after subcutaneous administration the regional glands, axillary and inguinal, become rapidly stained. It has been observed, however, that the intravenous administration of india ink may lead to pigmentation of the mediastinal glands, particularly those around the root of the lungs, but this does not invalidate the previous generalisation. The evidence is strongly in favour of the view that in this case cells laden with pigment have been removed from the circulation by the lungs, and have thence migrated along the lymph pathways to the regional glands.

When india ink particles reach the gland via the lymph stream, the endothelial cells behave just like the Kupffer cells of the liver: ink particles enter into the cells much more rapidly than the soluble dyes and appear within the cytoplasm in a few minutes even when the suspension used consists exclusively of particles whose size is at the limit of visibility. This factor comes into play in the development of/

of vital staining in lymphatic tissue and thus the particles within a few minutes of their entry into the gland are attracted to and ingested by the free macrophages and sinus endothelium and in a short time, if more ink particles reach the gland, the entire lining of the lymph pathways through the gland from periphery to medulla becomes outlined in black (fig. 37). The reticulum cells in the cortical lymph nodes are probably just as capable of ingesting the ink particles if the opportunity be afforded them, but owing to the barrier of the sinus endothelium, which filters out almost all the carbon, these reticulum cells never become as engorged as the sinus cells.

Saccharated oxide of iron and benzopurpurin in suspensoid form are distributed in the same way as india ink, and fail to stain the lymphatic glands immediately after intravenous injection. As a result of further changes in the body redistribution of these substances occurs and they are subsequently laid down in the lymphatic glands in a manner similar to the soluble dyes.

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OMENTUM AND MESENTERY.

The structure of the omentum varies considerably in different animals, but the variations are quantitative rather than qualitative, and in spite of the apparent dissimilarity the organ presents the same essential histological features in all the mammals which I have studied. Structurally the omentum consists of a framework of connective tissue carrying a rich plexus of blood and lymphatic vessels enclosed between two sheets of mesothelial cells.* Over the main trabeculae of the omentum the mesothelium forms a continuous sheet, but between these strands the organ presents the structure of a net, the cords and intersections of which are clothed on all sides by mesothelial cells. Along the main vessels adipose tissue tends to accumulate in well nourished animals, while from the main trunks numerous small vessels and capillaries arise and form a complicated anastomotic network around and throughout the strands of the framework. (fig. 38) The larger blood vessels are generally accompanied by lymphatic vessels which drain into the lymph nodes along the stomach and colon. The mesentery is of similar structure but there are no net-like portions and the membranes form a thin continuous sheet. In many animals histiocytes tend to be aggregated particularly in the neighbourhood of vessels/

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* In order to avoid confusion with the cells lining blood or lymph vascular channels the term "endothelium" will be restricted to the latter elements, while the serosal lining cells of the peritoneum and pleurae will be referred to as "mesothelium".

vessels and nerves, where they are included within the term "adventitial cells" (Marchand 1898). Many of these adventitial cells are small and round with darkly staining nuclei and closely resemble lymphocytes. Among these are a few larger cells with paler staining nuclei whose protoplasm at the periphery forms blunt pseudopods. The latter are the fully developed histiocytes, the former are believed to be reserves of primitive cells, but intermediate forms are of frequent occurrence. In the omentum and mesentery these adventitial cells are more numerous than elsewhere, and in rodents they form especially pronounced perivascular aggregations. In the rabbit and mouse large collections of these cells also occur scattered throughout the omental and mesenteric tissues without related blood vessels but frequently connected with the finer lymphatic channels; these form the so-called "taches laiteuses". (figs. 40,41,42)

It is essential, in order to study the process of vital staining in the normal omentum and mesentery, that intravenous or subcutaneous but not intraperitoneal injections be used. The intensity of the staining of the omentum increases with succeeding doses of dye, and this increase is due not merely to the greater dye content in individual cells but also to the greater number of cells participating in the staining. The larger type of cell, the histiocyte or "trailer" cell of Buxton and Torry (1906), shows vital staining first, but with increasing dosage some of the intermediate cells begin to take up the dye, and after intense staining even some of the small cells are stained while the number of stained cells of intermediate size is increased. In my/

my opinion therefore the large cells arise by differentiation from the smaller cells situated in the tâches laiteuses and around the vessels, and this development is accompanied by an increased avidity for vital stains. The number of fully *differentiated histiocytes in the omentum at any time is an index* of the functional need for such cells and as will be seen later their number becomes enormously *increased in inflammatory* conditions of the peritoneal cavity. The undifferentiated cells are morphologically indistinguishable from lymphocytes except perhaps in their mitochondria, which are more numerous and more scattered in the cytoplasm. It seems unnecessary to designate these cells by another name and we shall therefore refer to them as lymphocytes.

The endothelial cells of the blood and lymph capillaries exhibit only minute granules of dye, which are best seen by examining small portions of the tissue in the fresh state, as they may be no longer visible in fixed and counterstained preparations. They are found with dyes of group (a), while after these of group (b) granules are generally absent. The fibrocytes of the areolar tissue between the mesothelial layers contain smaller and more uniform granules than the histiocytes and are morphologically similar to those already described in the subcutaneous tissue.

The mesothelial cells covering the peritoneal surfaces store the vital stain in a characteristic manner. Intracellular dye granules appear first in a small group near the nucleus, and as staining proceeds, the number of granules increases and the group assumes/

assumes the shape of a small crescent at one side of the nucleus. (fig. 43) In very deeply stained animals this may extend further and form a complete ring of small deeply stained granules encircling the nucleus. These mesothelial granules are more abundant and are larger with dyes of group (a) than with those of group (b). As we have seen elsewhere the demarcation of cell types is rather less selective with the former than with the latter class of dyes. It is also noteworthy that the degree of staining in the mesothelial cells varies in different situations. The intracellular dye granules are least abundant over the bowel and most abundant on the surface of the spleen and ovary (for details see Cunningham 1922).

It has already been pointed out that suspensoid substances injected intravenously are at first confined to the blood vascular channels and are taken up by the cells lining them in certain situations and by a varying number of the circulating monocytes and polymorphs. Within a few minutes of the injection of india ink, carbon particles may be found adhering to the walls of the capillaries of the omentum and mesentery just as they do elsewhere, but it is not certain that any true phagocytosis occurs. After twenty four hours an occasional adventitial cell will be found with ingested ink granules, and during the next few days ink containing cells increase in number and appear even in the avascular tâches laiteuses. These cells become more numerous if repeated injections of india ink are given over a long period, and/

we have observed that they are relatively more numerous in rabbits than in mice. (figs. 39,40) Even when first observed the ink bearing cells appear to be in the normal resting condition with extended pseudopodia, and it is difficult to understand how the carbon particles reach them. It is possible that some of these cells may be migrated ink-containing monocytes or capillary endothelial cells as Foot (1921) suggests. On the other hand if such migration occurred, one would expect to find easily in spread preparations stained cells within the smallest vessels and in process of leaving them. In my experience such appearances are conspicuously absent. Further the stained cells seen in the tissues do not appear to be in a mobile state. Accordingly it is suggested that the capillary walls in the mesentery and omentum are more permeable than those of the general areolar tissue and permit the passage of very finely divided particulate matter, just as in the bone marrow. Saccharated oxide of iron at first behaves in a manner similar to india ink, and iron containing cells are found scattered throughout the masses of adventitial cells within 8 hours of a single injection. Later, however, the distribution of iron in the tissues becomes altered and these later changes will be discussed later.

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SUPRARENAL GLAND.

The suprarenal glands are much less active in their reaction to vital stains than the liver, spleen, marrow or lymphatic glands. Dyes of group (a) e.g. trypan blue, accumulate within the endothelial cells of the capillaries especially in the cortical zone, but similar cells though fewer in number are found in the medulla. Here too it has been observed that adjacent endothelial cells may vary greatly in the intensity of their reaction to the vital dyes. While the amount of dye taken up by the suprarenal endothelial cells increases with increased dosage, it is always less than that in the liver endothelium in the same animal, and thus these elements appear to occupy an intermediate position between the so-called "specific endothelia" of the liver and marrow and the general blood vascular endothelium.

The specific secretory elements of the suprarenal medulla have not been observed to take up vital stains, but in the cortex the epithelial cells of the outer or glomerular zone exhibit fine granules of trypan blue. The intermediate or fasciculate zone usually contains practically no dye granules but the innermost layer of cells of the zona reticularis also exhibit fine vitally stained granules, but of less intensity than those of the outer zone. With isamine blue, on the other hand, the site of maximum intensity of vital staining is the inner zone of the reticular layer of the cortex, while the outer or glomerular zone is much less deeply stained. A similar discrepancy of results has been observed between/

between other vital stains of rapidly acting and slowly acting type, but the reason for this difference has not been ascertained. Similar results have been observed in rabbits and guinea-pigs.

Suspensoid substances are stored in the endothelial cells in a similar manner to soluble dyes. The endothelium lining the capillaries of the suprarenal is comparable to that of certain other organs, e.g. the pituitary gland and kidney, etc., in that it tends to store india ink and other suspensoids for a short period (fig. 44) but rapidly frees itself and may later show but little trace of ink in any part. This early deposition is particularly evident if very large doses of ink have been administered (fig. 44) when the entire capillary bed of the organ may be outlined in black granules. After large doses the amount of carbon persisting in the gland may be quite considerable, but it is always confined to the capillary endothelium. The ink is usually distributed fairly regularly throughout the cortical capillaries, with a rather heavier deposit in the outermost layers and towards the junctional zone with the medulla. In the medullary sinusoids the deposit of carbon granules is generally very irregular. Here and there heavily laden cells are found in the capillary walls while adjacent cells are free. Entry of carbon into the specific epithelial cells of the cortex or medulla has not been observed.

The immediate distribution of iron in the suprarenal gland after the intravenous injection of the saccharated oxide is similar to that of carbon as just described, but appears to be even/

even more fleeting. Later, when the iron has undergone certain transformations as a result of the action of phagocytic cells elsewhere, iron storage is seen in the epithelial cells in a manner similar to isamine blue, i.e. especially in the inner zone of the cortex. (fig.45)

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PITUITARY GLAND.

The blue staining of this organ is due to the presence of vitally stained granules in endothelial and perivascular cells and in the glandular epithelial cells. Vital staining of the capillary endothelial cells is irregular and only a few of the lining cells contain blue granules while the majority show no trace of the dye. The most heavily stained elements are large stellate cells with coarse irregular blue granules; these appear to lie just outside the capillary walls in most cases (fig. 46), and while they have been generally regarded as analogous to the Kupffer cells of the liver, I do not consider that they are of this nature. In my opinion these cells belong neither to the capillary nor lymphatic endothelium, but are perivascular histiocytes (adventitial cells) similar to those in the stroma of the pancreas and other organs.

Cells with stained granules are more abundant in the anterior than in the posterior lobe; the granules of the epithelial cells are as in other organs smaller and less deeply stained than those in the endothelial and perivascular cells. In the anterior lobe it has not been determined whether the vitally stained granules of the parenchyme cells are in any way related to the specific eosinophile and basophile granulations; they appear to occur in both types of anterior lobe cells but are larger and of more irregular shape than the

specific granulations. In the posterior lobe or <u>pars nervosa</u> dye granules occur in endothelial and perivascular cells as in the anterior lobe but are more scanty. The staining of this part of the organ is due chiefly to fine intracellular granules in irregular and stellate cells which normally contain yellowish pigment granules, the so-called glial cells of the neurohypophysis (Schuleman 1912; Rachmanow 1913). The superficial blue staining noted around the base of insertion of the hypophyseal stalk is due to fine dye granules in these cells which spread out over the surface of the nervous tissue at this point)Rachmanow 1913), but I have observed that there is also a diffuse blue staining of the underlying nervous substance which extends a short distance internal to the layer of cells with stained granules. The meaning of this diffuse staining has already been discussed (see p. 58).

THYROID AND PARATHYROID GLANDS.

Vital staining of the thyroid and parathyroid glands is due to the presence of stained cells in the stroma around the vesicles. No granular staining has been seen in the parenchyma cells of either gland. The vitally stained cells appear to lie between the blood capillaries and the gland acini, being sometimes closely applied to the former; sometimes to the latter. Williamson and Pearse (1926) have described certain endothelial cells in the thyroid and parathyroid as part of the special lymphatic sinusoid in which the thyroid gland unit lies. They state that the staining character of these cells varies from coarse granular basophilia to marked diffuse acidophilia depending on the phase of activity in the adjacent thyroid cells, and that, since dells which store vital stains in granular form occur in similar situations, they are identical and can be regarded as analogous to the Kupffer cells of the liver. According to my observations the vitally stained cells of the thyroid are not part of the lymph sinus endothelium but are histiocytes closely applied to the blood or lymphatic capillary walls.

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TESTIS.

The colouration of the testis is due to vital staining of granular type in cells which occur throughout the stroma of the organ. In the fibrous tissue of the capsule these elements are uniformly distributed and correspond to histiocytes elsewhere, but in the interstitial tissue between the tubules the vitally stained cells are gathered together in small clusters and so seem to correspond to the interstitial cells of the testis. The number of these elements and the intensity of their staining varies somewhat in different animals, but we have not attempted to correlate these variations with conditions of functional activity of the germinal epithelium as suggested by Goldmann(1909) and Leopold (1921). It is noteworthy, however, that the present study did not reveal practically any cells morphologically resembling the interstitial cells of Leydig in which vital staining of considerable degree was not present.

Takamori (1921) considered that the interstitial cells were of two varieties, (a) typical histiocytes with coarse vitally stained granules, and (b) the true lipoid-containing interstitial cells of Leydig which are to be distinguished from the histiocytes since they stain vitally only after high dosage and show only scanty fine granules. This distinction is not, however, valid since we know that histiocytes elsewhere may actively store lipoid material and that cells when loaded with a large amount of one substance as a rule fail to take

up/

up intensely another injected subsequently (see p. 331).
We do not therefore consider that the interstitial cells can
be separated from histiocytes by this criterion.

No vital staining is found in the cells of the seminiferous
tubules either in the spermatogonia or in the cells of Sertoli,
though both of these elements contain small granules which
stain supravitally with neutral red. (Goldmann (1909, Plato,
(1897), Gatenby (1929).

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OVARY.

The surface of the ovary is covered by a layer of somewhat columnar cells which are directly continuous with the mesothelial cells of the peritoneum, and it has been questioned (see Gatenby, 1916; Cunningham, 1922) whether the former should be regarded as of mesothelial origin or whether they are true germinal epithelial cells. After intraperitoneal or intravenous injections of trypan blue, the cubical or columnar cells covering the ovarian surface contain fairly abundant small blue granules situated chiefly in the deeper infranuclear part of the cells. At the junction with the peritoneal serosa the transition to flattened cells occurs gradually, and the amount of intracellular staining also diminishes as the morphology of the cells changes, until the typical small perinuclear crescents are found (see p. 138). The evidence of vital staining is therefore in favour of the mesothelial nature of the cells covering the ovarian surface. Throughout the ovarian stroma there are scattered vitally stained cells sometimes occurring in small clumps; it is not clear whether these are the so-called interstitial cells of the ovary, analogous to those of the testis, or whether the latter are present in addition to the vitally stained histiocytes; if so they are very inconspicuous and we do not consider that it is justifiable to regard them as separate elements. Recently Benthin (1923) has expressed a similar view. The primitive germ cells (primordial follicles) and ripening ova show no evidence of vital staining, but in mice in the larger follicles, especially those nearly mature, fine vitally staining granules are found in small numbers in/

in the inner zone of theca cells. In these cells the granules are less numerous and are of finer type than in histiocytes. The inner cells of the corpora lutea have also been found to contain rather scanty dye granules of very fine type like those of the suprarenal cortical cells. In rabbits and guinea-pigs, the follicle cells show much less evidence of vital staining than in mice.

Ribbert (1904) considered that the vitally stained cells which he observed in the follicles of carmine stained animals were mononuclear leucocytes (histiocytes), but Goldmann (1909) states definitely that the vitally stained granules are present in the inner cells of the theca interna in maturing follicles. Borell (1919), on the other hand, claims that vital staining is scarcely appreciable in healthy maturing follicles, but becomes marked when the ovum degenerates. He regards the vitally stained cells as derived from the inner theca cells and points out that whereas in the formation of corpora lutea after discharge of the ovum, the lipoid storing cells fail to take up carmine, in atretic follicles on the other hand affinity for the vital carmine stain is closely parallel to the increasing lipoid infiltration of the theca cells. In the later stages of involution, however, Borell states that vitally stained histiocytes invade the inner zone of the follicle along with other connective tissue cells and capillaries, and that finally the vitally stained cells of different origins cannot be distinguished from one another. He also emphasizes
that/

that whereas rabbits and mice present similar appearances there are considerable differences in rats as regards the distribution of vital staining in the ovary. In my experience vital staining of the theca cells in mice by trypan blue is not confined to atretic follicles, though it may be more abundant in them; nor can the stained cells be identified with histiocytes since the morphology of their vitally stained granules is quite different from those of the latter type of cell. As mentioned previously certain differences in degree of staining have been observed between rabbits and mice.

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UTERUS.

The bi-cornuate uterus of the mouse becomes very deeply coloured with all the soluble vital stains and frequently remains deeply stained after the rest of the body has become almost completely decolorised. This is due to the presence of numerous histiocytes in the deeper layers of the endometrium, in the intermuscular stroma and in the subserous connective tissue. In these situations the vitally stained cells often form quite large masses; they store the stain with unusual intensity and retain it with peculiar tenacity. We have also observed, as Goldmann (1909) pointed out, that if an animal previously stained becomes pregnant, decolorisation of the tissues in general seems to occur more rapidly than usual, the dye apparently becoming transferred from other sites to the pregnant uterus which becomes very deeply stained. It is probable that transport of the dye is *via* the blood and lymph rather than by migration of stained cells from other localities. The sites of implantation rapidly become more deeply coloured than the remainder of the uterine wall, and can thus be recognised at an earlier period than in the normal animal. This observation may be of use in experimental embryology to locate the site of early fertilised ova. As previously mentioned the foetus does not share in the vital staining of the mother.

Details of the distribution of vital staining of the pregnant uterus and foetal membranes are given by Goldmann (1909) and Wislocki (1920).

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PANCREAS AND SALIVARY GLANDS.

In the pancreas vitally stained granules are not present in the secretory cells either in the acini or islets of Langerhans. In the interacinous tissue histiocytes with abundant dye granules are present and are closely applied to the basal membrane of the acini; in the resting state, their expanded processes extend between neighbouring acini (fig. 47). The vitally stained cells can be seen to lie definitely between the capillary endothelium and the gland cells, i.e. they are perivascular histiocytes and do not belong to the capillary endothelium (fig. 47). Vitally stained cells are more scanty in the islets of Langerhans, but an occasional histiocyte can be found between the capillaries and the islet cells. In salivary glands also vital staining is confined to the histiocytes in the interstitial tissue.

While supravital staining of the pancreas has not been carried out in the present study, it deserves mention on account of the important results obtained by Bensley (1911). He showed that supravital staining with janus green could be utilised to demarcate the islets of Langerhans in the pancreas of guinea-pigs. A solution of janus green 1-15,000 in physiological saline was injected into the aorta immediately after death, and perfusion was continued until the pancreas was of deep blue colour. In the absence of abundant free oxygen, the dye rapidly became reduced to a red (safranin) colour by the action of surviving tissues. This colour change occurred more rapidly in the acinous tissue than/

than in the islets, and a stage was reached at which the zymogenous tissue was stained red while the islets of Langerhans were of deep blue colour. This differentiation of colour could be preserved by injecting a solution of ammonium molybdate _via_ the ducts. It was found possible in this way to obtain specimens on which the total number of islets could be counted, and Bensley states that the pancreas of a guinea-pig may contain up to 56,000 islets. Neutral red may be used in a similar way for this purpose, and Bensley points out that when this dye is reduced to a colourless compound the colour may be restored by direct oxidation in the air. The staining of the pancreatic cells by janus green and neutral red is not due to colouration of mitochondria and segregation apparatus, but to the diffuse staining of cytoplasm and nuclei, similar to that shown in fig. 2 , (see p. 48).

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KIDNEY.

The degree of vital staining of the kidney varies greatly with dyes of different types. Dyes of group (a) e.g. trypan blue, vital new red, lithium carmine, etc. are excreted rapidly by the kidney and give rise to extensive intracellular dye deposits. Dyes of group (b) of less highly diffusible nature.such as isamine blue, may be scarcely excreted at all by the kidneys. After a single intravenous injection of trypan blue of the usual size, the dye appears in the urine in 15 minutes, the red impurity being excreted first. The maximum concentration of dye in the urine occurs during the first 12 hours and excretion continues in diminishing amount during the next few days. After about three hours coloured granules appear in the cells nearest the glomerulus and as the staining increases in intensity granules appear in cells further down the tubules, until ultimately even the medullary portion of the primary convoluted tubules contains dye granules. Intracellular staining increases in intensity during the first 48-96 hours, although the excretion of dye is by then diminishing. Maximal intracellular vital staining then persists for several days; thereafter it gradually fades, the granules disappearing first from the more distal cells in which they last appeared, but decolorisation is not complete for several weeks. The vitally stained granules appear first in the cytoplasmic zone between the nucleus and the so-called brush border of the cells; as staining proceeds, the granules fuse together and darken in tint; they/

they also pass deeper into the cell body, until in very heavily stained cells even the basal part is occupied by large deeply stained granules. Von Möllendorff (1918) states that the vitally stained granules are not formed from the mitochondria, but appear first in cytoplasmic zones in which the latter are absent. Mitochondria can be demonstrated in the vitally stained cells, apparently taking no part in the formation of the stained granules. Ludford (1928) has recently claimed that the granules stained by trypan blue are found in cytoplasmic areas in close relationship to the Golgi apparatus and he considers that this structure may be intimately connected with the absorptive functions of the cell. In some mice, the parietal layer of Bowman's capsule is lined by cubical cells morphologically resembling those of the proximal convoluted tubule with which they are continuous. These parietal glomerular cells become stained in a similar manner to the tubule cells (fig. 49). The endothelial lining of the glomerular capillaries and the visceral layer of flattened covering cells have not been observed to store soluble vital stains. The cells of Henle's loops do not usually store trypan blue, lithium carmine, or any of the other soluble stains used, nor have I been able to recognise with certainty the presence of vitally stained granules in the cells of the distal convoluted tubules, after the usual doses. In very intensely stained animals, however, tiny granules may occur in these situations, but they are so scanty and so minute that they are only apparent in very thick sections or in teased preparations./

preparations. Von Möllendorff especially has studied the excretion of a large series of dyestuffs by the kidney, and has confirmed the findings of Ribbert (1904) and Suzuki (1912) that vital staining is confined to the cells of the proximal convoluted tubules, but recently McMaster and Elman (1928) have stated that in animals injected with litmus vitally stained granules are found in the lining cells throughout the renal tubules from the glomerulus to the junctional tubules. They consider that the colour of the granules may be justifiably used as an indicator of the reaction and point out that this varies in different segments, the zone next the glomerulus always containing blue granules, while the zone next the junctional tubules always contains red granules. McMaster and Elman state further that the transition from alkaline to acid intracellular reaction occurs in the cells of Henle's loops during the secretion of neutral urine, but claim that the site of transformation moves proximally or distally according as the urine secreted is acid or alkaline.

That the reaction of the urine changes as it travels along the renal tubules is borne out by the following observations. Lithium carmine is frequently precipitated during its passage down the urinary tubules, apparently as a result of the increasing acidity of the fluid in the lumen, and in some cases the distal convoluted, junctional and collecting tubules may be almost obstructed by masses of precipitated dye. Baker and Dodds (1925) have shown that haemoglobin may be precipitated from solution in the tubules during excretion, owing to increasing acidity and salt concentration, and may give rise to intrarenal obstruction/

obstruction; this can be avoided by keeping the urine alkaline. Dunn and Polson (1926) have also shown that the zone of damage in "uric acid" nephritis corresponds to the broad ascending limb of Henle's loops and distal convoluted tubules and consider that the localisation of the destructive effect is probably due to the concentration of the urine with subsequent precipitation of "uric acid" where the reaction of the fluid becomes acid.

After a very small dose of trypan blue, e.g. 0.5 c.c. of 0.01 per cent solution for a mouse, vitally stained granules are found only in the proximal cells adjacent to the glomerulus and may not be present in every tubule. This is probably related to the intermittent circulation of the glomeruli (Richards and Schmidt, 1924) but when a larger quantity is given and the excretion of dye continues over a long period all the proximal convoluted tubules contain a number of dye granules, though the degree of staining may vary considerably. Other dyes of group (a) e.g. Lithium carmine, vital new red, are also excreted rapidly by the kidney and lead to similar intracellular deposits. When intracellular renal deposits are produced by isamine blue, they tend to occur throughout a greater length of the convoluted tubule than do trypam blue deposits of equal size. This difference is probably correlated with the greater slowness with which isamine blue enters cells generally; the dye is thus distributed throughout a greater length of tubule before absorption takes place. Other dyes of/

of highly colloidal type, e.g. benzopurpurin, produce even more minute and scanty renal deposits than isamine blue. After repeated intravenous injections of benzopurpurin in suspensoid form as previously described (see p. 31) redistribution of the dye occurs, with the liberation of a soluble form which stains the blood plasma. Very minute and scanty granules then appear in the cells of the primary convoluted tubules; frequently these can be recognised only in fresh preparations.

In general it may be said that the evidence of intra-vitam staining has been interpreted by most of the recent observers as confirmation of the filtration theory, though Marshall and Crane (1924) consider that they have obtained evidence pointing to definite secretion of phenol red by the tubule cells. Richards and his co-workers (1922, 1924) however, have shown by withdrawing the fluid from single glomeruli by means of a fine pipette, that in frogs phenol red and other dyes after intravenous injection are excreted in the glomerular filtrate. Hayman (1925) has shown further that if a dilute solution of the dye be injected into the glomeruli of a normal unstained animal, dye storage occurs in the cells of the convoluted tubule exactly as in cases where the dye is injected intravenously. These experiments would appear to offer conclusive evidence that the appearance of intracellular vitally stained granules is due to the reabsorption of the dye from the dilute filtrate in the lumen of the tubules. De Haan (1922) states on the basis of ultrafiltration experiments that the excretion of dyestuffs through the glomeruli cannot be harmonised with the theory

of a protein free filtrate, and suggests that the glomerular tuft is normally permeable to a small amount of protein from the blood, all of which is normally reabsorbed in the tubules. Wearn and Richards (1924) in their analysis of the fluid from single glomeruli point out that the glomerular filtrate is protein free as long as the circulation in the tuft is active, but when the glomerulus enters a resting phase and the circulation through the tuft is slowed, the filtrate often contains a small amount of protein.

It must be borne in mind that all the recent work on the vascular supply of the kidney is against the presence of a second supply of blood to the medulla by separate "arteriolae rectae" arising from the arcuate arteries. As Huber (1907) and Morison (1926) have shown the arteriolae rectae to the medulla are derived entirely from the vasa efferentia of the glomeruli. The blood in the capillaries around the tubules both in cortex and medulla is thus blood which has passed through the glomerular circulation and is therefore probably in a concentrated condition especially favourable to the absorption of fluid and other substances from the tubule cells.

In the interstitial tissue of the kidney there are scanty vitally stained cells. No staining with soluble dyes is found in the endothelial cells of the glomerular tuft, but between the tubules a few vitally stained cells are present in very close contact with the lining of the intertubular capillaries. These are more numerous in the junctional zone of cortex and medulla than

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in the outer cortex. In most cases it is impossible to decide whether, as appears probable, these are actually endothelial cells or whether they represent perivascular histiocytes. In the tip of the renal pyramid the more decided blue colour noted (p. 54) is due to the presence of vitally stained cells in the interstitial tissue; probably the majority of these are histiocytes. The results of intravenous injection of suspensoid substances support the views above expressed as to the nature of the vitally stained cells in the interstitial tissue. India ink and sugar of iron are both taken up by the endothelial cells of the glomerular capillaries to a small extent and the ingested particles may persist in this situation for several weeks. In the intertubular vessels soon after injection the deposit of particulate matter is heaviest at the boundary zone of cortex and medulla, and the injected substance is taken up by and is stored within what appear to be endothelial cells in the capillary walls. If these cells were perivascular histiocytes it is difficult to see how the particulate matter would reach them in sufficient quantity at such an early period. The deposit of suspensoids in this situation is more abundant than that of soluble vital stains, and it is possible that the relatively concentrated condition of the blood in the intertubular capillaries may accentuate the precipitation of circulating suspensoids on the capillary walls in this situation. The tip of the renal pyramid, however, is less affected by suspensoids than by

soluble/

soluble dyes; probably this is attributable to the majority of the vitally staining cells in this situation being extravascular histiocytes which are unaffected by the particulate matter.

The amount of deposition of particulate matter varies widely in different animals of the same species under apparently identical conditions. In most cases, however, the kidney becomes free from most of the foreign material within a few days, but traces may persist in the glomeruli and in the phagocytic cells between the tubules for prolonged periods.

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ALIMENTARY SYSTEM.

The staining of the alimentary tract previously described (see p. 55) is due chiefly to the presence of vitally stained histiocytes in the interstitial tissue. In no situation have the epithelial cells been observed to contain dye granules, though in some cases the faeces may be quite deeply coloured. As already mentioned no evidence has been obtained to show that any of the dyes used are excreted in the bile, and since staining of the intestinal contents is more marked at a lower level, presumably excretion of dye into the intestinal lumen occurs in the lower segments, especially the colon. Throughout the alimentary canal vitally stained histiocytes are scattered in the connective tissue of the wall and are more numerous in the submucosa. Vitally stained reticulum cells are also present in the stroma of the mucosa and villi. In mice the lymphoid-follicles form small prominent more deeply stained swellings in the wall, in these the reticulum cells are vitally stained and there is often a certain accumulation of histiocytes in their outer layers. The serosal cells covering the intestinal wall contain rather less numerous vitally stained granules than those elsewhere. Goldmann (1909) regarded the vitally stained "pyrrhol cells" in the intestinal wall as connected in some way with digestion and the transport of food materials, but according to Kuczynski (1922) the normal variation of individual animals would explain the results.

The variations in intensity of staining in different

segments of the bowel are not to be accounted for by obvious differences in the numbers of vitally stained cells. Again, the deep colouration of the caecum after the administration of trypan blue and the relative absence of staining after isamine blue are similarly unaccounted for by differences in the numbers of vitally stained histiocytes in the two cases. Since both dyes are excreted through the bowel wall it is possible that differences are due to diffuse staining of varying intensity in the epithelium which cannot be detected with certainty on microscopic examination.

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MAMMARY GLANDS.

Trypan blue is excreted in the milk of lactating mice and rabbits in small amount, yet sufficiently to colour the intestinal contents of the suckling young. With this dye no intracellular deposits are found, nor have they been observed with the other dyes used in this study. Evans and Scott (1921) state that dye granules appear in mammary gland cells after certain closely related substances, e.g. dianil blue 2R (C.I.No.465). I have observed an analogous condition in mice after intravenous administration of saccharated oxide of iron. After some days the epithelial cells of the mammary acini and to a less extent of the ducts are found to contain finely granular iron in considerable amount(fig. 64).

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MUSCULAR TISSUES.

The blue colour of muscles is due to the presence of vitally stained histiocytes in the connective tissue between the muscle fibres. These cells are sometimes closely applied to the capillary walls, in other places they form an almost continuous chain of cells between the muscle fibres and around the larger vessels and nerves. Where the connective tissue is more abundant the vitally stained cells are also more numerous - the so-called adventitial cells (fig. 50 ·). Normal sarcolemma cells remain quite unstained and the sarcous substance never exhibits granular vital staining.

The deeper staining noted in the tongue and heart muscle is due to the greater prominence of vitally stained cells in the intermuscular septa. In the heart the lining cells of the endocardium may contain fine blue granules in very intensely stained animals.

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BONES.

As has been previously mentioned (see p. 53) the centres of ossification in the ends of the long bones and in the short and flat bones of young animals possess a marked affinity for the dyes and after repeated administration of small doses become much more deeply stained than the cartilages or fully formed bones. The colouration of these areas is due to two factors:- (a) diffuse staining of the newly deposited bone and of the epiphyseal cartilage, and (b) the presence of many dye containing cells which form a continuous chain in close apposition to the bony lamellae. Many of these cells are stellate or spindle shaped; some appear to be the osteoblasts, others are free histiocytes. The bone-cells in the lacunae of the Haversian systems also show scanty fine dye granules resembling those of fibrocytes. Vitally stained connective tissue cells are also found along the shafts of the long bones where they form the endosteum, but here they are more elongated and spindle shaped and contain less dye than those at the epiphyseal ends. Presumably in this situation they are less active physiologically. The multinucleated bone cells, osteoclasts, have not been observed to contain any dye deposits, and we would agree with Shipley and Macklin (1917) that these must be highly specialised cells and cannot be regarded as simple phagocytes or scavenger cells.

NERVOUS SYSTEM.

The general absence of vital staining in the central nervous system has already been commented upon. In the true nervous elements no vital staining has been observed at any time with the acid dis-azo dyes. The staining of the choroid plexuses is due to the accumulation of numerous fairly fine dye granules in the cubical lining cells, and to the presence of a few large histiocytes in the fine stroma around the blood vessels (fig. 53). The ependymal cells lining the ventricles are in general unstained, but as the cells become larger and pass gradually into the lining cells of the tela choroidea, they come to contain dye granules in increasing number and size. The staining around the base of the infundibular stalk has already been described, and the absence of granular intracellular staining in this situation has been discussed (see p. 58). The only other area of the central nervous system which constantly shows evidence of vital staining is the pineal body. The staining of this organ is due to the presence of small intracellular dye granules both in the endothelial cells of its vascular capillaries and in the true gland cells (see Mandelstamm and Krylow, 1928).

In the peripheral nervous system no staining of the true nervous elements has been observed. Frequently the sheaths of the peripheral nerves appear deeply stained owing to the presence of histiocytes which are more abundant in the perineural connective tissue than in the subcutaneous tissue.

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The so-called vital staining of nerve fibres by methylene blue discovered by Ehrlich does not fall into this category, nor does it even appear to belong properly to the category of supravital staining, since it is obtained so long *post mortem* that the vitality of the nerve fibres does not seem to be necessary for the result.

The pale blue staining of the soft membranes is due to the presence of scanty histiocytes along the adventitial coats of the blood vessels which run on the surface and also in the substance of the nervous tissue, and also to the presence of fine blue granules in the flattened lining cells of the pia arachnoid. The intracellular granules in the lining cells resemble those seen in the mesothelial cells of serous cavities after moderate degrees of vital staining. Essick(1922) has claimed that the lining cells of the pia arachnoid give rise to macrophages in mild inflammatory conditions of the membranes, but it is possible that the more actively phagocytic cells found in such circumstances are derived from the histiocytes in the perivascular tissue (Kubie and Schultz, 1925, Kubie, 1927).

The dura mater is much more deeply coloured than the pia arachnoid and shows numerous darker blue flecks. The colouration is due to the presence of many histiocytes in the substance of the membrane , and these cells are aggregated in the perivascular tissue particularly around the ramifications of the blood vessels (fig. 51). The fibrocytes also appear to contain a considerable amount of dye, but I have not been able/

able to detect on the internal surface lining cells which present the morphology of mesothelial cells. As Mallory (1920) suggests, the lining cells appear to be simply flattened connective tissue cells (fibrocytes) which have not acquired the special characteristics of mesothelium. The intensification of vital staining of the dura at the points of exit of the peripheral nerves appears to be due to the presence of an increased number of histiocytes in these areas.

No noteworthy differences have been observed in the various animal species studied in respect of vital staining in the nervous system.

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DISCUSSION AND SUMMARY.

It has already been seen that certain cells have the capacity _in vivo_ for storing in their cytoplasm a series of substances some of which are soluble and others particulate. The process of storing dyes and coloured particulate matter has been defined as vital staining (see p. 76). One must conclude that this property indicates important functional capacities on the part of these cells and that normally they are engaged in absorbing and metabolising substances brought to them in the tissue fluids. It may be presumed that under pathological conditions they are likewise capable of absorbing and possibly of neutralising soluble substances of toxic nature, as well as dealing with organized structures, such as micro-organisms and tissue constituents. Therefore their behaviour and distribution have been extensively studied. As regards the use of soluble and insoluble substances as vital stains certain striking differences exist between these two classes, which are to be explained principally on mechanical grounds. Accordingly the results obtained with the soluble class will be considered first, special attention being paid to those obtained by intravenous administration which is the method most likely to bring about uniform staining in the different tissues. So far as the distribution of vitally stained cells in the mammalian body is concerned, it may be said that in all organs except the nervous system, there are cells (histiocytes) which possess in pre-eminent degree the capacity for vital staining, so that stained granules appear in their cytoplasm even when a relatively

weak solution of the dye is presented to them. Other cells again have less avidity for the dyes, and show staining only when the dye is offered to them in greater concentration. The histiocytes are referred to by various workers under different names (see p. 62) and special names have been applied to collections of these cells in particular situations, e.g. the perivascular and perineural aggregations of histiocytes and more primitive round cells have been called adventitial cells. Perhaps also the interstitial cells of the ovary and testis are to be classed with the histiocytes.

Aschoff and Landau have proposed the term "reticulo-endothelial metabolic apparatus" or "reticulo-endothelial system" in order to group under one head all the cells throughout the body which act as histiocytes. Their classification of vitally stained cells (slightly modified) is as follows, the elements being considered in order of ascending activity, as has been verified in the present work.

I. The endothelium of blood and lymphatic vessels generally. These cells take up the dyes only after very prolonged and intense administration, and then only in the form of most minute granules which are best seen by examining the fresh cells at a very high magnification. These granules are rarely visible in fixed and stained tissues.

II. The fibrocytes or ordinary connective tissue cells. These elements are stained fairly readily. They respond more actively to certain dyes than to others, e.g. more to vital

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new red than to trypan blue. The dye granules are usually fine and rather uniform in size. The mesothelial cells of serous sacs and the endothelial cells of the venous sinusoids of the spleen store vital stains to a similar extent, i.e. much less than the cells of groups III and IV.

III. The reticulum cells of the spleen both in the pulp and Malpighian bodies, and those of lymphatic glands and of lymphoid tissue generally. These cells are fairly readily stained, more so than those of group II, but less than those of group IV.

IV. (a) The endothelial cells which line the lymph sinuses of lymphatic glands, the sinusoidal blood capillaries of the liver, bone-marrow, suprarenal and pituitary glands. The reticulum cells of the marrow fall into this group in the rabbit and guinea-pig, but are less active in the mouse in which they belong to group III. All these cells are very active in storing vital stains of all kinds, but a certain amount of variation is found between those of different organs. The Kupffer cells of the liver are always the most active.

(b) The histiocytes or amoeboid wandering cells of connective tissue, also known as clasmatocytes, macrophages, etc.. These cells are very active in storing vital stains with which they are brought into contact.

(c)/

(c) Certain free mononuclear cells found in the spleen pulp and occasionally in the blood of internal organs. These are generally regarded as being derived from the cells of groups III, IVa and IVb, and to them various names have been applied. They are included within the group of "endothelial leucocytes" (Mallory) and have been called blood histiocytes (Kiyono).

Aschoff excludes from the true reticule-endothelial system the cells of groups I and II. He designates groups III and IV(a) the reticulo-endothelial system in the narrower sense, while these together with groups IV(b) and (c) constitute the system in the wider sense.

In view of the active part played by the reticulo-endothelial system both in the normal state and in pathological conditions in relation to the storage of lipoids, phagocytosis of micro-organisms etc., it is important to examine in more detail the basis of this classification. In the first place vital staining is a property of certain vascular endothelia. In some organs the capillary endothelium is endowed with special qualities, which are abruptly assumed without obvious transitions from the non-specialised endothelium. Thus the reticulo-endothelial system is highly developed in liver, spleen, and bone-marrow. It must be noted, however, that varying degrees of functional activity are found in different situations, e.g. the endothelium of the suprarenal and pituitary capillaries is less active in storing vital stains than that of the liver or bone-marrow, but more active than that of the general capillary bed. In the lymphatic system similar differences

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exist; lymph vessel endothelium behaves like the general blood capillary endothelium, but the sinusoidal endothelium of lymphatic glands abruptly assumes a pronounced vital staining activity in marked contrast to that of the afferent and efferent lymphatics. Secondly, in addition to these cells which are obviously endothelial there are found in the lymphoid and haemopoietic organs vitally stained stellate interstitial cells, the reticulum cells, which are disposed over the reticulum fibres. Although these cells do not form a lining to blood or lymphatic channels they cannot on other grounds be sharply separated from the previous group. As Kon (1908) and Corner (1920) have made clear, endothelial cells in certain situations, e.g. liver, marrow, lymph gland, spleen, suprarenal, kidney, etc. do actually produce the reticulum fibres on which they lie, and to them therefore the term reticulo-endothelium may justly be applied. A sharp separation between such capillary endothelium on the one hand and reticulum cells on the other cannot therefore be made, and their affinity is shown also by their behaviour towards vital stains. The third group of vitally stained cells, the histiocytes, are scattered almost everywhere throughout the connective tissues of the body in the stroma of the organs and in the loose areolar tissue, with a more pronounded accumulation around the smaller blood vessels and nerves.
It must be emphasised that, when we consider the minute histology of the different organs as regards the distribution of vitally stained cells, it is not always easy to decide

whether such cells lie in a capillary wall or just outside it, i.e. whether we are dealing with vitally stained endothelium or with adventitial histiocytes. I have encountered this difficulty especially in the kidney, but am inclined to the view that there are endothelial cells especially in the lining of the intertubular capillaries which are more active than their neighbours and which may on occasion behave like histiocytes. In the pancreas, salivary glands and thyroid on the other hand, the vitally stained cells do not seem to belong to the capillary endothelium but can generally be clearly seen to lie outside the wall, i.e. they are adventitial cells (histiocytes). It must also be emphasised that cells of the same type do not all exhibit at any one time a uniform capacity to store vital stains; thus after intravenous injection of dye it is the rule to find that in the organs one of several adjacent histiocytes is intensely stained and another is almost devoid of staining, while a third shows intermediate degrees of dye storage, just as has been observed in the histiocytes of subcutaneous tissue (see p.61-66). Accordingly from this and other observations it is justifiable to conclude that the functional capacity of such cells varies from time to time. The question therefore arises as to the relationships of the different members of the reticulo-endothelial system to each other. Some writers (Sabin, Doan and Cunningham, 1925) would separate into one class the endothelial cells and derive from them the histiocytes, while they place in another class the reticulum cells from

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which they consider the monocytes, myeloblasts and granular cell series develop. Against this view is the fact that as regards vital staining capacity and other characters transitions are met with between the reticulum cells and the endothelial cells on the one hand, and between monocytes and histiocytes on the other. Further, no essential difference in functional activity has been observed between these groups such as serves to distinguish the various classes of granular leucocytes (Muir, 1901).

It must be clearly understood that the distribution of vitally stained cells as described above refers only to the use of soluble dyes. After intravenous injection of particulate matter, e.g. india ink, the distribution of the stained cells is restricted, since in general these substances do not pass through the capillary walls. They are found in the reticulo-endothelial cells lining the blood-vascular system and do not usually reach the reticulo-endothelial cells of the lymph glands or the histiocytes of areolar tissue owing to the barrier interposed by the endothelium of the capillary blood vessels. In view of the relatively enormous extent of the interstitial tissue in organs, subcutaneous tissue, intermuscular fascia, etc., it is probable that the histiocytes constitute a relatively large proportion of the total number of cells in the reticulo-endothelial system in the wider sense. It has also been shown that the capacity of histiocytes to store soluble dyes and other substances cannot readily be exhausted by any dosage tolerated by the animal, and since/

since similar cells are constantly being formed in the tissue to replace physiological wastage, there must exist in the tissues a widespread reserve of undifferentiated cells which can rapidly produce new histiocytes in greatly increased numbers if occasion demands. Proof of this physiological wastage in internal organs is afforded by the continuous circulation of reticulo-endothelial cells from these organs to the lungs, while new cells arise to take the place of those desquamated. The subsequent fate of the substances transported intracellularly in this way is not known with certainty, but it has been suggested that the lungs may play an important part in the metabolism of lipoids and other substances (Stewart 1923). These are points which must be borne in mind in considering the possibility of throwing out of action the cells of the reticulo-endothelial system as a whole, i.e. the so-called reticulo-endothelial blockade, by injecting intravenously more or less inert substances such as india ink or saccharated oxide of iron, or by the use of soluble dyes. For these reasons above stated and others which will form the subject of a separate communication (see p. 383) I believe that effective inhibition of the functions of the reticulo-endothelial system, i.e. blockade, is a physiological impossibility. Although cells which have ingested large amounts of dye are not necessarily functionally paralysed, as has been shown by double-staining experiments, it has been noted that a proportion of the stained cells may fail to take up a subsequently injected substance. Further, it/

it has been repeatedly found that heavily dye-laden cells, while still able to absorb more dye in situ, are no longer capable of the rapid reactive migratory movements which characterise normal histiocytes. Such cells frequently fail to migrate in response to the stimulus of local irritation, which is then met by the development of new unstained cells from the omnipresent tissue reserve to which attention has been drawn.

The reticulo-endothelial system does not, however, include all the cells which show definite capacity to store dyestuffs in the form of granules, for in addition to the elements comprising this system one finds dye granules in the cells of the renal convoluted tubules, in the epithelial cells of the suprarenal glands and choroid plexus, in the interstitial cells of the testis and theca cells of ovarian follicles, and in some animal species in the hepatic cells. These are not included by Aschoff in the reticulo-endothelial system, but the fact that they may in conditions of intense administration exhibit a considerable degree of vital staining shows that this property is a very widely developed one and that vital staining is a relative term. In this connection I would emphasize that the mechanism by which dye storage is effected is not as yet understood. At present it is not justifiable to assume that the mechanism of dye concentration with the formation of intracellular deposits is the same in the epithelial cells of the kidney, liver, etc., as in the histiocytes and other cells of the reticulo-endothelial system. As has been

pointed out, epithelial cells may be permeable to dyes and transfer them from the blood into the secretion of the gland without giving rise to intracellular deposits, while on the other hand granules of the same dye may be present in the cells of other glands of the same animal without giving rise to colouration of the gland secretion. A striking example of this is afforded by a lactating rabbit injected with trypan blue; the mammary gland cells contain no dye granules, but the milk is coloured; on the other hand the liver cells contain granules of the dye but the bile is unstained. Further it is known that the liver excretes many dyes into the bile without the formation of intracellular deposits, e.g. brilliant vital red used in blood volume determinations, and dyes of the phenolphthalein group now used in liver function tests.
From what has been said it is clear that the formation of intracellular dye granules must depend on something more than the penetration of the dye into the cell, and that the additional factors concerned vary in different cells in the same animal and as between different animals.

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III. The late results of intravenous injection of colloidal iron.

Methods.

Liver.

Spleen.

Bone Marrow.

Lymphatic Glands.

Omentum, Mesenteries and Connective Tissues.

Mammary Glands.

Stomach.

Intestines.

Kidneys.

Lungs.

Suprarenal Glands.

Pancreas.

Brain.

Discussion.

During experiments on intravitam staining with insoluble suspensoid preparations, saccharated oxide of iron was found to be well adapted to intravenous injection and the distribution of iron after administration by this route was fully investigated. Saccharated oxide of iron is at first taken up by certain cells of the reticulo-endothelial system. Its distribution immediately after intravenous injection is similar to that of other finely divided particulate suspensions but iron oxide does not behave as an inert substance after deposition in the tissues and the further changes which ensue do not appear to have been adequately investigated hitherto. A series of experiments was accordingly undertaken in order to trace the ultimate fate of the injected iron, and to follow the alterations in its distribution and sites of storage with increasing lapse of time, and the results form the subject of the present study. These experiments differ from those recorded by Polson (1928; 1929) in that there is no flocculation of the iron solution in contact with the blood plasma; consequently pulmonary embolism does not occur and hence the lungs do not constitute a storehouse from which iron is absorbed slowly to be deposited in other organs.

Methods.

Mice were used chiefly, animals of about 20 grammes' weight being chosen as far as possible, but in some cases older animals weighing up to 30 grammes were employed. In a few experiments white rats were also used. A standard diet of brown bread, oats and milk was employed. The sexes were segregated throughout the experiments. A wide range of doses of saccharated oxide of iron was employed, in order to find the maximum safely tolerated dose, both total amount and concentration of the iron solution being varied. Dosage was always calculated per 20 grammes body weight, and the animals were weighed before each injection after fasting overnight. It was found that 0·3 c.c. of a 10 per cent. solution by weight of sugar of iron * in distilled water was the maximum safely tolerated dose, with the sample of sugar of iron employed at that time even when injection was performed as slowly as possible. Some animals tolerated considerably larger doses, e.g. 0·5 c.c. of a 10 per cent. solution, or 0·3 c.c. of a 20 per cent. solution, but these doses were found to kill a certain number of the injected animals, and could not therefore be employed. It was observed that after repeated injections, the mice appeared to tolerate larger doses than did animals not previously injected. The toxicity of different

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* This preparation contains 2·8 per cent. of metallic iron.

samples of sugar of iron appeared to vary, and some had to be discarded owing to their irregular effects.

It was previously mentioned that the saccharated oxide of iron used in these experiments formed an opalescent suspensoid solution which was actually particulate. Other samples of saccharated oxide of iron have been found to yield clear transparent brown solutions free from suspended particles; solutions of this character have been employed in recent work, and have in general yielded similar results. In a few experiments iron ammonium citrate has been administered intravenously in single or repeated doses. This salt is stated to form a non-colloidal solution, but the results following intravenous administration are strikingly similar to those after colloidal iron. Since this substance is fairly toxic it was possible to administer only small doses, but daily repetition seemed to lead to increased tolerance. A 1 per cent. solution neutralised with N/10 Na $H.C.O_3$ was administered in doses of 0·5 to 1·0 c.c. to rats of 150 to 250 grammes.

Intravenous injections were made into the tail veins of mice and rats and the animals were then killed at intervals from 10 minutes up to days and weeks after a single injection. Repeated injections were also given, at weekly intervals, beginning with 0·3 c.c., increased after the fourth dose to

0·45 c.c. of 10 per cent. solution and then to 0·3 c.c. of 20 per cent. solution, eight doses being administered to the animals in the longest experiments. To other animals a series of smaller doses, e.g. 0·3 c.c. of a 1 per cent. solution were given at intervals of a day or longer. Animals were examined weekly, one or more being chloroformed and the remainder reinjected; in some cases several animals were killed at varying intervals after the same number of injections. In all 80 mice and 10 rats have been used, the longest period of survival being 14 months after the last injection.

Histological Technique.

In the majority of cases the animals were killed when in good condition, but a few died of intercurrent disease. From some animals portions of omentum, mesentery and subcutaneous tissue from different situations were spread out on large cover slips and fixed while wet in 10 per cent. formol alcohol. Tissues were fixed in 10 per cent. formol saline, also portions of the various organs were at once placed in a warm mixture of potassium ferrocyanide and hydrochloric acid, and the reaction for the presence of free iron was noted. In some the lungs were inflated with formalin and the trachea ligatured; the peritoneum and pleural cavities were filled with 10 per cent. formalin through a fine glass pipette, and the animals were then fixed whole in formalin for 24 hours in

order to coagulate the blood in the great vessels, so that their cellular contents might be examined in sections. Tissues were embedded in paraffin and sectioned at 6 - 7 μ; for special purposes thinner sections were employed. Sections were stained by haematoxylin and eosin and others were examined for iron by pouring over them a mixture of equal parts of 4 per cent. potassium ferrocyanide and 4 per cent. hydrochloric acid, heated to 50° - 55°C. The iron reaction is thus obtained instantaneously and very sharply defined staining results. Treatment with ammonium sulphide was not found to demonstrate any additional iron. As a counterstain carmalum or acetic fuchsin was used, and sections were mounted in Gurr's medium as this has been found to preserve the prussian blue reaction indefinitely, whereas specimens mounted in balsam frequently become decolorised after months or years. The spread preparations of subcutaneous tissue, omentum and mesentery were treated with the iron reagents at 37°C., as warmer solutions frequently caused the tissue to leave the cover-glass, and were then counterstained with carmalum.

While the immediate effects of intravenous injection of saccharated oxide of iron have already been described it is convenient to recapitulate the results briefly here. Like other insoluble suspensoids, iron oxide is taken up by the reticulo-endothelial cells lining the blood vascular channels

and by a proportion of the circulating blood leucocytes which then leave the peripheral circulation and are retained temporarily in the internal organs. After a short time the iron becomes concentrated within the cells which have ingested it and the diffuse bluing of the cytoplasm gives place to a more intense granular blue staining on applying the prussian blue reagents. In course of time the cells appear to act further upon the intracellular iron and set free within the blood vessels a soluble iron compound in the plasma which causes the latter to assume a distinct diffuse blue colour, on treating sections with hydrochloric acid and ferrocyanide of potassium. Rarely traces of this are found 24 hours after injection, but, as a rule, it appears about 72 hours after injection and increases in amount during the following weeks. This soluble iron then continues to circulate in the plasma and gives rise to iron deposition in the organs of quite a different distribution from that initially produced by the intravenous injection.

The histological findings in the various organs after varying periods will first be described, the significance of the observations and their bearing on pathological problems will then be discussed. It is to be noted that mice are particularly suitable animals in which to observe the distribution of iron over long periods, for their organs and tissues

rarely exhibit any natural siderosis; the tissues of rabbits, on the contrary, are extremely variable in their iron content.

LIVER.

For the first 24 hours after intravenous injection the iron is confined to leucocytes in the sinusoids and to the endothelial cells and Kupffer cells lining their walls; its distribution is then fairly uniform throughout the lobules. During the following 96 hours two series of changes appear. In the first place, the engorged Kupffer cells and endothelial cells commence to migrate as described previously, some passing into the blood stream as free 'blood histiocytes' while others wander into the stroma of the portal tracts and Glisson's capsule where they accumulate as small foci of heavily iron-laden cells. (figs.56,58,59) This migration of laden cells is found after vital staining with any of the commonly used dyes or suspensoids, and when the ingested material is insoluble and inert, e.g. carbon particles, the hepatic connective tissue will continue to show its presence for very long periods. Iron under such circumstances is not, however, so inert as carbon and, while its presence is recognisable histologically for many months in macrophages lying in the portal tracts, the intensity of the intracellular iron reaction gradually diminishes and finally may practically disappear.

The second series of changes seems to result from the appearance in the blood plasma of soluble or loosely bound iron. The hepatic cells, which at first were completely free from all trace of iron, begin to exhibit in some animals definite accumulation of iron in their cytoplasm after about 72 hours, in others not until about 10 days. The cells first affected are generally those situated toward the centre of the hepatic lobules, so that the central veins and infralobular veins come to be surrounded by a zone of parenchyma cells in which the cytoplasm gives a faint diffuse prussian blue reaction. Sometimes the peripheral cells of the lobule are also affected or are affected even before the central cells, while those of the intermediate zone are still free from iron. It is a striking feature that individual cells in the affected zone may exhibit a very much more pronounced iron reaction than their neighbours, and in such cells the iron assumes a finely granular form earlier than in less affected cells. The iron content of the liver cells increases progressively during the next few weeks until usually all the cells are affected, (fig.56) but it remains most pronounced in the region in which it first appeared, viz. the central and peripheral zones while the intermediate zones are less affected. Six to eight weeks after a single injection the iron content of the liver cells undergoes diminution probably owing to continued excretion of the iron, but in the experiments of longest duration the

hepatic parenchyma cells were still found to contain histologically demonstrable iron.

With samples of saccharated oxide of iron yielding clear brown solutions a similar sequence of events is observed, but the iron is not taken up by the reticulo-endothelial cells as rapidly as is the suspensoid preparation. Iron ammonium citrate is also taken up first by the Kupffer cells but required to circulate for about 12 hours before intracellular granules appear. These differences are to be expected since it was previously pointed out that suspensoid substances are taken up much more rapidly than dyes in solution. With both soluble oxide and ammonium citrate of iron, however, storage in the liver cells does not usually appear until about ten days after injection, i.e. after an interval similar to that required after suspensoid preparation. These observations would suggest that in order to enter the hepatic and other parenchyma cells iron has to be linked in some way, perhaps in combination with proteins (Cloetta) and that this may be brought about by the activity of the cells of the reticulo-endothelial system.

After repeated injections of iron oxide at weekly intervals the amount of iron stored in the liver becomes very great. Following each injection there is the same cycle of changes described after a single injection: the iron is at first stored in the endothelial and Kupffer cells, which in

turn migrate from the sinusoids into the portal tracts etc. The amount of iron in these situations thus increases with each fresh invasion of iron-containing cells, and there is a continuous migration of iron-containing phagocytes along the portal tracts to the hilum of the liver where they may be seen after many weeks in large numbers around the main vessels and ducts, apparently making their way into the lymphatics draining the liver. Their rate of migration, however, is very slow; months after the last injection of iron the portal tracts still contain many iron phagocytes, (figs.57,58,59) and small masses of similar cells are found lying in the capsule beneath the peritoneal surface of the organ. The larger the amount of iron administered the longer these cells persist in the portal tracts and stroma of the liver. After a time some of the cells disintegrate and liberate their iron which is partly taken up by new phagocytic cells but may be partly absorbed into the fibrous stroma, giving rise to localised iron incrustation of the connective tissue fibres.

The hepatic parenchyma cells exhibit during the continued administration of saccharated oxide of iron a progressive increase in their iron content, and soon active storage is going on in the cells throughout the entire lobule. At first diffuse, iron in the finely granular form appears after about 15 days and becomes progressively more abundant within the

liver cells. After about 4 weeks a definite orientation of the iron granules within the cytoplasm is seen and accumulation becomes more pronounced towards the inner part around the bile canaliculi, which later become strikingly outlined by the bright blue granules (fig. 59). After the cessation of injections of iron, accumulation of iron in the hepatic cells continues and seems to reach a maximum about 3 - 4 months after the last injection. In one group of animals killed at intervals from 3 - 6 months after the last of a series of injections an approximately equal degree of iron storage in the hepatic cells was observed. The number of animals surviving beyond this period is too small to furnish a graded series but in the longest experiment, i.e. survival for 14 months, although this animal received only a very small dose (4 injections of 0·3 c.c. of 1 per cent. iron), the hepatic parenchyma cells still exhibit quite definite intracellular iron, generally in the form of fine granules heaped up along the bile canaliculi and rendering these structures clearly visible. In this case the iron is most abundant in the hepatic cells in the centre of the lobules but is absent from the bile duct epithelium, and there are no pronounced collections of iron phagocytes in the stroma of the liver, either beneath the capsule or in the portal tracts, only a few cells with a faint iron reaction being present in these situations. The sinusoids are lined

by new Kupffer cells free from even traces of iron. Presumably, as is seen in animals killed after a shorter duration, iron phagocytes were at one time present in the stroma but sufficient time has elapsed to enable an almost complete removal of the deposited iron to occur.

In the longer experiments, i.e. 6 - 8 months, the epithelial cells of the smaller bile ducts also give a sharp prussian blue reaction and in some animals contain very definitely granular iron (fig.59). In these animals, too, the nuclei of the hepatic cells are found to contain one or more iron reacting bodies of round or oval shape. When multiple they are often of unequal size and may be situated close to one another or lie quite apart (fig. 59). Morphologically they resemble plasmosomes and are similar to the bodies described by Lauda and Haan (1925) in haemolytic anaemia in rats.

It is noteworthy that in spite of the continued invasion of the portal tracts and capsule of the liver by iron-containing cells, many of which disintegrate and liberate their iron, and despite the long continued and very marked degree of iron storage by the liver cells, nothing in the nature of cirrhosis has been observed. In two animals a condition of lymphatic leukaemia with lymphomatous masses in the liver was found, but this must be regarded as a purely accidental finding unrelated to the experimental procedures.

SPLEEN.

As occurs with other suspensoids, the initial distribution of iron in the spleen is most abundant around the Malpighian bodies and the interfollicular pulp contains relatively few iron-storing cells. This phenomenon has already been discussed.

After a few days a gradual change in the distribution of iron in the spleen is apparent. In the Malpighian bodies iron accumulates very strikingly in the reticular cells particularly in those of the outer zone, while in the centre of the follicles the reticular cells are stained a much paler shade by the prussian blue reagents. (fig.60) At the same time the iron-containing cells in the pulp zone immediately around the Malpighian bodies appear to diminish in number, and in some animals this is so striking a feature that it almost suggests a direct migration of the iron-storing cells into the Malpighian bodies. Against this is the fact that the iron phagocytes in the outer zone of the lymphoid follicles are of stellate shape with long processes which can be traced for some distance between the lymphocytes. This suggests that they are not recently migrated cells as such cells would be rounded in evidence of their recent wandering. Usually, however, in spite of this transfer of iron the pulp immediately around the Malpighian bodies remains the richest part of the spleen in iron-containing cells for many weeks after a single

injection. Owing to the wide variation in the size of the spleen in apparently healthy mice, it is difficult to make a comparison on purely histological grounds between the total amount of iron in the spleen at different intervals, but the general impression from the experiments is that after the first few days there is no further increase in the total iron content of the spleen, and that by the end of 12 - 16 days the amount of iron present after a single injection has diminished. The endothelial cells of the venous sinusoids remain remarkably indifferent to the proffered iron and no definite iron storage by these elements has been observed soon after a single injection. It does not seem that this can be explained on an anatomical basis, since the blood must pass through the sinusoids either directly from the ellipsoids or after passing through the pulp. If it were merely due to the rapid removal of the iron by the pulp cells, one would expect to find iron storage in the endothelial cells of the sinusoids in those animals in which the interfollicular pulp remains comparatively free from iron owing to short circuiting of the blood directly into the sinusoids without passing through the pulp. In such animals, however, there is no evidence of increased iron storage in the sinus endothelium.

The soluble saccharated oxide is deposited in the spleen in the same way, but the accentuation at the periphery of the

Malpighian bodies is less pronounced. With iron ammonium citrate this feature is quite absent and the iron is scattered in cells throughout the pulp like soluble vital stains, e.g. trypan blue.

When a series of injections of saccharated oxide of iron are given the same cycle of changes is again observed after each injection, and iron accumulates progressively in the spleen. The iron is laid down first in the zone around the Malpighian bodies and in the pulp as described previously; later, more iron accumulates in the reticular cells of the Malpighian bodies which come to give a very intense iron reaction. These cells seem to be more numerous in the outer part of the follicles but as time goes on the reticular cells throughout the Malpighian bodies nearly all give an equally intense coarsely granular iron reaction. In the longest experiment (14 months) the Malpighian bodies actually appear to contain more iron than the pulp in relation to their size, and the number of stellate cells appears to be much greater than in earlier experiments. In some of the lymphoid follicles the iron-containing cells are more numerous towards the centre, (fig.61) and are clustered around the central arteriole, an appearance again suggestive of a progressive invasion of the Malpighian bodies. It appears likely that these cells are migrating toward the terminal lymphatics in the stroma

around the central arteriole. In the pulp the amount of iron present increases with each injection; the reticular cells become enormously engorged and the pulp spaces contain many mononuclear phagocytes stuffed with concentrated iron. These cells tend to collect into small groups in the pulp or along the trabeculae, and they may disintegrate there and liberate masses of concentrated iron; this may be partly taken up by new cells but it may also lead to iron encrustation of the connective tissue fibres of the trabeculae.

While the endothelial cells lining the venous sinusoids failed to store iron in the short experiments, iron appears in finely granular form in these cells about four months after a series of injections and persists in considerable amount in most of the animals surviving 4 - 8 months. In animals surviving beyond this period, iron storage in the sinus endothelium appears to diminish, and after 14 months (one animal) is almost absent. The number of animals is too small to permit of a definite statement, but it is noteworthy in this connection that in conditions in man in which iron storage by the sinus endothelium is known to occur, e.g. acholuric jaundice, the amount of iron is subject to wide fluctuations in relation to the attacks of blood destruction.

BONE MARROW.

In the early stages iron is deposited to a small extent in the endothelial cells of the sinusoidal capillaries, and in the stellate reticulum cells of the marrow, but the amount is relatively small, and is much less striking than in the liver and spleen. As previously described, the bone marrow of the mouse is much less active in storing soluble dyes and suspensoid preparations than that of the rabbit or guinea-pig. After a few days the amount of iron in the endothelial cells diminishes, while that in the reticulum cells increases and a few rounded iron-containing phagocytes are found along the capillaries. These are probably derived from the endothelium of the sinusoidal capillaries, which have migrated in response to the stimulus of phagocytosis, but some may be derived from reticulum cells which have become rounded under the same stimulus. In the later stages of experiments limited to a single intravenous injection, only traces of iron are found in the endothelial cells after one or two weeks, but the reticulum cells are found to retain the iron for a much longer time.

When repeated injections of iron are given the picture in the marrow is somewhat intensified, but the storage of iron is rarely very marked, and is always much less striking than in other parts of the reticulo-endothelial system. In

some animals I have observed a definite hyperplasia of the megacaryocytes of the marrow, but whether this is in any way related to the experimental procedures is not known, as it has not been constant. A similar condition has occasionally been found after administration of india ink. The immature leucocytes of the marrow exhibit no trace of iron at any stage.

With soluble oxide or ammonium citrate of iron even less iron storage in the marrow is found. Polson (1929) observed that the deposition of iron in the marrow was practically negligible after feeding or subcutaneous administration, and it would appear therefore that the bone marrow is not greatly concerned with the storage of iron apart from conditions of increased blood destruction.

LYMPHATIC GLANDS.

In some animals after about 24 hours, in others not until about 72 hours, the lymphatic sinus endothelium, the free macrophages in the lymph pathways and the reticular cells of the cortical nodes begin to exhibit a faint iron reaction which at first is rather diffuse but later becomes granular. This reaction, which becomes progressively more intense with passage of time, follows the appearance of 'soluble' iron in the blood plasma and tissue lymph. Within 72 hours after a single injection of saccharated oxide of iron the whole reticulo-endothelial system of the gland contains iron in a diffuse form and the lymph in the vessels and pathways of the gland gives a faint diffuse prussian blue reaction. After 2 - 3 weeks the lymph in the peripheral simuses of the glands gives a much more intense reaction with the prussian blue reagents, a nd the reticulo-endothelial cells contain iron in finely granular form (fig. 62). It seems reasonable to suppose that the absence of iron in the reticulo-endothelial cells in the early stages of the experiment is due to the impermeability of the general blood-vascular capillary endothelium to the colloidal iron, since, as has been pointed out previously (1929), substances passing through the blood capillaries of lymphatic glands are not available to the reticular cells of the cortical nodes or to the sinus endothelium

In animals receiving repeated injections of iron the changes in the lymphatic glands are intensified and a progressive accumulation of iron in the reticulo-endothelial cells of the gland goes on for months after the cessation of iron administration.

With iron ammonium citrate the lymphatic glands are sooner affected than after the saccharated oxide, probably owing to a more rapid passage of iron into the tissue lymph. The deposition is, however, similar, and it would thus appear that with the simpler iron compounds the form in which iron is administered does not play an important part in determining the final sites of deposition. It is therefore difficult to understand why Polson (1929) should have failed to find iron in lymphatic glands other than those directly concerned with absorption from lungs the seat of iron emboli (tracheo-bronchial group) and from the intestine (mesenteric and coeliac groups).

The histiocytes in the capsule of the gland and in the surrounding connective tissue also come to exhibit a well-marked iron reaction. The iron content of the lymphatic glands appears to reach a maximum about 6 months after the last of the series of injections; thereafter it slowly diminishes, but a considerable amount is still present even after 14 months. The presence of much iron in the reticulo-endothelial cells of the lymphatic gland does not appear to

lead to any ill effects and no fibrosis has followed the experimental procedures. The lymph glands would thus appear to be one of the more permanent storage depots for excess iron, and from them iron is liberated slowly to maintain the state of equilibrium with the blood and tissue fluids as excretion slowly proceeds.

OMENTUM, MESENTERY AND CONNECTIVE TISSUES.

The general structure of the mesentery and omentum and the distribution of histiocytes in these situations have previously been described. The relative permeability of their capillary walls to finely divided particulate matter in general has also been emphasised; accordingly, saccharated oxide of iron finds its way into the perivascular histiocytes within a few hours of intravenous administration. The histiocytes in the interstices between the fat cells around the omental and mesenteric vessels thus store the iron in a form similar to the intravascular histiocytes, e.g. the Kupffer cells. After 72 hours, as the soluble or diffusible iron appears in the plasma, iron storage begins to appear in the histiocytes lying farther from the vessels; ten to fourteen days later these cells give a very distinct diffuse iron reaction, and by the thirtieth day after injection iron in granular form is seen even in apparently quiescent cells lying in avascular portions of the omental and mesenteric tissues and in the tâches laiteuses. No iron storage has been observed in the mesothelial cells of the peritoneum and other serous cavities.

Repeated injections lead to a great intensification of the above picture, but the essential features are the same. It is interesting to observe that the diffuse iron reaction

appears in the histiocytes of the omental and mesenteric tissues considerably earlier than in those of the subcutaneous tissue. A similar condition is found in the histiocytes of the adipose tissue around the kidneys, pancreas and other abdominal viscera. It appears clearly that the capillaries in these situations are more permeable to iron and to vital stains than those of the intermuscular and subcutaneous tissues. In the latter situation the condition of the histiocytes has been investigated both in spread preparations and in paraffin sections: iron in the diffuse form appears in those cells after about thirty days and after longer intervals, e.g. four months, is found in granular form, but the intensity of the staining is less than that in the abdominal histiocytes except where they are in relation to iron-storing parenchyma cells, e.g. breasts. As was observed in connection with vital staining by soluble dyes, the histiocytes in the stroma of other organs, e.g. salivary glands, thyroid etc., give rise to a certain degree of coloration on treating the tissues with the prussian blue reagents, but no storage of iron in the parenchyma cells of these organs has been observed.

MAMMARY GLANDS.

In female mice the effect of iron administration has been investigated only in adults which had previously littered: no control experiments were performed on virgin mice nor has the effect of further pregnancy and lactation been tested. The cells of the mammary gland appear to possess a very strong affinity for iron, and begin to exhibit iron storage within a few days. After sixteen to twenty days the cells of the gland acini are filled with coarsely granular iron to a degree unequalled in any of the other organs which do not receive the iron directly, i.e. the mammary acinar cells give a much more intense iron reaction than do the hepatic cells or renal cells at the same time. The cells of the acini are much more active in storing iron than those of the ducts but the latter also exhibit definite iron accumulation (fig.64). Iron storage in the breast epithelium is very persistent and is maintained with great intensity up to at least eight months after the last injection.

STOMACH.

Iron deposition in the stomach is at all times scanty and is practically confined to iron storage in the histiocytes of the stroma. These cells occur singly and in little groups scattered throughout the various layers, and are more numerous in the glandular than in the squamous portion of the stomach. They tend specially to occur in small foci in the submucosa, and are rather scanty in the stroma of the mucosa itself. About four weeks after administration of iron, a faint diffuse bluing is visible along the free borders of the epithelial cells and glands in the gastric mucosa, but no granular intracellular iron has been found at any time. This appearance is similar to that observed along the villi of the duodenum before granular iron appears in the covering cells, but since it is not succeeded by a stage of granular intracellular iron it cannot with certainty be ascribed to iron absorption. It is possible that the diffuse reaction in the gastric mucosa may be evidence of iron excretion, but no definite statement can as yet be made. No further evidence of iron storage in the stomach is found in the later stages, though the amount of iron in the interstitial histiocytes is considerably increased.

INTESTINE.

The intestinal tract is at first practically free from iron after intravenous administration but about 14 days later iron appears within the stellate reticulum cells of the intestinal lymph follicles and interstitial histiocytes throughout the bowel wall. The large bowel, particularly the region of the caecum and first part of the colon, contains at this time distinctly more numerous iron-storing histiocytes than the small intestine, both in the interstitial tissue of the mucosa among the glands and in the submucosa. Similar cells are present but less abundant in the inter-muscular and subserous layers. At this stage no iron in granular form is found within the epithelial cells at any level in the bowel. Between the 20th and 25th days from the first injection, a faint iron reaction is seen along the free borders of the epithelial cells of the villi in the duodenum and upper part of the jejunum, and the material in the lumen of the glands in these situations may also give a faint prussian blue reaction. In the stellate reticulum cells of the intestinal lymph nodes and the histiocytes of the interstitial tissue iron is more abundant than at earlier stages and is assuming a granular form, but there is still no definite intracellular iron in the intestinal epithelium at any level. By the 30th day the epithelial cells along the free margins

and tips of the villi in the duodenum and upper jejunum contain numerous fine granules of iron, while the cells at the bases and in the glands are much less richly supplied(fig.65). The iron granules are situated in the free margin of the cells between the nuclei and the lumen of the bowel; the lymph in the terminal lacteals of the villi gives a distinct prussian blue reaction, and the histiocytes around these lacteals also give a strong iron reaction. Iron storage commences in the first part of the duodenum immediately beyond the pyloric ring, and is richest in the duodenum and first part of the jejunum; it gradually diminishes in intensity towards the ileum, in the lower part of which it is completely absent. In the caecum and lower levels of the large intestine I have not found any corresponding degree of intra-epithelial iron storage, though this is said to be the chief site of iron excretion (Hochhaus and Quincke, 1896). As regards the origin of the iron in the lumen of the small intestine, nothing definite can as yet be said but the appearances in the upper levels of the small bowel seem more suggestive of iron reabsorption than of iron excretion and correspond to the findings of Hochhaus and Quincke, and Cloetta (1900). If the iron is in course of reabsorption it may have gained entrance to the lumen of the duodenum either by ingestion in the food or by excretion from a higher level. As has been described, the evidence in favour of iron

excretion in the stomach is not conclusive. It is also possible that iron may reach the intestinal contents in the bile as a result of excretion from the liver since in these animals the hepatic cells show finely granular iron orientated in the neighbourhood of the excretory bile canaliculi. Iron arriving by this route might reach the first part of the duodenum above the entrance of the bile duct and even the gastric mucosa by regurgitation of duodenal contents, though whether this occurs in the mouse is not at present known.

With regard to the role of the iron-containing histiocytes in the interstitial tissue of the gastro-intestinal tract, I do not consider that any special importance can be attached to them in relation to absorption or excretion of iron. As has been previously pointed out, similar cells are readily demonstrated in these situations by vital staining with soluble dyes, and it is probable that the storage of iron is merely evidence of their general tendency to take up and concentrate substances of this kind which are present in the circulating blood. I have found no evidence that histiocytes play any part in excretion of iron or vital stains by direct intracellular transport as Hochhaus and Quincke suggest, the number of such cells in the interstitial tissue of the intestinal wall is not notably increased over that normally present, and I consider that the evidence points to a purely passive rôle in this connection.

KIDNEY.

The initial deposit of iron in the kidney has already been described as similar to that of other suspensoids, and consists in a deposition in isolated cells in the capillary walls both of the glomeruli and intertubular capillaries, especially about the junction zone of cortex and medulla. This is less striking with the soluble forms of saccharated oxide and has not been observed after iron ammonium citrate. In these situations the amount of iron gradually diminishes though here and there isolated cells may be found containing iron granules for many months after the last injection of iron. The condition of the cells of the convoluted tubules of the kidney is of great interest. In the early stages these cells exhibit no trace of iron, but within a period which seems to vary with different animals and with different samples of saccharated oxide, iron appears within the tubule cells. It is probable that the iron is excreted in the glomerular filtrate and is reabsorbed in the tubules, and it is noteworthy that this occurs only subsequent to the appearance of loosely bound iron in the plasma. After intravenous injection of iron ammonium citrate a similar sequence is observed in so far as no iron is found in the tubule cells until after several days at least. This appears to support

further the suggestion made previously that during this interval the iron is entering into some combination in which it is adapted to continuous circulation in the body fluids and which can be absorbed by parenchyma cells. It is of interest in this connection that some writers consider the glomerular filtrate normally to contain a small amount of protein which is completely reabsorbed in the tubules; this is stated to be necessary to explain the mode of excretion of certain vital dyes (de Haan, 1922). From the above observations it would appear that even relatively simple iron salts such as iron ammonium citrate are unable to escape directly through the glomerular membrane. As regards the subsequent appearance of iron in the glomerular filtrate it has not been determined whether this depends on the iron first being acted upon by the tissues in some way that renders it suitable for excretion, or whether its presence in the circulation increases the permeability of the glomerular tuft. In the mechanism of renal filtration some factors other than the size of the molecules must be concerned, for as we have seen simple dialysable iron salts do not pass through the glomerular filter whereas haemoglobin with an enormous molecule does so with great rapidity. Unless the amount of haemoglobin passed through is considerable no excretion of haemoglobin in the urine follows, an indication that complete reabsorption in the

tubules has occurred. In these experiments it would appear that all the iron passing through the glomerular filter is reabsorbed by the tubule cells since the amount of iron present in the urine is too small to be detected by simple chemical tests such as the prussian blue reaction or ammonium sulphide. In some cases as early as 24 hours after a single intravenous injection of saccharated oxide of iron the contents of some of the first convoluted tubules and the free margins of their cells give a faint diffuse iron reaction which becomes more distinct during the next 48 hours. After 72 hours, a faint diffuse prussian blue reaction is given by the cells of many of the proximal convoluted tubules, and in a few cortical tubules fine granules appear. During a further period of 20 - 30 days this tubular iron reaction usually becomes more distinct and the iron in the cells assumes a more definitely granular form; it persists for several weeks in similar intensity and diminishes gradually, but traces of iron persist even several months after a single injection. It is noteworthy that in kidneys the seat of patchy chronic nephritis of spontaneous origin, the iron reaction appears earlier and is more pronounced than in healthy kidneys. Presumably this is related to increased permeability of the glomerular tufts.

After repeated injections of iron the histological picture of iron storage in the kidney is merely intensified,

but not to a proportionate extent. The deposit in the glomerular and intertubular capillaries is more marked and more persistent, but the chief difference is seen in the more pronounced evidence of iron reabsorption in the convoluted tubules. (fig.66) In the most intensively treated animals in the series, i.e. mice receiving 8 intravenous injections of saccharated oxide of iron, the cells of the convoluted tubules continue to exhibit granular iron throughout the duration of the experiments. At the end of 14 months, which represents a large part of the average lifetime of a mouse, a few of the endothelial cells of the glomerular and intertubular capillaries and the cells of the convoluted tubules still exhibit traces of granular iron, though this animal did not receive the maximum dosage employed.

The histiocytes of the perirenal connective tissues at first contain no iron but after a few days a faint diffuse prussian blue reaction appears in the cytoplasm; twelve to twenty days later the iron has become very finely granular. When repeated injections are given iron storage by the histiocytes of the surrounding connective tissue becomes progressively more intense and assumes a coarsely granular form.

LUNG.

As noted previously, saccharated oxide of iron has proved to be stable in contact with the blood plasma, no gross precipitation occurring, therefore there is practically no immediate deposit of iron in the lung tissues. The endothelial cells of the alveolar capillaries are almost entirely devoid of phagocytic properties and it may be stated definitely that no clear evidence of iron storage by them has been observed at any stage of the experiments. The epithelium of the alveoli and bronchial mucosa likewise shows no trace of iron during the earlier stages. In addition to the normal blood leucocytes, which at first contain iron, (fig.67) there are found in the lung capillaries larger cells very heavily laden with iron which seem in some cases to form actual capillary emboli. These appear within an hour but are much more numerous after from 5 - 24 hours: they are desquamated reticulo-endothelial cells such as were observed in the blood of the hepatic veins and sinusoids (fig.68)

These desquamated reticulo-endothelial cells persist *in situ* for long periods and it is probable that all those which actually form capillary emboli finally die and disintegrate in the lungs. Small amorphous masses of dense iron pigment with no related cell body can be observed in the lung capillaries of animals in the experiments of longer duration but the probable date of their arrival in the lungs

cannot be determined since desquamating reticulo-endothelial cells are found in the internal organs for long periods even after the cessation of injections of iron.

When repeated injections of iron are given and the animals are observed at intervals over a long period, the most notable change in the lungs is shown by the normal "dust" cells, which are produced in response to the natural anthracosis in town-bred mice. These cells at first contain no iron, but after twelve to sixteen days they exhibit a faint diffuse bluing in some parts of the lung. This iron reaction is always diffuse at first and indeed it has only rarely been observed to become even finely granular. With increased duration, the coloration of the cells becomes deeper, but is generally still diffuse even after several months. It is noteworthy that all the lung phagocytes do not give an equally intense reaction: in some parts of the lungs the cells are only faintly coloured or are quite free from iron, in other parts the prussian blue reaction is intense. In the longer experiments some of the alveolar lining cells have been observed to give a diffuse iron reaction similar to that of the "dust" cells; they are generally swollen and project into the air vesicles suggesting that they are in a stimulated condition and are about to desquamate to become alveolar phagocytes; such cells may also contain carbon particles (fig. 68)

while *in situ* in the alveolar walls. The epithelium of the bronchial mucosa does not at any time participate in the storage of iron.

SUPRARENAL GLANDS.

In view of the sinusoidal character of the suprarenal capillaries, their lining cells exhibit surprisingly little affinity for saccharated oxide of iron, and only an occasional endothelial cell in the cortex or medulla is found to give a strong iron reaction. A few such cells are generally present in the glands at all stages indicating that, as in the kidney, those cells which have actually stored the iron intracellularly retain it for very long periods. This intracellular iron goes through the same transformations as in active reticulo-endothelial cells in other organs, i.e. it soon becomes intensely concentrated and granular; after four to six months it becomes faint, indicating gradual excretion.

In the cortex, the epithelium of the glomerular zone first exhibits a faint diffuse iron reaction 72 hours after a single injection. In some animals this is more marked than in others, but in no case does it become very pronounced or approach the degree found in the liver cells. Later the iron changes to the finely granular form. The cells of the zona fasciculata take no part in iron storage, but the zona reticularis, while more slow to begin the accumulation of iron, does eventually store it in an amount exceeding that of the glomerular zone. The cells adjacent to the medulla are usually the first to be affected, beginning to react about

twenty to thirty days after the first injection, and as time goes on the amount of iron in this situation increases and the cells further out in the zona reticularis become involved. (fig.69)
In the longer experiments, i.e. 6 - 8 months after repeated administrations iron accumulation in this zone of the suprarenal cortex is marked. It thus corresponds to the gradual accumulation previously described as occurring with dyes of the less diffusible type, e.g. isamine blue. I have not observed any trace of iron storage in the cells of the medulla, but Polson (1929) records iron in this situation in one animal although he never found iron storage in the cells of the suprarenal cortex.

PANCREAS.

The endothelial cells of the pancreatic capillaries do not appear to possess histiocytic propensities and do not store iron after intravenous injection of the saccharated oxide. In the experiments of long duration especially after repeated injections of iron, the histiocytes in the stroma of the pancreas around the vessels and ducts and between the acini accumulate iron just as do the histiocytes elsewhere, and in common with those of the other abdominal viscera they exhibit this reaction earlier and more markedly than do those of the subcutaneous tissues.

Even in the longest experiments after repeated injections the parenchyma cells of the pancreas have not been observed to contain any trace of histologically demonstrable iron, either in the zymogenous cells or in the islets of Langerhans. In all the experimental animals the pancreas has appeared healthy microscopically; no fibrosis or atrophy has been observed.

BRAIN AND NERVOUS SYSTEM.

In the early stages, especially after large doses of iron, there may be some deposit on the walls of the brain capillaries, but this is rapidly removed and thereafter no iron is found in the nervous tissue. The cells lining the choroid plexuses appear to possess little affinity for iron, and do not exhibit a degree of iron storage comparable with their avidity for certain vital stains. In the longer experiments only a few tiny iron granules were found in the lining cells, but the histiocytes present contain a rather greater amount of iron and those of the pia arachnoid generally are similarly affected. The nervous elements never exhibit any trace of iron, either in the brain, spinal cord or peripheral ganglia.

DISCUSSION.

The above experiments are of importance in relation to the mode of disposal of excess iron which is not due to blood destruction. Since each intravenous injection amounted to at least 1 mgm. of iron the maximum number of injections corresponded in all to approximately 11 mgms. of iron per 20 grammes body weight. Owing to the difficulties involved in the estimation of minute quantities of iron few investigations on the amount normally present in mouse organs have been recorded. Hall (1894), estimating the total iron content of mice excluding skin and alimentary tract but including the blood, found that young mice contain 1·25 mgm. and old mice 1·81 mgm. of iron, an average of 1·53 mgm. per mouse. It will be seen therefore that a relatively very large amount of iron has been administered in proportion to the size of the animals.

The deposition of the iron immediately after injection is similar to that generally observed after the administration of suspensoids and other foreign substances which act as vital stains. But the feature which it is desired to emphasize is that the iron deposited in the reticulo-endothelial cells does not merely behave as an inert substance like acid dyestuffs or carbon particles. With iron there are later transformations under the influence of cellular activity which lead to a redistribution. If the tissues be examined immediately after an intravenous injection of

saccharated oxide of iron, free iron is found in the blood in the vessels; this is always in finely granular form corresponding to the finely particulate nature of the solution. The free iron is rapidly removed, as it is taken up and stored in the cells of the reticulo-endothelial system which lie along the blood vascular channels, so that after 24 hours no trace of it is found in the plasma. A portion of the iron is also taken up by certain of the circulating leucocytes. The ultimate fate of the latter is uncertain but it is probable that after a temporary sojourn in the lung capillaries most of them finally accumulate in the spleen pulp and are destroyed there. The first evidence of the later transformations is the increased concentration of the ingested iron; fairly diffused throughout the cytoplasm to begin with, it soon becomes concentrated into smaller but more intensely staining granules. About 72 hours after injection the blood plasma again gives a faint iron reaction, but this is now diffuse and cannot be resolved into granular form even with the highest powers of the microscope. It is best seen where the corpuscles have settled to one side of a vessel leaving a zone of clear plasma which then turns faint blue on treatment with warm hydrochloric acid and ferrocyanide of potassium. This diffuse plasma reaction gradually increases in intensity and becomes very distinct fourteen to twenty days after an injection. When repeated injections

are given it continues to be present throughout the treatment and persists for several months after the cessation of iron administration. This diffuse reaction is evidently due to the presence of loosely bound iron in the plasma, and it is reasonable to suppose that it is derived from the iron taken up by the phagocytic cells which has been liberated again owing to their metabolic activity probably after being linked in some way to protein. This loosely bound iron is diffusible and passes through the capillary walls into the tissue fluids, from which in turn it gains entrance to the lymphatics and so reaches the lymphatic glands. Since iron appears within the hepatic cells, renal tubule cells, lymphatic glands and histiocytes only subsequent to the appearance of this loosely bound iron in the blood plasma and lymph it is reasonable to infer that the two processes are correlated. The transfer of iron from the engorged Kupffer cells to the liver cells appears to occur only by diffusion, and there is no direct transfer of granular iron. The rapidity with which this process occurs varies in different animals under similar conditions; in some it commences avout 72 hours after a single injection but in others it is absent at this early stage and appears only later - after seven to ten days. It is of course impossible to say when iron first appears in the liver cells in any given animal, but by comparing the degree

of iron storage in animals killed at varying periods after administration the conclusion has been reached that the interval required to produce iron storage within the hepatic cells presents wide individual variations. There is no definite explanation for the differences in site of initial deposition in the liver, in some animals periportal, in others central. Since all the animals were on a standard diet it does not appear that the differences can be attributed to variations in the nutrition of the animals as suggested by Schwarz (1928). Iwanaga (1925) states that colloidal iron ("hemosil") injected intravenously is first taken up by the hepatic cells and that it appears only later in the Kupffer cells and other reticulo-endothelial elements. I have not observed this phenomenon with any of the samples of colloidal iron I have employed either in the form of suspensoids or true colloidal solutions, nor does it occur with iron ammonium citrate. In all my experiments the iron is first deposited in the cells of the reticulo-endothelial system and is only afterwards transferred to the liver cells.

Although the amount of iron in the different organs has not been estimated by chemical methods, it is clear from the histological observations that the greatest amount remaining in the tissues after some months is present in the liver. Very high degrees of iron storage have been attained, and even the cells of the bile duct epithelium come to contain a

considerable amount of granular iron. In spite of this marked siderosis and the accumulation of iron-containing phagocytes in the stroma of the liver, no cirrhosis has been observed, and it must be concluded that siderosis alone is insufficient to produce cirrhosis, at least in mice, in the absence of some toxic factor the nature of which is unknown. The migration of iron-containing cells and the transference of iron to the lymphatics of the liver is of interest and suggests that probably a similar process is responsible for the very marked iron storage in the glands draining the liver and pancreas in haemochromatosis.

The complete absence of iron storage in the parenchyma of the pancreas in spite of the long continued subjection to excess iron in the circulating blood and tissue fluids is very striking. It is clear that excess of iron alone does not as a rule lead to storage in the pancreatic cells, and the additional factor which produces this change in the human subject remains undetermined.

The spleen has been found to retain a considerable amount of iron in the pulp for long periods; in addition a large amount of iron has been found in the lymphoid follicles in the later stages of the experiments. After haemolysis experimentally produced and in human pathology iron storage in the spleen pulp is a frequent finding, but the reticulum cells of the Malpighian bodies rarely participate in this

process. The explanation of this surprising finding which was common to all the animals surviving more than a few days is at present obscure. In Polson's experiments (1929), on the other hand, the Malpighian bodies remained free from iron although the pulp contained a large excess. It is also difficult to assess the significance of the late participation of the splenic sinusoid endothelium in iron storage, since these cells have previously been observed to remain relatively unaffected by soluble vital stains. This would seem to be another example of the selective affinity possessed by certain elements of the reticulo-endothelial system.

Iron storage in the lymphatic glands in all situations was also a marked feature of the longer experiments with saccharated oxide of iron. Since repeated intravenous administration of iron ammonium citrate also produces a similar widespread distribution of iron in the lymphatic glands it is difficult to explain Polson's results unless they are due to differences in the animal species employed. The liver, spleen and lymph glands in the mouse would thus appear to be the chief storehouses of excess iron not immediately utilisable in metabolism, and iron appears to be transferred from other sites to these organs. The liver takes up the greatest part of the iron and retains it longest, the maximum amount of intrahepatic iron being reached about four to six months after

the last of a series of injections; thereafter the iron content of the liver cells slowly declines.

It has long been known that iron absorbed from the intestine is stored principally in the liver and spleen. As Muir and Dunn (1915) showed also, iron liberated from the destruction of blood following injections of haemolytic serum is retained in the body, being stored very rapidly in the liver, spleen and kidneys. They considered that the character of the iron reaction shown by the liver and kidney cells depended on the absorption of free haemoglobin from the blood as contrasted with that due to phagocytosis of damaged corpuscles in the Kupffer cells. Further, they observed that siderosis of the renal tubule cells occurred in the absence of haemoglobinuria. Muir (1928) has since found that the injection of homologous haemoglobin free from stromata leads merely to siderosis of the kidney; under these conditions the liver parenchyma and the reticulo-endothelial cells generally do not take up the free pigment at all, so that no iron storage is observed in the Kupffer cells or hepatic cells. In the case of the kidney it is clear that the haemoglobin is excreted in the glomerular filtrate and is reabsorbed in whole or in part in the tubules in whose cells it is broken up with retention of the liberated iron in the form of haemosiderin. It is possible, however, that the

hepatic cells possess no capacity to break down haemoglobin. Their siderosis in haemolytic anaemia would then be due, not to direct absorption of haemoglobin, but to the storage of iron liberated secondarily from reticulo-endothelial cells which had ingested damaged corpuscles and broken down their haemoglobin. Muir and Dunn showed further that the iron deposited in the organs in haemolytic anaemia was rapidly utilised as new blood formation proceeded but the manner in which the iron was transported from the sites of temporary storage to the haemopoietic organs in which it was again built up into haemoglobin remained obscure. In my own experiments the presence of a great excess of iron in the organs led to a flooding of the blood stream with loosely bound iron in amounts large enough to cause the blood and tissue lymph to give a distinct diffuse prussian blue reaction. This would suggest that under normal conditions iron may be transported in the form of loose combination with the plasma proteins but since the amounts present are so minute they cannot readily be demonstrated.

The presence of iron in the epithelium of the duodenum and jejunum is also surprising. Hochhaus and Quincke (1896) and Cloetta (1900) observed a slight degree of iron storage in this situation in normal guinea pigs but not in mice. They showed that iron granules could readily be produced in the duodenal epithelium of mice by feeding with organic or

inorganic iron, and concluded that such granules were indicative of iron absorption. These views have been generally accepted. In my experiments the treated mice showed iron storage in the duodenal and jejunal epithelium only after about 30 days whereas control mice on a similar diet failed to show iron granules in this situation. Since a considerable amount of iron is excreted in the faeces the iron content of the food of the treated animals might be increased owing to contamination by excreta. Iron excreted in the stomach would also pass into the small bowel and give rise to a similar appearance. Though no definite evidence of iron excretion such as intraepithelial iron granules has been found in the gastric mucosa such evidence is also lacking in the caecum and colon which are generally accepted as the chief sites of iron excretion, and therefore iron excretion by the stomach cannot on this ground be denied. Further, other substances such as morphine are known to be excreted in the stomach and reabsorbed in the intestine. In haemochromatosis the stomach is intensely brown, and the pigmentation of the mucosa is due largely to the presence of iron. It may be that this accumulation is evidence of attempted iron excretion but that owing to the abnormal affinity of the tissues, the iron is fixed and retained. As regards the etiology of haemochromatosis, these experiments would seem to bear out

the contention of Muir and Dunn (1915) that the essential factor in this disease is the fixation of iron and that this is the cause and not the result of decreased elimination. As these workers state: "in haemochromatosis there appears to be an increased affinity of the cells for iron which leads to its fixation over a long period of time, though we cannot explain how this is brought about."

In the present series of animals the tissues have for long periods been overloaded with iron far in excess of their capacity for immediate elimination, yet nothing analogous to the lesions of haemochromatosis has been observed. The tissues seem to deal with a great excess of iron not derived from blood destruction just as they do with the iron resulting from increased haemolysis and no ill effects follow the presence of marked excess in the tissues. The rôle of copper and other heavy metals in producing fixation of iron in the tissues is not yet proven, and can only be settled by further investigation.

IV. The nature of the normal lining of the pulmonary alveoli, and the origin of the alveolar phagocytes in the light of vital and supravital staining.

The structure of the normal alveolar lining.

Vital staining of the normal lung.

Supravital staining of the normal lung.

Experimental investigations on the origin of the alveolar phagocytes.

(a) anthracosis produced by inhalation of a sooty atmosphere.

(b) pulmonary irritation produced by intratracheal injection of saline suspensions of insoluble particles.

(c) intratracheal injection of soluble dyes;
neutral red;
trypan blue.

(d) the role of the monocytes : are the alveolar phagocytes derived from monocytes ?

Discussion and Summary.

The following study deals with the behaviour of the lungs to vital and supravital staining under normal and pathological conditions. This involves a discussion of disputed questions concerning the nature of the lining of the pulmonary alveoli and the source of the "dust cells." The origin of the large mononuclear phagocytes which serve to remove from the air passages and pulmonary alveoli foreign particles which have gained entrance thereto has provoked discussion and controversy for over half a century. Some of the early investigators (Slavjanski 1869, Metchnikoff 1905) believed that these cells were derived from the circulating mononuclear leucocytes of the blood or monocytes, and this view has recently been again upheld by Foot (1927) on the evidence supplied by supravital studies with nile blue sulphate and by a silver impregnation method alleged to give specific differentiation of the monocytes. Other workers (Tchistovitch 1889, Herxheimer 1903, Briscoe 1908) attributed their source to the alveolar epithelial cells, and this has again been strongly supported by Sewell (1918) Westhues (1922, 1925) F. Gross (1927) Carleton (1927) and others. Within recent years it has been alleged, chiefly by American writers (Haythorn 1913, Permar 1920, Foot 1920), that the source of these cells is the endothelium of the interalveolar capillaries. The histiocytic nature of the alveolar phagocytes

has been advocated by Kiyono (1914) Lang (1925) Policard (1926) Fried (1927) and Gardner and Smith (1927). In a preliminary communication (1923) I presented evidence in support of the epithelial origin of the "dust cells"; the present study based on further experimental work confirms and extends the conclusion that under conditions of mild stimulation, such as result normally from the inhalation of particulate matter, the cells which become free in the alveoli and ingest the foreign particles are derived almost exclusively from the respiratory epithelium.

The structure of the normal alveolar lining.

In order to understand the present position of the controversy it is necessary to give a brief account of current views on the structure of the alveolar lining and of the experimental methods by which the problems have been studied. It was first suggested by Kölliker (1881) that the alveolar lining was composed of two types of cell, small cubical nucleated cells and large flat non-nucleated squames. This view has since been supported by many writers and appears to be generally accepted (see Ogawa 1920, and the descriptions and figures by Stöhr 1915, and Bailey 1925 in their textbooks of histology). According to this view the alveolar lining is believed to consist of two types of cell (a) small cuboidal granular lipoid-containing cells which occupy chiefly the intercapillary spaces and while

not strictly confined to the alveolar angles are most frequently met with in this situation; a small part of their protoplasm interdigitates with the processes of the other cells of the lining. These elements have recently been designated "septal cells" (Lang 1925). (b) Large flattened non-nucleated plates are alleged to form the greater part of the alveolar lining; these plates are said to cover the capillaries and sometimes to cover the intercapillary spaces also, at least on one side. Some writers (Foot 1920, Stewart 1923) allege that there is, in addition to the above two kinds of cell, a third variety which represents a transitional form. According to my own observations also, this transitional form is represented by (c) flattened nucleated cells very closely applied to the capillary walls and separated from the latter only by the reticular and elastic fibrils. These cells possess flattened protoplasmic processes which join with those from similar cells and with the non-nucleated plates. Most writers who have studied the respiratory epithelium have remarked on the difficulty or impossibility of separating the non-nucleated squames by maceration or by teasing or scraping the cut surface of the lung. Probably the number and importance of these non-nucleated squames has been over estimated, and while I am not prepared to deny their existence I believe that they form a relatively inconsiderable part of the alveolar lining. The

latter is composed chiefly of nucleated cells, the majority of which are stretched and flattened on the alveolar walls. When the lung is in its normal condition of expansion, these cells are kept stretched and are difficult to recognise, so intimately are they applied to the alveolar walls, but in collapse of the lung they become more conspicuous and resemble closely the cuboidal nucleated cells of the intercapillary spaces. When attempts are made to dissociate the squames by maceration or by scraping the lung surface, the dissolution of the intercellular cement substance by releasing the tension allows these cells to retract, so that the free elements no longer appear as flattened plates but shrink superficially, gaining at the same time in thickness. We believe that a similar change can be observed in the separation of these cells under pathological conditions. Miller (1923, 1928) holds similar views on the structure of the respiratory epithelium and describes flattened nucleated cells, the desquamation of which into the air spaces in pneumonia leads to the formation of alveolar pores. In places, however, the nuclei of these flattened cells appear to degenerate, losing their staining capacity in fixed preparations; in this way non-nucleated plates may be formed, but they do not constitute the major part of the alveolar lining. Granel (1919) Fauré-Fremiet (1920) and others have shown that the cuboidal nucleated cells of the alveolar

lining are rich in lipoids, and thus frequently present a vacuolated appearance in paraffin sections. Stewart (1923), confirming their findings, suggests that these lipoid-containing cells may play a part in relation to lipoid metabolism generally.

Striking corroboration of the epithelial nature of the lining cells of the air spaces is afforded by the recent work of Young (1928) who found remarkable proliferative changes in the marginal alveoli after intrapleural injection of certain substances, e.g. calcium salts. On the subpleural portion of the alveolar wall the flattened lining was rapidly replaced by a layer of cubical or columnar cells in which active proliferative changes were seen. Along the lateral walls these cubical cells passed by gradual transitions into the normal flattened lining. In Young's preparations it is possible during the earliest stage of the reaction to follow clearly the swelling and rapid proliferation of the normal flattened nucleated cells, but no trace of the non-nucleated squames can be detected. Seeing that this process of metaplasia starts in the alveoli under the pleura, there is no possibility that it can be due to a downgrowth of epithelium from the terminal bronchioles as Lang (1925) suggested to account for the obvious epithelial layers seen in partially collapsed alveoli under pathological conditions. Equally striking proliferative changes were found

in the serosal mesothelium, but this does not invalidate the evidence in favour of the epithelial nature of the alveolar lining cells. It is now generally accepted that serosal cells are much more closely akin to epithelium than other mesenchyme derivatives since, together with the renal and tubo-uterine epithelium, serosal cells originate embryologically in the mesenchyme of the intermediate cell mass. My own experiments on inflammatory exudates in serous sacs are in agreement with the view that serosal cells are highly differentiated and do not give rise to phagocytic cells to any considerable extent.

Most writers agree that the non-nucleated squames and the cuboidal nucleated cells are separate elements, and that the plates are not merely flanges or processes of the nucleated cells, but the occurrence of transitions between these two types in the normal lung is not generally accepted.

Recently very novel views have been advanced by Policard (1926) who claims that the epithelial lining of the air spaces undergoes degeneration during the latter part of intra-uterine life, and that a new lining is furnished by the mesenchyme cells of the lung stroma. According to this theory the air spaces come to be lined secondarily by mesenchymal cells of histiocyte type which produce fine protoplasmic extensions to cover the alveolar surface while the cell body and nucleus lie in the intercapillary spaces. The squames are then said to be mere processes of the histiocytes, and the "dust cells"

derived from the alveolar lining are thus brought into line with the other phagocytic cells of the body and are of mesenchymal origin. Rose (1928) claims that embryologically the lung is of dualistic origin, the bronchi being developed from endoderm and the alveoli from mesenchyme into which the bronchi grow. He regards the septal cells as mesenchymal histiocytes, and states that he was unable to recognise any epithelial lining on the alveolar walls in normal or pathological conditions either in man or lower animals. While such views may be theoretically acceptable as regards the role of the cuboidal cells in giving rise to phagocytes in the alveoli, they take no account of the striking epithelium-like proliferations which the alveolar lining undergoes in many pathological conditions, e.g. in Young's experiments (1928) and in "jagziekte" disease of sheep (Cowdry 1925). There is no evidence that histiocytes ever proliferate to form a continuous layer of cubical or columnar epithelium-like cells as a result of single or repeated stimuli. Accordingly the balance of evidence would appear to be against the views of Policard and Rose.

Vital staining of the normal lung.

It is impossible in a large city to obtain animals whose lungs do not show at least a trace of anthracosis. Mice are less objectionable than larger animals in this respect, as they

can be obtained of fairly uniform size and weight at an early age. I have generally employed in this work mice of about 20 grms. weight and as usual the dose of vital stain has been calculated on this basis. If a mouse receive a subcutaneous injection of trypan blue, 1 c.c. of a 0·5 per cent. solution per 20 grms. weight, the lungs after 48 hours are of normal pink colour, only the walls of the bronchi and vessels being stained blue. This coloration is due partly to the presence of vitally stained histiocytes in the interstitial tissue (fig. 73) and partly to diffuse staining of the elastic and connective tissues. The lung parenchyma shows practically no staining of any of the constituent cellular elements. After a series of injections producing intense vital staining of the subcutaneous tissue and other organs, the lung substance becomes of faint blue colour. The histiocytes of interstitial tissue are as usual deeply stained; in addition one can find here and there minute blue granules in the alveolar walls which sometimes are definitely within the flattened nucleated cells and cuboidal cells of the alveolar epithelium, but frequently their exact situation cannot be determined owing to the extreme tenuity of the lining. Occasionally cuboidal cells more deeply stained are found especially in the alveolar angles; these are more rounded than the majority and usually contain in addition granules of naturally occurring carbon pigment (figs. 74, 77). Most of the free "dust cells" however, do

not exhibit any vitally stained granules. The presence of minute dye deposits in the epithelial cells of the alveoli is not a unique occurrence, since as has been already pointed out epithelium in many organs, e.g. suprarenal cortex, choroid plexus, etc. usually contains small dye granules in similar conditions of intense administration. Even in very heavily stained but otherwise normal animals, the alveolar epithelial dye deposits rarely attain any considerable size, thus differing sharply from the deposits in the cells of the reticulo-endothelial system. The behaviour of the alveolar cells under conditions of stimulation will be discussed later. In heavily stained animals the most striking feature in sections of the lung is the occurrence in the lung capillaries of large vitally stained mononuclear cells and masses of dye granules (figs. 72,74). The nature of the latter can frequently be made out by serial sections since they are continuous with the cytoplasm of vitally stained cells. These are the desquamated cells from the reticulo-endothelial system previously described as "blood histiocytes" (p. 91) which have been carried to the lungs but have failed to pass through the interalveolar capillaries where they form small emboli (Kiyono 1914, Wentzlaff 1924). It is generally believed that most of these desquamated reticulo-endothelial cells disintegrate in the lung capillaries and this is borne out by the occurrence of masses of stain with no

related cell body. The fate of these cells and their contained foreign material will be further considered below.

Other soluble vital stains of group (a) have yielded similar results. With dyes of group (b) there is usually no evidence of vital staining in the alveolar epithelium but the histiocytes of the interstitial tissue around the bronchi and vessels contain vitally stained granules, and blood histiocytes are found in the capillaries. It is important to note that dyes of group (b) e.g. isamine blue, tend to flocculate in contact with the blood plasma (see p. 24) and if injected intravenously in strengths over 0·5 per cent. usually produce rapid death from embolism. Even weaker solutions of isamine blue, e.g. 0·4 per cent., sometimes give rise to small flocculi but without causing death. These are removed mechanically by the lung capillaries, and constitute a serious source of error in interpreting the appearances, since they are liable to be mistaken for true vital staining in the alveolar walls. Permar (1920) used this dye by the intravenous route and possibly some of the results described are due to the factor just mentioned. In guinea-pigs and rabbits similar appearances are met with, but certain differences have been noted. In guinea-pigs small haemorrhages in the lungs are not uncommon, and occasionally areas of chronic inflammatory reaction are found in the interstitial tissue; these features are known to be of frequent

occurrence among stock animals (Branham 1922). The amount of lymphoid tissue in the lungs of guinea-pigs is also very variable; in some, large peribronchial sheaths are present, in others such elements are scanty. As Seeman (1925) pointed out, the lungs of guinea-pigs normally contain a considerable number of granular leucocytes, and as has been noted previously (p.124) these cells exhibit well-marked vital staining of their specific granules when the animals are killed with abundant dye in the plasma (figs. 74,77). At longer intervals after injection this granular staining disappears. It has also been observed that, if the blood is taken into citrate saline and the leucocytes concentrated by centrifuging, films from the surface layer show no vital staining of the neutrophile and eosinophile leucocyte granules although such staining is subsequently found in sections of the fixed tissues of the same animal. It would appear that in the diminished concentration of dye in the blood citrate mixture the stain may be washed out of the leucocyte granules, just as it disappears when the concentration of dye in the plasma falls owing to excretion. It is also possible that the staining is an agonal phenomenon occurring during cell death in the presence of much free dye (Gross 1911), but against this hypothesis is the observation that leucocytes with stained granules are sometimes found in the alveoli. According to Siengalewicz and Clark (1925) trypan blue does not pass readily

into the fluid of exudates in serous sacs, but my observations render it doubtful if this statement is always true for the pulmonary alveoli. Sewell (1918) also noted staining of the specific granules of leucocytes in the lung with trypan blue, and stated that this was less pronounced in animals killed at longer intervals after injection of the dye. In addition there is also a larger amount of true vital staining in the cells of the alveolar lining than in mice similarly treated. In rabbits vital staining of the granular leucocytes has not been observed in the present experiments; in these animals too, vital staining in the alveolar lining is more pronounced than in mice. In both rabbits and guinea-pigs the difficulties already noted in mice arise in interpreting the appearances, and it is frequently not possible to decide in which cells fine blue granules are situated.

It has recently been suggested that the cuboidal cells of the alveolar walls are not of epithelial nature but are extravascular mesenchymal histiocytes. Lang [1] (1926) on the basis of tissue culture experiments traces the origin of the mononuclear phagocytes of the alveoli to these cells which he designates "septal cells" and which he regards as histiocytes because they possess phagocytic properties. Carleton [2] (1927) using the same methods has criticised Lang's views and points out that although the alveolar lining cells grow out and become

actively phagocytic *in vitro* this alone cannot be accepted as proof of their histiocytic nature since their behaviour in other respects conforms to that of epithelial cells. In my opinion also the evidence is completely opposed to Lang's view. That these cells fail to react to vital stains in the circulating blood except in conditions of very intense administration is sufficient to show that they are not ordinary histiocytes. Further, it cannot reasonably be suggested that they are primitive mesenchyme cells such as those in the omentum which are capable of developing into histiocytes upon stimulation, since in various pathological conditions the alveolar lining cells give rise to a complete layer of epithelium-like cells around the alveolar walls as previously mentioned.

I consider that the small intracellular dye granules of the cuboidal nucleated cells observed in some animals after very intense administration of vital stains are analagous to those of the hepatic, suprarenal, pituitary and other glandular cells, and cannot therefore be regarded as proof of the histiocytic nature of the stained elements. The subsequent behaviour of these cells after stimulation cannot be adduced as evidence in favour of such a hypothesis; the important point which concerns us here is that the alveolar lining cells while *in situ* exhibit none of the specific properties by which histiocytes may be identified, and contrast strikingly in their behaviour with the

true histiocytes of the interstitial tissue around the bronchi and vessels.

The intravenous injection of india ink leads to a varying amount of deposition in the lungs according to the dilution of the ink and the rate of administration (see p. 27). In all cases a certain amount of carbon is deposited during the first few hours after injection (Wislocki 1924). As mentioned previously there is at first a general precipitation of carbon on the capillary walls in all situations, but especially in the lungs. This precipitated carbon is clearly extracellular (fig. 70), and later (after 24 hours) the greater part of the deposit may have been washed away and the capillaries are then practically free from pigment. I have not observed definite phagocytosis on the part of the capillary endothelium. As described in connection with the effect of suspensoids on the blood cells (see p. 96) ink-storing leucocytes tend to accumulate in the pulmonary capillaries, where they are retained for some hours at least. During this period they may become flattened and closely applied to the endothelium; they have thus frequently been mistaken for phagocytic endothelial cells (Foot 1927). Later they disappear from the lungs, and their subsequent fate has already been discussed (p. 95). At longer intervals after injection, i.e. from 24 hours onwards, the lung capillaries contain an increasing number of heavily stained

cells, the blood histiocytes already mentioned (fig. 74). The nucleated cells of the alveolar epithelium remain free from ink at all times, but care must be taken not to confuse appearances due to naturally occurring anthracosis. With other suspensoids this difficulty does not arise, and it may be definitely stated that suspensoid substances do not gain entrance to the alveolar epithelium directly.

After intravenous injection saccharated oxide of iron is not usually precipitated in contact with the blood, and the massive capillary coating and embolism seen after india ink is therefore absent. The colour of intracellular iron does not mask cell structure as does carbon, and when the prussian blue reaction is developed the presence of even small amounts of iron can be recognised. Oxide of iron has the further advantage that it is less liable to be confused with naturally occurring pigment. As previously mentioned (p. 97) the percentage of vitally stained monocytes is higher than after india ink (fig.71); saccharated oxide of iron has therefore several advantages as a vital stain to distinguish monocytes in the lung tissue. The fact that although saccharated oxide of iron at first behaves like any other insoluble suspensoid but that it later undergoes changes which result in the liberation of a soluble iron product into the blood plasma may be advantageously employed for studying the fate of materials

deposited in the lungs by the blood histiocytes. This process of mobilisation of iron in soluble form is well marked about 10 days after the commencement of a series of injections of iron. The soluble iron diffuses through the capillary walls, and is then stored in the peribronchial histiocytes, the cytoplasm of which thus give a prussian blue reaction which at this time is diffuse but several weeks later becomes finely granular. Iron storing histiocytes persist in the interstitial tissue in this situation long after iron-containing blood histiocytes have ceased to be found in the capillaries; this is discussed later. The alveolar epithelium may also give a diffuse iron reaction and this is especially the case in cells in the neighbourhood of impacted iron-containing histiocytes, and particularly where the epithelium is in a condition of stimulation from commencing ingestion of inhaled dust (fig. 72). Thus it comes about that a proportion of the "dust cells" also give the iron reaction. The iron in the epithelium and dust cells assumes the granular form at a much later date than that in the extravascular histiocytes. A similar sequence of events has been observed after intravenous injection of benzopurpurin used as a suspensoid which is taken up by the cells as such and thereafter slowly passes into a soluble form (see p.32). Hence localised staining of the alveolar cells adjacent to impacted dye-laden blood histiocytes occurs. With more readily

soluble dyes such as trypan blue, on the other hand, this localised staining is not so prominent a feature; possibly owing to the greater solubility of this dye the remains of disintegrated cells are removed more readily.

Supravital staining of the normal lung.

The general methods of supravital staining have already been described (p. 34). The reaction of the pulmonary cells to neutral red may be investigated in a variety of ways. Immediately after the death of the animal the lung tissue may be scraped or teased in saline containing the dye in appropriate concentration (1/20,000 to 1/100,000) or the lungs may be distended by injecting a similar solution through the trachea; portions are then fixed for sectioning while others are scraped or teased in the same fluid and examined fresh. It is also possible to produce almost immediate vital staining of the cells by injecting intravenously 0·5 c.c. of a 1 per cent. solution of the dye in distilled water (it is insoluble in saline in this concentration); but the tissue must be examined within a few minutes since the dye is rapidly decolorised by the action of the living tissues.

In teased or scraped preparations of the lung stained supravitally with neutral red, various types of cells can be distinguished. Granular leucocytes are frequently present in

considerable numbers especially in guinea-pigs, and their specific granules become definitely stained. The endothelial cells of the blood capillaries and the epithelial cells of the bronchial mucosa also exhibit tiny scattered orange-red granules. Some of the dust cells show abundant neutral red granules and vacuoles in addition to their ingested carbon, but others are practically free from the dye. The epithelial cells of the alveolar lining are detached during the preparation of the specimen, and the flattened nucleated squames undergo the shrinkage with increase of thickness already referred to; they are then difficult to distinguish from the cuboidal cells. The protoplasm of the latter often contains small refractile droplets which may be so abundant as to fill the cytoplasm; these are usually present but are less numerous in the flattened cells. The nature of these droplets is obscure; they are distinctly more refractile than the granules and vacuoles of the segregation-apparatus of histiocytes. In the high dilutions of neutral red used for supravital staining their coloration is feeble but in the higher concentrations (1/10,000 to 1/1000) they stain more deeply but present a homogenous character without the more intensely stained granules seen in the segregation-apparatus of histiocytes. After fixation they are said to stain with nile blue sulphate, neutral red and other fat stains, and they are blackened by osmic acid (Stewart 1923). In contrast with the

granules of histiocytes they persist as free globules when the cells are disintegrated by crushing, and when in the free state they retain their affinity for neutral red. In my experience rupture of histiocytes stained with neutral red leads to rapid blanching and disappearance of the stained granules; Sabin and her co-workers (1925) have also observed this phenomenon. It is not possible, however, to differentiate clearly the two types of intracellular granules stained with neutral red in the cytoplasm of intact cells. According to McMaster and Elman (1928) the stained granules which appear in the cytoplasm of the renal epithelial cells after administration of litmus can also be expressed from the cells and appear to be composed of a fairly stiff gel-like material. The evidence on the whole suggests that the refractile droplets are of lipoid nature and for this reason the cuboidal nucleated cells are referred to by Granel (1919), Fauré-Fremiet (1920) and other French writers as "les cellules à graisse." It is therefore clear that the neutral red reaction of the alveolar lining cells is not of the same nature as that of histiocytes. As has been pointed out (p.178) it is questionable whether the mechanism of vital staining is the same in epithelial cells as in those of the reticulo-endothelial system.

Permanent preparations of supravitally stained tissues may be obtained by fixation in the alkaline Zenker-formol mixture of

Gardner and Smith (1927) followed by rapid dehydration in mixtures of alcohol and benzol. Even with this method there is a certain amount of diffusion of the dye, and the results on the whole are not very satisfactory. In sections of lungs fixed 10 to 20 minutes after <u>intravenous</u> injection of neutral red, the dust cells free in the alveoli are usually unstained but those closely applied to the alveolar walls exhibit scattered neutral red granules of varying size. The histiocytes of the interstitial tissue around the vessels and bronchi exhibit the extensive neutral red granulation of such cells elsewhere, and the leucocytes show staining of their specific granules as previously described. The reaction of the alveolar lining cells to neutral red administered by the intravenous route consists in the appearance within both cuboidal and flattened cells of a variable number of stained granules usually of small size; in the former a few larger globules may also be present. These neutral red granules are found in a greater proportion of the lining cells and are more numerous than are trypan blue granules after even the most intense administration. They are not identical with the refractile droplets previously described, since the latter are dissolved out in the paraffin process.

As regards the significance of neutral red staining and its relation to true vital staining it has been previously

emphasised that neutral red stains granules and other structures in many cells which fail to store the acid dis-azo dyes. The results obtained with the former are therefore less specific than with the latter class. In support of this it may be mentioned that in mice injected intravenously with neutral red the hepatic cells which in this animal never store trypan blue contain neutral red granules in much greater abundance than does the alveolar epithelium. Accordingly the presence of small neutral red granules in the latter cells is not proof of their histiocytic nature. The effect of intratracheal injection of neutral red will be considered later (p. 266).

Experimental investigations on the origin of the alveolar phagocytes.

Before proceeding to consider the results of experimental studies on the origin of the alveolar phagocytes in conditions of mild stimulation induced by the inhalation of smoke or by the intratracheal injection of insoluble particulate matter, it is convenient to state at this point certain general conclusions which I have reached and which form a basis for the experimental use of intravitam staining as a means of tracing the origins of cells taking part in inflammatory reactions.

Conditions governing the use of intravitam staining as an aid to the identification of cells taking part in inflammatory processes.

I. If an animal receive a first dose of trypan blue during the production of an inflammatory exudate, no vitally stained cells can be expected until about six hours have elapsed, as this period is necessary for the formation of true intracellular dye deposits (see p.60). This statement does not, of course, refer to the staining of the specific granules of the leucocytes which may occur very rapidly and is of quite a different nature. Failure to find vitally stained cells after this period may be due to a variety of causes; the cells may possess no capacity to stain vitally, or they may be in such a situation that the circulating dyestuff is not available to them.

II. By repeated injections of a suitable dye, e.g. trypan blue or isamine blue, the cells of the reticulo-endothelial system can be selectively demarcated. The histiocytes of the connective tissues throughout the body are thus stained. If pulmonary irritation be induced after an interval sufficient to reduce the amount of circulating dye to a negligible level but before decolorisation is complete, the presence of vital staining in any considerable proportion of the cells of the exudate permits

the conclusion that such cells are derived from those which were stained prior to the formation of the exudate. In order that this conclusion may be justified it is essential that the condition specified above be fulfilled, viz. that there be not sufficient dyestuff free in the body fluids to account for the staining of the cells after emigration or proliferation.

III. The procedure detailed under II when combined with an intravenous injection of a contrasting dye (lithium carmine or vital new red) given under the conditions specified in I extends the information obtainable regarding the orgin of the cells of an imflammatory exudate. It may be concluded that cells which contain only the second dye have arisen directly from unstained precursers, and that they have acquired the capacity to stain vitally owing to their altered functional activity.

IV. The ability of cells in a pulmonary exudate to store vital stains may be conveniently tested by administering the dyes intratracheally.

V. Since a considerable proportion of the circulating monocytes store suspensoid substances, the relation of these cells to an inflammatory exudate may be ascertained by injecting intravenously a suitable dose of india ink or saccharated oxide of iron. The absence of vital staining

from the cells of the exudate would suggest that monocytes do not participate in the inflammatory reaction to any considerable extent. The objection may be raised that india ink is unsuitable for experiments on the lungs owing to the risk of confusing pigment particles with those of carbon inhaled naturally. This danger is not great, since the ink particles in monocytes are much more dense and opaque than the fine carbon particles in recently formed dust cells; in the larger pre-existing dust cells the morphology is so different from that of monocytes that confusion is unlikely. Further, the results have been controlled by comparison with those obtained after intravenous injection of saccharated oxide of iron and of benzopurpurin suspensoid. Difficulty might arise in distinguishing vitally stained blood histiocytes from monocytes, but under the conditions of the experiment the former are present only in negligible numbers. Evidence regarding the relation of the blood histiocytes to the cells of the exudate is provided by the conditions of II (above).

From the foregoing considerations it is clear that in order to obtain evidence from intravitam staining as to the participation of different types of cells in inflammatory lesions, e.g. in the lungs, it is essential to compare the

results obtained by a variety of methods and by combinations of these.

Anthracosis produced by the inhalation of a sooty atmosphere.

In this experiment two series of mice were used together with an adequate number of controls. (1) One group were subjected to intense intravitam staining receiving 5 to 12 injections of isamine blue 1/150 1 c.c. per 20 grms. weight, at weekly intervals, and after a varying number of days were exposed to a sooty atmosphere in order to produce anthracosis. (2) In a second set of mice trypan blue was employed instead of isamine blue, because cells take it up from the plasma and become stained more rapidly. Daily injections of 1/200 or 1/500 were given according to the duration of the experiment during the production of the anthracosis, in order to maintain an adequate supply of dye in the plasma while the cellular reaction was taking place. These animals were accordingly not vitally stained before the first exposure to soot.

A smoky atmosphere was produced by burning turpentine under a tin connected by means of an air-pump with a bell-jar under which the animals were exposed. In this way the concentration of soot could be maintained at any desired intensity and fresh air could be brought into the jar as required. After a few preliminary trials it was found that exposure to

a suitable smoky atmosphere for a period of 2½ hours led to a definite sooting of the alveoli, and by repeating this dose for six days a well-marked anthracosis could be produced without serious ill effects. The first group, i.e. animals vitally stained with isamine blue,were divided into two sets and both were subjected to this smoky atmosphere for varying periods up to 15 hours over six days. During this time some animals, group 1 a, received no further vital stain, and therefore had no dye in their plasma, while others, group 1 b, received a daily injection of isamine blue in order to maintain a constant supply of dye in the plasma during the period of formation of the anthracotic cells. The animals were killed after varying periods and the tissues were fixed in Zenker's fluid (see p. 25), a small quantity of the fixative being injected into the trachea which was then ligated prior to opening the thorax. The lungs and other organs were then immersed in a large amount of the same fluid and were subsequently embedded in paraffin.

The experimental procedure has induced a well marked anthracosis in the lungs of the experimental animals. The inhaled carbon is deposited first on the bronchial walls, and tends to be especially heavy at the bifurcations of the bronchial tree. A sufficient quantity of soot has also penetrated to the air sacs and alveoli to set up a well-marked phagocytic reaction, but owing to the relatively slow cellular response to particulate matter deposited by inhalation, the formation

TABLE I.

Group 1a.—Mice vitally stained with isamine blue, no dye in plasma.

Four mice received repeated injections of isamine blue and after an interval of one to four weeks were subjected to the sooty atmosphere for varying periods. In all cases the other tissues were examined to determine the presence of adequate vital staining; the degree of staining of the histiocytes around the bronchi and main vessels of the lungs served as controls in individual sections.

Injections of dye	Interval between last injection and first exposure	Number of exposures and total duration	Survival after first exposure	Histological findings in lungs
5	4 weeks	1 exposure of 1 hour	3 hours	Slight deposit of soot in lung, chiefly on bronchial walls. No evidence of phagocytic reaction in bronchi or alveoli. No vitally stained cells seen in alveolar walls.
5	4 weeks	1 exposure of 3 hours	5 hours	More definite deposit of soot in alveoli, but no evidence of phagocytic reaction. Carbon lies precipitated on the alveolar walls clearly extracellular. No vitally stained cells seen.
7	1 week	6 exposures of 2½ hours daily = 15 hours	15 days	Marked generalised anthracosis, still much soot extracellular. Many more free phagocytes than normally, but these are so heavily laden with soot that no cellular details are visible. No vitally stained cells seen in alveoli. Migration of phagocytic cells to lymphatics is commencing.
12	2 weeks	6 exposures of 2½ hours daily = 15 hours	17 days	Marked anthracosis, still some carbon free but greater part is intracellular. Cells are all free and rounded up, no cytoplasmic details visible. Migration of carbon laden cells to lymphatics. Patches of irregular pneumonia present but mononuclear cells of exudate are not vitally stained.

TABLE II.

Group 1 b.—Mice vitally stained with isamine blue; abundant dye in plasma.

Five mice received repeated injections of isamine blue and were exposed to a sooty atmosphere for fifteen hours in six periods of $2\frac{1}{2}$ hours daily. Thereafter they were killed at intervals from 24 hours to 18 days after the last exposure.

Throughout the exposures and up to the date of death isamine blue was administered subcutaneously at frequent intervals so that a plentiful supply of dye was maintained in the plasma while the reacting cells were being formed.

Number of injections of dye	Period of survival		Histological findings in lungs
	After first exposure	After last exposure	
14	7 days	1 day	Well marked anthracosis, but much soot on bronchial walls especially at bifurcations. A considerable number of phagocytic cells already formed and others in process of formation by desquamation from the lining. Blood histiocytes seen in alveolar capillaries, taking no part in the reaction. No vital staining of the reacting cells. Practically no migration of carbon bearing cells into lymphatics.
10	15 days	9 days	More marked anthracosis, less free soot and more abundant phagocytosis than in previous animal. No vital staining of reacting cells seen. Commencing lymphatic migration of carbon bearing cells, and some carbon is seen in the cells of peribronchial lymph nodes.
21	22 days	16 days	Anthracosis less marked, but lymphatic migration is much more pronounced than in previous animals. Still traces of free carbon on alveolar walls, and a few phagocytes are seen in process of formation in alveolar walls. None of the reacting cells is vitally stained.
11	22 days	16 days	Marked anthracosis, but still soot free on alveolar walls. Many phagocytes in alveoli and on walls but no vital staining. Lymphatic migration is well advanced, and some of the peribronchial lymph nodes contain carbon.
11	23 days	17 days	Anthracosis less pronounced than in the three preceding animals, alveolar phagocytes still being formed on the walls, and a little extracellular carbon remains. No vital staining of the reacting cells is seen anywhere.

Table III.

Group 2.—Mice not previously stained: injections of trypan blue during exposure to sooty atmosphere.

Four mice received an injection of trypan blue about three or four hours before each exposure to a sooty atmosphere lasting 3 to 4 hours. The number of injections and exposures is shown in the table. The animals were killed after the intervals shown below; blood from the heart was collected in citrate saline, and after centrifuging, the leucocytic layer was examined for vitally stained cells, and the presence of free dye in the supernatant fluid was noted.

Number and duration of exposures to soot	Number of injections of dye	Period of survival after first exposure	Histological findings in lungs
1 exposure	1	18 hours	Slight amount of soot in alveoli, but no cellular reaction. No vitally stained cells in alveoli. Histiocytes of interstitial tissue show early vital staining.
2 exposures	2	26 hours	Distinct increase of soot in alveoli, very early cellular reaction, but most of soot extracellular. Reacting cells appear to be alveolar lining cells. No vital staining of these cells but interstitial histiocytes are well stained.
4 exposures	4	5 days	Well marked anthracosis, with pronounced cellular reaction. The phagocytes are seen in all stages of formation and appear to be derived chiefly from the cuboidal cells; majority are free from dye, but a few show traces of vital staining. Blood histiocytes are present in the capillaries, but take no part in the reaction. Migration of cells to the lymphatics is commencing. The interstitial histiocytes are brilliantly stained.
5 exposures	5	9 days	Well marked anthracosis, cellular response less pronounced than in previous animal. Practically no vital staining of the reacting cells. Lymphatic migration more advanced. Blood serum contains less dye than previous animal, but is still distinctly coloured.

of the alveolar phagocytes is difficult to follow, and staining in the cells if present is generally obscured by the intense phagocytosis of carbon. In the animals of group 1 (a and b), the long interval which has elapsed in most instances since the soot was deposited has allowed nearly all the cells which have reacted to attain the character of fully developed free alveolar phagocytes, and only occasionally can one find a cell apparently in process of migration. In group 2 where the interval after exposure to the sooty atmosphere was shorter, the formation of the free phagocytes can be seen more clearly. In such cases the reacting elements belong to the alveolar lining cells, and appear to be derived chiefly from the cuboidal nucleated cells of the alveolar angles and intercapillary spaces (fig. 75). There is apparently also some participation of the flattened cells in the process. It is clearly brought out that the inhaled soot which has reached the alveoli is removed by intracellular transport; the pigment-laden cells are in part expelled _via_ the air passages in the sputum, and in part they migrate along the lymphatics to the mediastinal lymph nodes.

Pulmonary irritation produced by intratracheal injection of saline suspensions of insoluble particles.

As the previous studies had failed to yield decisive results, a further series of experiments was undertaken in

which a fluid suspension of insoluble particulate matter was injected into the trachea of vitally stained animals. By this means a much more rapid mobilisation of reacting cells is produced in the lungs than follows the inhalation of particulate matter in a dry state. A weak suspension of finely ground carmine or india ink was usually employed as the alveolar irritant, and minimal quantities of the substances were injected in order to avoid masking slight degrees of vital staining in the exudate cells.

A series of 21 guinea-pigs from the laboratory stock were used in this experiment. Eight pigs were used as histological controls, their tissues being examined by a variety of methods after exsanguination. Thirteen pigs were subjected to intense vital staining with trypan blue; of these five served as vital staining controls, while the remaining eight were anaesthetised and a small quantity of carmine or india ink suspended in saline was injected into the trachea. The animals were killed at short intervals, and the presence of abundant dye in the plasma was determined as before. In all cases the lungs were partially inflated with 10 per cent. formalin in saline and the trachea was ligated before opening the thorax. The organs were fixed in formalin, and paraffin sections were prepared.

As a result of the intratracheal injection of finely divided particulate matter in a fluid medium, a large number

of new mononuclear phagocytes appear in the air sacs and alveoli of the experimental animals. One of the most striking results is the extraordinary rapidity with which particulate matter in a fluid medium is taken up by phagocytic cells, in contrast to the relatively slow ingestion of particulate matter deposited in the lungs by inhalation. This is well known, and was emphasised by Mavrogordato (1918). Within half an hour finely divided particles of carmine in a fluid medium are already actively taken up by the preformed dust cells and by some of the alveolar lining cells _in situ_. After three to five hours phagocytosis of the finer particles is almost complete. India ink and saccharated oxide of iron are taken up with equal rapidity. The active cells are clearly derived from the lining cells of the alveoli, but it is desirable to emphasise that not only the cuboidal cells of the intercapillary spaces, the "septal cells" of Lang, but also the flattened nucleated cells take part in the process, the steps of which appear to be as follows. Some of the lining cells begin to absorb the particles of carbon, carmine, etc., while still _in situ_. The nucleated squames, at first closely applied to the alveolar wall in a flattened state, soon separate themselves from the wall, at first retaining their stretched and flattened shape. The next stage appears to correspond to what has been described as occurring when attempts are made to separate the squames by maceration or by scraping the cut surface of the lung. As soon

TABLE IV.

Guinea-pigs intensely stained with trypan blue, abundant dye in plasma throughout. The animals were anæsthetised and a small quantity of a fluid suspension of finely divided particulate matter was injected into the trachea, the animals being held vertically.

Animal No	Material injected	Survival Period	Histological findings in lungs
G.P. 5	1·5 c.c. ½ per cent. carmine	Died under ether, lungs fixed after 30 min.	Much free carmine in alveoli. Phagocytosis by preformed dust cells well marked. Commencing ingestion by flattened and cuboidal cells, many of which show desquamation. Most of these exhibit slight degree of vital staining. A few polymorphs with vitally stained granules in alveoli, some show commencing phagocytosis. No monocytes observed.
G.P. 10	1 c.c. ½ per cent. carmine	3½ hours	Coarser masses of carmine in alveoli still free, but most of finer granules intracellular. Preformed dust cells contain much carmine. Many new phagocytes in process of formation from the lining cells; these show traces of vital staining. Polymorphs very scanty.
G.P. 13	1·5 c.c. 1 per cent. india ink	1½ hours	Injected ink has penetrated well into alveoli; most abundant in subpleural alveoli. Most of the carbon particles are already intracellular, but the majority of the phagocytic cells are still attached to the alveolar walls and are seen in various stages of desquamation. A few small epithelial sheets attached to wall by delicate strands, these contain carbon particles, also faintly vitally stained. The desquamating cells show traces of trypan blue. No polymorphs seen in alveoli.
G.P. 4	1·5 c.c. 1 per cent. ink	3 hours	General features similar to G.P. 13, ink almost completely intracellular. Many phagocytic cells free in alveoli but a still greater number are attached to the walls and are seen in various stages of desquamation. Some of the free cells are becoming more rounded, others are still sharply angular. The free and attached cells show no greater degree of vital staining than in G.P. 13.
G.P. 3	1·5 c.c. 1 per cent. ink	12 hours	General features similar to G.P. 4, but majority of the carbon laden cells are now separated from the walls and lie free in the air space. Only a few cells of flattened type show commencing desquamation, and most of the free cells are rounded or oval. Vital staining of exudate cells is scanty and bears no relation to the stage of formation of the phagocyte. Polymorph granules well stained, no monocytes seen in alveoli.
G.P. 2	1·5 c.c. 1 per cent. ink	18 hours	This animal was stained with trypan red which did not show up well in sections. There is a well marked phagocytic reaction with more numerous polymorphs than usual in some places, and these are accompanied by monocytes. Only few of these blood cells contain much carbon. Most of the alveolar phagocytes are rounded up, but a few are still attached to the walls by long processes. No vital staining seen in any of the cells.
G.P. 6	1·5 c.c. 1 per cent. ink	30 hours	No free carbon particles in alveoli. Almost all the reacting cells are completely separated from the walls and are rounded up. In some areas a greater amount of ink has penetrated into alveoli and has brought about marked polymorph and monocyte migration. Both types of cell contain a small amount of ingested carbon, but as usual the greater part of the pigment is within large mononuclear cells. No increase of vital staining of exudate cells in comparison with those of earlier stages.
G.P. 1	1 c.c. 50 per cent. ink	96 hours	Posterior aspects of lower lobes show deep blue patches, extending into lung substance about 2 mm. These subpleural alveoli contain relatively little ink, the bulk of which is in the central part of the lungs, but the free phagocytes in this zone are much more deeply stained than those in any other experiment of this series. In the central part the alveoli contain very numerous phagocytes, but these as usual show only traces of vital staining. In some parts small giant cells are present and show varying degrees of vital staining. Polymorphs are almost completely absent, and no monocytes can be recognised.

as the intercellular cement substance is loosened so that the cells become detached from their neighbours they retract markedly apparently from release of the tension and appear free in the alveolar space as sharply angular cells with oval or rounded nuclei (fig. 76). They contain within their cytoplasm the ingested particles which provided the stimulus for their migration. It must be emphasised that generally the process of separation is extremely rapid so that only occasionally the actual steps by which it is accomplished can be followed. Carbon particles in the form of india ink appear to be less irritating than some of the other substances used; after injection of this substance into the trachea desquamation of cells is less rapid, and the stages in the formation of the alveolar phagocytes are more readily observed. After separation from the wall the cytoplasm of the cells becomes more abundant and the sharp angles became rounded. The nucleus does not increase proportionally and remained relatively small. Sometimes the lining cells desquamated in a small sheet, and formed small multinucleated cells as early as 1¾ hours after the introduction of the irritant (fig. 78); in the longest experiment in this series, i.e. 96 hours, such cells are fairly numerous in some parts of the lung (fig. 79). In some alveoli the appearances suggest that separate cells have fused to form giant cells but it is not possible to state definitely

the modes in which all the giant cells have arisen. The cuboidal nucleated or "septal" cells also play a part in the formation of the mononuclear phagocytes. These cells do not seem to possess much capacity to ingest foreign matter as long as they are in the alveolar wall, probably because the surface they normally expose to the alveolus is small but they readily undergo changes which lead to their active participation in the reactive process. When about to migrate into the air sacs they become increasingly vacuolated and swollen (figs. 77,86) so that they project from the space in which they lie. They then gradually detach themselves and enter the alveolus as free cells of oval or round shape. Occasionally they appear to migrate into the alveolus on the opposite side of the septum from that to which they actually belong, and they could then be seen passing between the cells of the lining, as elongated or constricted cells like leucocytes in the act of migrating from blood vessels (fig. 80); these are the cells which Permar (1920) described under the impression that they arose from the endothelium of the interalveolar capillaries. After a short time the phagocytes derived from the two different types of alveolar lining cells become indistinguishable; both appear as round or oval cells with a relatively small nucleus and bulky cytoplasm containing ingested foreign matter and frequently globules of fatty material. In the experiment of longest

duration in this series, i.e. 96 hours, the majority of the phagocytes are still within the air vesicles; little or no evidence of migration to the lymphatics is seen, but a small number of the heavily laden cells appeared to be in course of excretion by way of the air passages.

In spite of the previous intense vital staining of the animals and the continued administration of the dye so that the plasma contained a large amount of stain throughout the experiments, the degree of staining in the alveolar phagocytes is extremely variable not only as between one animal and another but also in different parts of the lungs of the same animal, though as a rule most of the cells show little staining. A similar result has been observed by others (Carleton[2] 1927) and seems to depend on the degree of staining of the alveolar cells prior to the intratracheal injection. This appears from the fact that in the experiments of $\frac{1}{2}$ to 4 hours' duration, i.e. those in which the interval was less than that required for the new formation of intracellular dye granules, the newly formed alveolar phagocytes exhibited minor degrees of vital staining at least in some part of the lung, though the degree of staining varied even in adjacent alveoli. In the sub-pleural alveoli the cells tend to contain more trypan blue than in the central part of the lung, irrespective of the amount of particulate matter which had entered the alveolus.

It is clear that in these short experiments the newly formed vitally stained phagocytes must have already contained dye granules when the particulate matter was introduced. In the longer experiments, 4 to 96 hours, no constant or noteworthy increase in the degree of vital staining is observed, as compared with the short period experiments. During the longer interval the flattened desquamated cells and the migrated cuboidal cells enlarge and become oval or rounded until ultimately they are indistinguishable from one another, but these changes are not accompanied by any progressive increase in the degree of vital staining. Yet here and there more intensely stained cells are found especially in the subpleural alveoli (fig. 81). Possibly this is the result of stimulation by the injected irritant at a time when the plasma is saturated with dye. It is also noteworthy that in some animals there were foci of interstitial and alveolar inflammatory change clearly of longer duration than the experimental pulmonary irritation; in these areas the mononuclear cells are not more intensely stained than those produced by the experiment. In some animals, e.g. G.P.6, killed 30 hours after intratracheal injection, a relatively large amount of ink has penetrated into some alveoli; in these areas the exudate is more abundant and the desquamated alveolar epithelial cells are accompanied by polymorphs and monocytes (fig. 82). Both the latter types of cell are also

phagocytic, but, as other experiments have shown, these cells tend to migrate relatively late; hence before their appearance in the alveoli most of the carbon has been ingested by the desquamated lining cells. The granules of the polymorphs are usually lightly stained with the trypan blue, and some of the monocytes also exhibit commencing dye storage. In these more severely affected areas only a few alveolar epithelial cells remain _in situ_; these are swollen and vacuolated but as a rule exhibit enhanced degrees of vital staining only if they happen to have taken up carbon particles.

The evidence provided by these experiments clearly points to the alveolar lining cells as the first line of defence in the lung parenchyma. The epithelial cells are rapidly shed and assume a phagocytic function; monocytes are only occasionally present and are always accompanied by polymorphs; but vital staining of the actively phagocytic cells is relatively scanty and bears no constant relation to the conditions of the experiment.

An attempt was next made to determine whether the traces of vital staining noted in the phagocytic cells of the alveolar exudate in the previous experiments were present in the cells prior to the introduction of the irritant or whether they arose as a result of stimulation by the ingested foreign material.

A rabbit of 2 kilos weight was vitally stained by 6 injections of trypan blue. The blood serum was examined at intervals, and after four weeks only traces of dye were present in the serum although the animal still exhibited well marked blue staining. 11 c.c. of 5 per cent. lithium carmine was then given intravenously, and immediately thereafter 2·5 c.c. of 1 per cent. india ink was injected intratracheally. The animal was killed after 26 hours.

Microscopically there is an active phagocytic reaction in the alveoli, but the majority of the cells are rounded and only occasionally are flattened cells seen in the act of desquamation. Many of the cuboidal nucleated cells are more swollen and vacuolated than in normal animals, but neither they nor the free phagocytes as a rule show any trace of carmine staining. Occasionally a free cell or a swollen vacuolated cell show a few faint carmine granules, but this is exceptional. None of the alveolar phagocytes show any trace of trypan blue, although the histiocytes of the lung stroma and elsewhere still contain a considerable amount of the dye. The latter also show definite carmine staining which is far in excess of that shown by both free and attached alveolar cells. From this observation it may be concluded that neither the free phagocytes of the alveoli nor their progenitors exhibit much capacity to absorb and store dyestuffs present in the circulating blood,

even when in a mildly stimulated condition. As noted in normal animals, the alveolar epithelial cells appear to store vital stains only when the cells of the body which are more directly concerned with intracellular dye storage, i.e. the reticulo-endothelial system, are practically saturated with the dye.

Intratracheal injection of soluble dyes.

The action of dyes introduced directly into the alveoli was investigated in order to determine whether the alveolar phagocytes were capable of storing the vital stains if brought into direct contact with them. In such experiments it is unnecessary to add particulate matter to the fluid injected to stimulate the alveolar cells, since as has previously been noted, the dye solutions are *per se* capable of producing a mild inflammatory reaction at the site of injection.

Intratracheal injection of neutral red.

In view of the extreme rapidity with which cells become free in the alveoli and exercise their phagocytic action (see table IV) the effect of intratracheal injection of neutral red was studied to see whether the desquamation of lining cells previously noted was accompanied by any noteworthy alteration in their reaction to supravital stains. Accordingly in some animals neutral red saline of various strengths was injected

into the lungs after death from exsanguination and the animal was incubated at 37°C. for 10 to 20 minutes; the lungs were then removed, and portions were fixed as before and fresh preparations were also examined. In other animals the lungs were first removed and perfused till free from blood; saline containing neutral red was then injected through the bronchi, and the lungs suspended in a further quantity of the same fluid were incubated for varying intervals up to 20 minutes at 37°C.

In sections of lungs fixed 10 to 20 minutes after intratracheal injection of neutral red, the appearances differ markedly from those after intravenous injection of this dye. Many of the dust cells exhibit pronounced neutral red granulation, but others though apparently equally exposed to the action of the dye fail to become stained. The alveoli contain many more free cells than are apparent in normal animals or in those after intravenous injection, and this is clearly due to the stimulating action of the dye solution. With the high dilutions (1/40,000) the free cells contain rather small and poorly stained granules as noted in the teased preparations (p. 247), but with stronger solutions (1/10,000 to 1/1000) the staining is more intense and the granules are larger and more numerous. It is not clear whether these represent merely staining of the refractile droplets previously described, or

whether there is also enlargement of the granules and vacuoles of a segregation-apparatus, due to the stimulating action of the strong solution of neutral red. As has been already pointed out it is not possible to differentiate between these structures with certainty in the cytoplasm of intact cells when stained with neutral red. The newly formed free alveolar cells appear to be derived from the lining cells since in alveoli in which free supravitally stained cells are abundant the epithelial lining cells are much less numerous than in normal animals. Nevertheless it is to be noted that a few lining cells are still present in some alveoli, and these give a much less striking neutral red reaction than do the free cells in the air spaces. Exsanguination has no appreciable effect on the number of neutral red stained cells which appear in the alveoli after intratracheal injection of the dye solution. Accordingly it is clear that the latter must be derived from cells in or on the alveolar walls. The appearances indicate that both flattened and cuboidal nucleated cells take part in the process.

From similar experiments Gardner and Smith (1927) have stated that since the cuboidal nucleated cells (septal cells) exhibit numerous irregular neutral red stained granules after intratracheal injection of the dye solution they must therefore be histiocytes. It must be pointed out, however, that these

authors used neutral red in a concentration of 1/1500 which not only causes a considerable increase in the size and number of the neutral red granules displayed in susceptible cells, but it is also capable of staining the refractile lipoid droplets which have already been discussed. Their evidence cannot therefore be accepted as proving the histiocytic nature of the septal cells. As regards the degree of staining noted in the free cells which have migrated from the alveolar walls in response to the stimulus provided by the intratracheal injection of dye solution, it must be emphasised that the extent of the segregation-apparatus as shown in actively phagocytic cells by supravital staining is merely an indication of the functional activity of the cells at the time of examination, and does not permit of any inference as to the origin or normal condition of the reacting cells.

Intratracheal injection of trypan blue.

A guinea-pig was intensely stained intravitam by repeated introperitoneal injections of vital new red (see p. 22). After 16 weeks a minute fragment of subcutaneous tissue was removed and examined in spread preparations, and the presence of fairly numerous intensely stained intracellular granules in the histiocytes was determined. Then 1 c.c. of 1 per cent. trypan blue was injected intratracheally, and the animal was

killed after 24 hours. The blood plasma was found to be definitely blue stained showing that absorption of the dye had occurred. Microscopically the alveoli contained numerous mononuclear cells, the majority being of rounded shape and lying free in the air space. In addition to the carbon the dust cells nearly all show granules deeply stained with the blue dye (fig. 83). Here and there a cell can be seen exhibiting trypan blue granulation flattened along or otherwise attached to the alveolar wall, but this is exceptional. In alveoli containing many free phagocytes the normal lining cells are scanty and may appear to be absent, but in others immediately adjacent in which only one or two free cells are present, the flattened and cuboidal cells *in situ* are easily seen and are usually free from dye granules (fig. 83). The histiocytes of the interstitial tissue of the lung and other organs contain numerous granules of the previously administered vital new red, but no trace of this dye is present in the alveolar epithelium or in the free cells of the alveolar exudate. From these results it may be concluded that while the free cells possess the capacity to store vital stains if the dye is present in their environment, contact with the dye solution is not in itself sufficient to bring about vital staining of all the alveolar lining cells.

It would appear from other experiments of this nature also

that the flattened nucleated cells and the cuboidal septal cells exhibit very little capacity to store soluble dyes when in an unstimulated state and in their normal position, but when in a stimulated condition especially when they are about to leave their normal site in or on the alveolar walls to enter the air space their capacity for vital staining may become enhanced. The flattened cells appear to desquamate very rapidly and their increased avidity for vital stains is seen usually only after they have become free in the alveoli. In the case of the cuboidal cells (septal cells) migration occurs more slowly probably on account of the anatomical situation of the cells in the intercapillary spaces; the alteration in their functional activity is shown by increased swelling and vacuolation of the cytoplasm which protrudes from the space in which it normally lies. During this time the capacity to concentrate soluble vital stains in the form of granules is increasing, and if the stimulated cells remain in contact with the alveolar wall for a sufficiently long period while intracellular granules are developing we have then the appearance of vitally stained lining cells _in situ_ (fig. 83). As has already been pointed out cells may perform a normal function as lining units, e.g. the endothelial cells of the sinusoids of the liver and spleen, but may on proliferation give rise to cells with two different sets of functions (a) those which continue to act as lining cells, and (b) those which develop

into highly phagocytic cells anchored to the wall but floating in the slowly circulating blood (see pp. 104-106). Similarly in the lung alveoli the cuboidal septal cells may give rise to two sets of cells with widely different properties (a) lining cells - the flattened nucleated squames, and (b) the phagocytic dust cells of the alveoli. Upon stimulation, however, the flattened squames may also become phagocytic and desquamate into the air space; when in the free state they become rounded and revert to a condition wherein they are indistinguishable from the phagocytes produced directly by the cuboidal septal cells. Just as has been observed in the sinusoids of the liver and spleen, the cells which continue to function as lining cells show relatively little capacity to store vital stains, whereas those which become actively phagocytic develop this capacity in high degree.

In view of the above results Fried's statement (1927) that the phagocytic alveolar cells are mesenchymal histiocytes cannot be accepted. From experiments of a similar nature to those described above and yielding similar results he has concluded that because the phagocytic cells become stained with soluble dyes, they must therefore be histiocytes. In Fried's experiments repeated intratracheal injections of dye produce marked hyperplasia of the alveolar lining so that the air spaces came to be lined by a complete layer of cubical cells

similar to those observed by Young (1928) in experimental hyperplasia and by Cowdry (1925) in the lungs of sheep suffering from "jagziekte." These cubical lining cells remained unstained although the free phagocytes in the lumen of the vesicles were heavily laden with dye.

Are the alveolar phagocytes derived from monocytes?

Attempts were made to ascertain whether any considerable proportion of the alveolar exudate cells were derived from the circulating monocytes. Accordingly experiments were carried out in which a proportion of these cells were demarcated by the intravenous injection of suspensoid preparations, e.g. india ink and saccharated oxide of iron.

(a) A rabbit was intensely stained by repeated intraperitoneal injections of trypan blue; 12 days after the last injection of dye the animal received an intravenous injection of india ink and immediately thereafter 2 c.c. of fine carmine suspension in saline was injected into the trachea. The rabbit was killed after five hours. The blood serum contained a small amount of trypan blue and there was a small amount of ink still free in the circulation at the time of death. The histiocytes of the interstitial tissue were heavily stained with trypan blue but contain no ink granules. The intravascular polymorphs contained no carbon particles but a considerable

proportion of the intravascular monocytes had taken up carbon particles. The injected carmine was widely distributed throughout the lungs and was chiefly in the alveoli, the bronchi showing only traces of pigment on their walls.

Microscopically the greater part of the carmine is already intracellular being contained within large rounded phagocytes with vacuolated cytoplasm, many of which show small granules of soot finely dispersed throughout their cytoplasm. This carbon is readily distinguished from phagocytosed ink as the latter forms much denser and more closely aggregated black deposits than the finely dispersed particles of anthracotic pigment seen in such animals (fig. 84). The alveolar phagocytes appear to be derived chiefly from the cuboidal nucleated cells, and the flattened cells seen so frequently in the guinea-pig are less obvious. All stages in the formation of free phagocytes can be seen, the cells swelling and becoming more vacuolated before finally desquamating into the alveoli. The intravenously injected ink is distributed irregularly throughout the lungs, some areas being free while in others the ink forms heavy deposits on the capillary walls amounting to capillary emboli (fig. 84). No actual phagocytosis by the capillary endothelial cells has occurred and it is clear that the ink has not passed through the capillary walls in this situation as it may do elsewhere (see p. 138); the injected

ink does not therefore gain access to the alveolar lining cells. Frequently the latter can be seen in the act of migration from the wall where they have been lying in contact with capillaries almost plugged with ink, yet the migrating cells contain no ink but only ingested carmine. In some alveoli a considerable number of polymorphs are present and are accompanied by a few monocytes which contain small dense carbon deposits (fig. 84) Only a few of these blood cells have taken up carmine granules; probably the greater part of the carmine was already intracellular when the polymorphs and monocytes entered the alveoli.

(b) A mouse received an intravenous injection of india ink and immediately thereafter under ether anaesthesia a small quantity of fine carmine suspension was injected into the nostrils, the animal being held vertically for a few moments. It was killed after 24 hours.

Microscopically the injected carmine is found in the alveoli where it has called forth a large number of phagocytic cells. The majority of these are rounded and lie free in the alveoli; a few contain finely dispersed carbon particles of natural origin, indicating that they are pre-existing dust cells which have subsequently taken up carmine. There is practically no deposition of ink on the capillary walls at this stage, and only a few of the circulating monocytes now contain carbon particles though it is probable that such cells were much more numerous soon after injection.

(c) A rabbit received 10 c.c. of 10 per cent. saccharated oxide of iron suspensoid in distilled water intravenously, and immediately thereafter under ether anaesthesia 2 c.c. of 1 per cent. diamine fast scarlet was injected into the trachea. The animal was killed after 30 hours. Microscopically the alveoli contain numerous large mononuclear phagocytes with abundant dye granules, but the majority of these are oval or rounded and are free in the air spaces (fig.85). Only rarely are vitally stained cells seen _in situ_ on the alveolar wall. Many of the cuboidal nucleated cells exhibit very pronounced swelling and vacuolation, but have not taken up the injected dye (fig.85). Many of the free phagocytes exhibit finely dispersed carbon grains in their cytoplasm, thus proving that this pigment is of natural origin. None of the large free cells in the alveoli show any trace of iron, and the alveolar lining cells are likewise iron-free. A few of the circulating monocytes still give a definite prussian blue reaction (fig.85). In places where a larger amount of dye has penetrated into the alveoli numerous polymorphs are present, and many show vital staining of their specific granules. In these areas monocytes are also present in small numbers and show commencing vital staining by the diamine fast scarlet. Many of these small mononuclears show traces of iron thus differing from the alveolar phagocytes of epithelial origin, but at this stage

of the reaction such cells are easily distinguished from the larger epithelial phagocytes by their size and general morphology.

From these three experiments it is clearly demonstrated that the alveolar lining cells are the source of the free phagocytes, and there is no evidence to suggest that the monocytes play any noteworthy part in the production of the alveolar phagocytes under these conditions. In areas in which the reaction has been more severe owing to the penetration of a larger amount of the irritant introduced into the trachea, polymorphonuclear leucocytes are found in the alveoli in considerable numbers and are accompanied by monocytes. The latter type of cell has not been observed to enter the alveoli except under these circumstances. In such cases it is important to note that many of the monocytes exhibit the specific vital staining employed to demarcate this class of cell, thus showing that the procedure has been successful in its purpose. Even apart from the evidence of general structure and site of origin, the absence from the reacting alveolar cells of the specific vital staining characteristic of monocytes demonstrates clearly that the former cannot be considered to arise in any noteworthy proportion from cells of monocyte type.

DISCUSSION.

Under natural conditions inhaled particulate matter acts as a mild irritant in the lungs and is disposed of by phagocytosis. Much discussion has centered round the origin of the phagocytic cells. The older view was that they were of epithelial origin, derived from the cells lining the alveoli. The possession of phagocytic properties by epithelial cells was, however, called in question by Haythorn (1913) and other American workers who stated that they were of endothelial origin and endeavoured to support this view by experiments with intravitam staining. Accordingly I have attempted to correlate the results of various vital staining methods in the hope that conclusive evidence might thereby be obtained as to the nature and origin of these cells.

From the experiments recorded above it is clear that when particulate matter gains access to the alveoli the first line of defence in the lungs lies in the cells which line the alveoli and terminal ramifications of the bronchial passages (atria and alveolar ducts). The histological evidence by itself strongly supports this conclusion, and the evidence afforded by vital staining is sufficient to rule out other sources of these cells.

The local histiocytes of the interstitial tissue of the lung are in no way concerned in the reaction. Since they

become heavily stained by the soluble dyes injected, e.g. trypan blue they are readily identified, and there is no evidence that such cells wander into the alveoli and take part in the phagocytic process at any stage. The participation of desquamated cells from the reticulo-endothelial system - blood histiocytes - is also completely negatived by the evidence presented as these cells also are heavily stained. The only possible sources from which the reacting cells might be derived are the alveolar lining cells, the endothelial cells of the interalveolar capillaries or the monocytes from the circulating blood. The evidence that the alveolar epithelial cells are the elements concerned is as follows. The flattened lining cells have been observed to ingest finely divided particulate matter while still _in situ_; they then rapidly desquamate into the air spaces in the form of angular cells which soon increase in size and become rounded. The cuboidal nucleated cells also take part in the process and migrate actively into the air spaces where they take up the injected particulate matter.

The effect of previous vital staining of the animal on the newly formed alveolar phagocytes is very variable and it is impossible to state why some of these cells show staining while in others it is quite absent. This variability has been noted by others (Carleton [2] 1927), but the underlying reason is certainly not that advanced by Permar (1920) who suggested

that it was due to rapid disappearance of the dye from the blood stream so that no dye was available to the cells at the time when they were altering their functional activities. In my experiments this contingency has been provided for, and in all the animals shown in table IV a large amount of dye was in circulation throughout the entire period of the experiment. In these animals fine vitally stained granules are seen in some of the exudate cells at an early stage, but the degree of staining is extremely variable even in animals killed at approximately the same interval after introduction of the irritant. It is probable that this irregularity in the vital staining of the alveolar phagocytes depends partly on the variability in the staining of the lining cells previously noted in the normal lung. In part also the variability depends on the length of time which elapses between the stimulation of the cells and their separation from the alveolar wall and thus from their source of available dye. Those which react rapidly and so leave the alveolar wall almost immediately have less opportunity to absorb and concentrate the dye than those which continue to lie in contact with the blood capillaries for a longer period. As previously pointed out an interval of at least several hours is necessary before intracellular dye granules become visible. Contact with the vital stain is not, however, in itself sufficient to produce vital staining in such

cells; this appears from the fact that after intratracheal injection of dye the lining cells _in situ_ are usually unstained while those free in the air spaces are heavily stained. Active storage of vital stain is therefore an indication that the function of the cell is undergoing transformation from that of a lining cell to one of active phagocytic character.

Nothing has been observed to support Permar's statement (1920, 1927) that the capillary endothelium takes part in the reaction.

The rôle of the monocytes is more difficult to assess, but the evidence provided by intravenous injection of suspensoid preparations does not point to any noteworthy participation of these cells in reactive processes in the lung under the conditions studied. Thus the newly formed alveolar phagocytes are quite free from the agent used when the intravascular monocytes are loaded with the injected suspensoid. In more severe inflammatory conditions leucocytes from the blood including monocytes also take part in the reactive process and migrate into the alveoli. These blood cells do not, however, constitute the first line of defence and they make their appearance only after a lapse of several hours. At this time the reactive process induced by the foreign material injected may be well advanced, and the greater part of the particulate matter may be already engulfed by phagocytes.

The conclusion that the alveolar phagocytes are derived from the nucleated cuboidal cells of the alveolar lining agrees with that of most of the previous investigators among whom may be mentioned Herxheimer (1903) Briscoe (1908) Sewell (1918) Westhues (1922, 1925) Seeman (1925, 1927) Kageyama (1925) and F. Gross (1927). Most of these writers employed either intra-vitam staining methods of some kind or intratracheal injection of foreign particles (carbon, oxide of iron, tubercle bacilli, quartz dust etc.). Aschoff (1924) in attempting to define the reticulo-endothelial system considered that the possession of phagocytic properties alone was too widespread a function of body cells to furnish a sufficiently discriminating criterion. Writers who have been unable to accept the conception of phago-cytosis by epithelium have on this ground disputed the epithelial origin of the pulmonary phagocytes and have thus been forced to the view that since the alveolar lining cells obviously become phagocytic they cannot be epithelial. Although elsewhere the defensive mononuclear phagocytes are usually of mesenchymal origin, phagocytosis by epithelial cells is by no means rare. In epithelial organs undergoing atrophy (Guieyesse-Pellissier 1911, Wegelin 1921) and under a variety of pathological condi-tions, e.g. "chronic mastitis," in the central nervous system (Lubarsch 1921) and in tumour growth, phagocytosis by epithelial cells is frequently encountered. In tissue cultures Smith (1921)

and Lewis (1925) have observed that both epithelial and connective tissue cells may actively ingest pigment added to or occurring naturally in the medium.

Summary.

The normal structure of the pulmonary alveoli in mice, guinea-pigs and rabbits has been studied by the aid of intra-vitam staining, in addition to the usual histological methods. My observations go to show that the normal lining consists of (a) flattened nucleated squames, which form the greater part of the alveolar lining; these cells interdigitate with the following two types of cells; (b) large flat non-nucleated squames, which form only a small part of the alveolar lining and appear to be derived from the cells of the first group; (c) the cuboidal nucleated cells of the intercapillary spaces, recently called "septal cells." This view is at variance with that currently expressed, according to which the alveolar lining is composed exclusively of non-nucleated squames and the cuboidal septal cells.

After intravitam staining with soluble dyes administered by the intravenous intraperitoneal or subcutaneous route the cells lining the pulmonary alveoli show little or no evidence of vital staining except in conditions of very intense administration. After intense treatment the epithelial lining cells

contain only a small number of fine dye granules; but the degree of staining varies greatly in different animals and also in different parts of the lung of the same animal. The histiocytes of the interstitial tissue of the lung on the other hand become very deeply stained even after small doses of trypan blue and other soluble dyes which are insufficient to produce any vital staining of the alveolar epithelium; the two types of cell can thus be sharply distinguished from one another.

After intravenous injection of suspensoid preparations a proportion of the mononuclear leucocytes of the blood ingest the particulate matter, but no trace of the injected substance is found in the alveolar epithelium or in the histiocytes of the lung stroma.

In order to study the phagocytes which appear in response to a mild stimulus a condition of experimental anthracosis has been produced in normal mice and in mice previously vitally stained in a variety of ways. Soot deposited in the alveoli by inhalation is removed by the action of mononuclear phagocytes but the cellular response is less rapid than that produced by intratracheal injection of particulate matter in a fluid medium. Accordingly the mode of formation of the reacting cells is difficult to follow in inhalation experiments and the amount of soot taken up by individual cells is generally so

great that vital staining if present is obscured. The appearances indicate, however, that the reacting cells are derived from those of the alveolar lining. The inhaled soot is subsequently removed chiefly by intracellular transport and is thus passed either into the bronchial lumen to be expelled in the sputum, or is carried into the peribronchial lymphatics to be deposited in the glands of the hilum and mediastinum.

Particulate matter in a fluid medium injected into the trachea penetrates into the alveoli and leads to a rapid mobilisation of mononuclear phagocytes which are derived chiefly from the lining epithelium. The flattened nucleated squames and the cuboidal nucleated cells (septal cells) both take part in the process; while still *in situ* they begin to ingest finely divided particulate matter, and the subsequent process of desquamation and morphological change to typical rounded free mononuclear phagocytes has been followed in detail.

Further evidence as to the origin of the alveolar phagocytes has been obtained by giving intratracheal injections of carbon suspensions etc. to animals which have been vitally stained by repeated intraperitoneal or intravenous injections of soluble dyes (trypan blue etc.). In order to obtain distinct vital staining of the reacting alveolar cells in such animals it is essential that they should have been previously highly saturated with the dye and that a large amount should also be

present in the plasma during the process of desquamation. Under these circumstances the alveolar cells occasionally show much more intense vital staining than is obtained in the normal lung, and this is no doubt explained by the stimulated condition of the cells. The degree of vital staining in the pulmonary phagocytes is extremely variable, and this is attributed to two factors, (a) the variation in the amount of vital staining in the normal alveolar epithelium before the intra-tracheal injection of the irritant, and (b) the length of time the reacting cells remain *in situ* on the alveolar wall after responding to the stimulus of the presence of particulate matter. It is clear that the longer a stimulated cell remains in intimate contact with the dye-containing capillaries in the alveolar wall before desquamating the greater will be the degree of staining in the resulting free phagocyte.

In animals in which as a result of intravenous injection of a suspensoid preparation (e.g. india ink or saccharated oxide of iron), a proportion of the monocytes have been demarcated, no evidence has been found to suggest that these cells take any part in furnishing phagocytes to the pulmonary alveoli in the mild reactions specially studied. It is probable that monocytes participate only when the reactive process is more intense, and that they are then generally accompanied by other blood leucocytes, e.g. polymorphs.

V. The cellular reactions following mild irritation of the peritoneum, with reference to the origin and nature of the mononuclear phagocytes.

Methods.

The reaction produced by intraperitoneal injection of broth :

in normal animals :

in mice vitally stained by subcutaneous injections of dye :
(a) during the reaction
(b) prior to the reaction.

The rôle of the circulating monocytes in peritoneal exudates :

The oxidase reaction of the free exudate cells:

The reaction produced by intraperitoneal injection of dyes of group (b); diamine fast scarlet, isamine blue, etc.

The absorption of dyes (diamine fast scarlet, isamine blue) from the peritoneum.

Changes in the peritoneal tissues, omentum and mesenteries, following intraperitoneal injection of dyes.

Changes in the peritoneal mesothelium.

The peritoneal reaction in animals stained by previous intraperitoneal injection of dye.

Discussion and Summary.

In the course of studies on intravitam staining it was observed that certain of the dyes used as vital stains produced a well marked cellular reaction at the site of injection, particularly when introduced into a serous sac. Hitherto such reactions have received little attention, and it has not been sufficiently realised that these substances *per se* produce a mild aseptic inflammation. This property is shared in varying degree by the majority of the dyes used as vital stains, and it may be stated that the capacity to produce marked cellular reactions is in inverse proportion to the diffusibility and ease of absorption of the dye solution. Thus dyes of group (a), e.g. trypan blue, vital new red, produce relatively slight and transient exudation of fluid and cells when introduced into the peritoneum in 1·0 per cent. solution, whereas dyes of group (b), e.g. isamine blue, diamine fast scarlet, give rise to serous exudation which persists for several days and is accompanied by marked cellular emigration.

It appeared that the reaction to these substances offered a favourable opportunity for studying the large mononuclear phagocytes which form so prominent a feature of a resolving peritonitis, and for investigating the relation of these cells to the mesothelium and to the histiocytes of the surrounding tissues. Accordingly this aspect of vital staining has been studied in more detail.

It was felt that additional information would be obtained by comparing the results with those following the intraperitoneal injection of other mild irritants in normal animals and in those previously vitally stained.

As in the earlier studies, the term "endothelium" is restricted to the lining cells of blood and lymphatic vessels; the peritoneal lining cells are referred to as mesothelium or serosal cells; "monocyte" refers to mononuclear cells of the blood and tissues which show the characteristic reaction to supravital stains, while "histiocyte" is applied to the vitally staining wandering cells of the connective tissues. The term "macrophage" refers to mononuclear cells in the serous exudate or tissues which are in an active or stimulated condition, as is demonstrated by their being highly phagocytic; when supravitally stained they exhibit a characteristic morphology similar to that of histiocytes in an active phase. The name, however, covers a group of cells exhibiting similar functional capacities and carries no implication as to their origin, which is believed to be attributable to various sources.

I have already described in detail the different agents used as vital stains, with their dosage, mode of administration and distribution in the body. It is convenient to recapitulate here some points which are

essential to the present work.

In the subperitoneal tissues of rodents especially in the omentum and mesenteries there are cellular collections around the vessels and in the intervening avascular portions - the adventitial sheaths and the tâches laiteuses. These consist chiefly of small round cells resembling lymphocytes and monocytes with a varying proportion of larger cells, many of which are histiocytes. When vital staining is produced by repeated subcutaneous or intravenous administration, the histiocytes readily become stained and are thus easily distinguished by their content of dye granules from the smaller cells and from the fibrocytes and mesothelium, both of which store only in small amounts vital stains circulating in the blood. As will be shown later, when dyes are introduced directly into the peritoneum, the staining of cells bathed directly in the dye is greatly increased.

The peritoneal cavity of normal mice contains a very small amount of fluid in which only a few cells are present. I have not found in normal mice the large number of free cells (over 100,000 per c.mm.) described by Nakahara (1925). The cells by all criteria fall into three groups: (a) cells with rounded deeply staining nuclei and scanty basophilic cytoplasm, numerous short rodlike and granular mitochondria

and one or two tiny neutral red granules - lymphocytes: (b) cells of slightly larger size with oval or reniform nuclei and more abundant cytoplasm; neutral red granules are of small size and grouped in the nuclear indentation, and mitochondria are usually clustered around them - monocytes: (c) cells similar to the previous group but with more numerous neutral red granules irregularly arranged and less prominent mitochondria. These I regard as young or immature macrophages, but consider that they cannot be sharply separated from the cells of group (b). Some such cells are considerably larger and are filled with neutral red granules; these are fully developed macrophages.

In animals vitally stained by subcutaneous or intravenous injections of dye, the "segregation vacuoles" of both the free monocytes and macrophages in the serous sacs are stained though less deeply than those of the histiocytes in the adjacent tissues.

METHODS.

The general plan of the investigation has been as follows. The mice required for each individual experiment were selected from the stock, and as far as possible young mice of about 20 gms. weight were employed. The material to produce the reaction was injected into the peritoneum and the ensuing exudate after suitable intervals was withdrawn with aseptic precautions by means of fine sterile glass pipettes. Sometimes the exudate was examined repeatedly in the same animals; at other times a series of animals was prepared and one or more killed at intervals. Various substances have been used to induce peritoneal reactions, but particular attention has been devoted to the results following the introduction of sterile concentrated meat infusion broth, and to the effects of dyes of the less rapidly absorbed type, e.g. isamine blue and diamine fast scarlet. These substances were injected in the dose of 1 c.c. per 20 gm. body weight, the dyes being used in 1 per cent. solution in distilled water. Suspensions of killed or living organisms were tried but were not found suitable owing to the greater participation of polymorphonuclear leucocytes from the blood which tended to obscure certain points on which further information was desired. It was also found that ingested cocci became deeply tinged with janus green and gave rise to confusion with mitochondria.

Similar experiments were performed on animals previously stained *intra vitam* by repeated subcutaneous or intravenous injections of dye, and on animals stained by intraperitoneal injections of dye after allowing a suitable interval for the subsidence of the primary peritoneal reaction. The peritoneal exudates were studied in the living state by the supravital technique with neutral red, alone or in combination with janus green, both in isotonic saline and by the dry-dye film method previously described.

It was found convenient to stain the living cells supravitally by taking up the fresh exudate into fine glass pipettes previously coated internally with a thin film of dry dye by passing through them the alcoholic dye solution already described (p. 35). The dye rapidly dissolved in the fluid and stained the cells supravitally; fresh preparations were then made on slides and sealed with paraffin. Films were also prepared on coverslips and fixed in Zenker-formol or dried in air; in this way a permanent record of the neutral red staining could be obtained. At the same time control preparations were always made without supravital staining; films on coverslips were fixed while wet in Bouin's fluid, corrosive sublimate, Zenker's fluid or 10 per cent. Zenker-formol, as demanded by the fixation requirements of the vital stains employed and were studied after suitable counterstaining, while

others were dried in air and stained by Leishman's stain and by Goodpasture's peroxidase method.

It is very important that the cells should be examined in the living state without the addition of supravital stains, as the latter may obscure faint vital staining of the "segregation apparatus": in fixed preparations traces of staining are not always preserved although readily observed in the living cells.

The cellular reaction to the intraperitoneal injection of concentrated meat infusion broth.

In this group mice receiving a single intraperitoneal injection of concentrated infusion broth were killed at intervals of 2, 4, 6, 9, 12, 18, 24, 30, 36, 48 72, 96 hours; in addition six animals received several injections at daily intervals, and the exudate was examined repeatedly at intervals up to six days after the last injection. The intraperitoneal injection of broth sets up a mild inflammatory reaction characterised by the outpouring of serous fluid and emigration of cells. During the early stages of the reaction, e.g. 2 - 6 hours, the cells correspond to the types normally present but a few polymorphonuclear leucocytes also appear. The majority of the cells are small mononuclears resembling lymphocytes, in stained preparations they possess scanty cytoplasm and the characteristic lymphocytic nucleus. In supravitally stained preparations the majority of these cells show no neutral red staining or only a few tiny granules in the broader part of the rim of protoplasm (fig. 88). Some of the larger cells possess a larger cytoplasmic rim in which in Leishman-stained films a few coarse dark purple granules may be present, but often the cytoplasm of the larger cells is deeply basophilic (fig. 90a). These large lymphocytes may

exhibit slight degrees of nuclear indentation and opposite this a small number of neutral red granules may be present in the form of a rosette or arranged irregularly. The mitochondria in these lymphocytic cells are generally short rods or granules. In the mouse the exudate at all stages contains a few mast cells; these present the characteristic appearances previously described and their granules stain vividly with neutral red and are negative to the peroxidase test. They do not appear to increase in numbers to any extent and are at no time very prominent. No special significance can be attached to their presence.

Even in the earliest stages examined (2 hours) a number of larger cells of macrophage type were present, these are probably cells normally present in the peritoneum. After supravital staining they show the characteristic coarse irregular neutral red granules of histiocytes, and the mitochondria when visible are longer and more rod-shaped than those of the lymphocytes, though it is to be noted that in the largest macrophages the mitochondria may be granular or no longer recognisable. In air-dried films Leishman-stained these cells are often burst and fragmented, an indication of their delicate character. The well preserved cells, however, show an eccentric nucleus with a finer chromatin structure than that of the lymphocytes and bulky cytoplasm, the staining

of which presents greatly varied degrees of basophilia. These fully developed large macrophages are scarcely more prominent 6 hours after injection and many of them appear to be degenerating. By the end of 6 - 9 hours, however, the lymphocytic cells have undergone further development and a greater number than at the earlier periods exhibit commencing neutral red storage, coincidentally with an increase in the amount of cytoplasm. After 12 hours the exudate is more cellular; polymorphonuclear leucocytes have increased greatly in number and form about 50 per cent. of the cells present. Mononuclear cells are also more numerous and have undergone further changes. While many cells of lymphocyte type are present, the majority of the mononuclears exhibit nuclear indentation, a more abundant and basophilic cytoplasm and increased storage of neutral red, the granules being in some aggregated in rosette form, in others scattered throughout the cell. No clear distinction can be made by supravital staining between lymphocytes, monocytes and macrophages; while typical examples of each can readily be identified, the three types are connected by all intermediate stages and it is clear that a progressive development is going on in the smaller cells, by which they are rapidly acquiring the functional characters of young macrophages (fig. 88).

Throughout the active stages of the peritoneal irritation new cells appear to be arriving in the cavity, where they undergo the changes described above. With a single injection of sterile broth the cellular reaction reaches a maximum at about 18 - 24 hours, though the amount of free fluid is less than at earlier stages; many polymorphs are still present, but over 50 per cent. of the cells are young mononuclears, and all transitions are met with between lymphocytes, monocytes and macrophages. As judged by their supravital staining reactions, the macrophages are in a highly stimulated active condition and store neutral red with great avidity (fig. 88).

At this time relatively few of the mononuclears are of very large size and the majority are still capable of much greater development, as is shown by the reaction to dyes. It is desirable to emphasise this point here, as it is of importance in the experiments reported in the succeeding paper.

After 24 hours the reaction following a single injection of broth retrogresses; the amount of free fluid in the peritoneum is very scanty, polymorphs diminish rapidly in number and at the end of 48 hours are almost absent. The mononuclear cells also diminish in number; some continue to develop and proceed to ingest the remains of polymorphs and other debris, while others leave the peritoneum and

return to the surrounding tissues. After 72 hours the peritoneum is usually as dry as normally, but there is still an excess of free cells in the sac; these consist chiefly of smaller mononuclear cells many resembling lymphocytes, together with a few large macrophages containing cellular debris. At the end of 96 hours after a single injection of broth the peritoneum is practically restored to normal.

The macrophages and other cells of the exudate which survive appear to return to the tissues around the cavity, where many of them again assume a smaller resting form. The stimulus of broth thus leads to a temporary hypertrophy of the mononuclear cells which is accompanied by greatly enhanced capacity for supravital staining. This does not persist, however, for long after the cessation of the stimulus; it is as if the cells were able to metabolise or otherwise dispose of the materials they had absorbed and stored in the structures which stain with neutral red - the 'segregation apparatus'. When, on the other hand, the more highly colloidal dyes are taken up and stored in the cytoplasm they are much more inert and difficult to deal with, so that the cells are able to dispose of the ingested material only very slowly. The persistance of the hypertrophied form of the cells for long periods after vital staining with isamine blue, etc. thus appears

to be due to the 'indigestibility' as it were of the materials stored in the cytoplasm.

During this inflammation the exudate contains at all stages a few desquamated serosal cells, sometimes occurring in small sheets. When free they become more rounded or oval, but do not appear to perform any special function. They may undergo mitosis but only very rarely are appearances seen suggestive of transformation into macrophages. The stimulus of broth is comparatively mild, and serosal cells are less frequent in such exudates than in those following injection of dyes.

When repeated injections of broth are given the reaction is intensified and prolonged. Fresh polymorphs reappear in the exudate as a result of the renewed stimulus, but in diminishing numbers with each repetition; the mononuclear cells undergo further enlargement and become more numerous, so that for a time there is a very high proportion of large macrophages; later many of these cells disintegrate and the proportion of smaller macrophages and monocytes again rises. After daily injection of broth over a week the stimulus appears to lose effect, the fluid is absorbed more quickly and produces a much less marked cellular response. It would appear that the peritoneum has become relatively tolerant to the broth.

<u>The cellular reaction in the peritoneum in mice vitally stained by subcutaneous injections of dye during the reaction.</u>

The previously described procedures were repeated in mice vitally stained with trypan blue or vital new red, one or two suitable doses being administered subcutaneously on the days immediately preceding and on the day of the injection of broth; in this way the vitally staining cells of the tissues were filled with dye granules and an abundant supply of free dye was present in the blood and tissue fluids throughout the reaction in the peritoneum. Six mice received trypan blue and four received vital new red.

The general character of the cellular exudate was similar to that already described, but many of the mononuclear cells were vitally stained.

In the early stages, 2 - 6 hours only a few of the larger cells showed dye granules, but as the reaction proceeded these appeared in the smaller mononuclears also. In some the stained granules were in the form of a rosette but in others they were irregularly disposed throughout the cytoplasm (fig. 89). The vital staining of the exudate cells appeared to correspond in every way with the supravital neutral red reaction already described, but the staining of the granules and vacuoles was generally pale, indicating that dye was available to the cells only in a low concentration.

It is clear from the histological picture at different stages that the majority of the mononuclears are practically unstained when they first appear as free cells in the peritoneum but that during their sojourn there they acquire the capacity to stain vitally and take up dye from the fluid of the exudate. Examination of the peripheral blood during the reaction fails to show any evidence of vital staining of the mononuclear cells, and it would appear that the capacity to stain vitally is not acquired so long as the cells are in the circulating blood. In the later stages of the reaction, it is not possible to distinguish by the degree of vital staining in individual cells those of the large macrophages which have been derived from fully formed histiocytes in the surrounding tissues, and those which have developed from smaller lymphocyte - and monocyte-like cells. Owing to the continued presence of free dye in their environment all the cells of the exudate which have come to exercise the functional activity of macrophages have had the opportunity of storing the dye, and have become at least faintly vitally stained.

Siengalewiez and Clark (1925) have stated that trypan blue does not pass into the fluid of exudates in serous sacs, but I consider that this is not strictly accurate and that the dye is present though in very low concentration - certainly much less than that in the blood.

Examination of the tissues, e.g. the omentum and mesentery also supports this view, for in these situations the histiocytes are more deeply stained than the large free cells of the exudate. It would thus appear that the majority of the larger exudate cells are not fully formed histiocytes which have migrated into the exudate in the later stages, but that they have been formed by growth and differentiation of the smaller cells which entered the cavity earlier.

Accordingly, further experiments were carried out with animals vitally stained some time before, in which free dye was no longer present in the blood and tissues in amounts great enough to lead to vital staining. It was hoped in this way to obtain definite evidence as to the participation of the preformed histiocytes.

<u>The cellular reaction in the peritoneum of mice previously vitally stained by subcutaneous injection.</u>

In this group 22 mice stained by repeated subcutaneous injection of dye were used. Eight animals received 4 or more injections of trypan blue, 4 received corresponding doses of vital new red, 6 were given 5 or more injections of isamine blue and 4 received similar treatment with diamine fast scarlet. In these animals the cells of the reticulo-endothelial system in the wider sense were distinctly stained, though, as previously described, the staining was always most intense at the site of injection. Intraperitoneal injection of sterile broth was the usual method of producing a cellular exudate but, in a few instances, washed homologous red cells were used. As before, the exudate was sometimes drawn off repeatedly for examination, the animal being killed when finally no fluid was obtained by puncture; other animals were killed at intervals during the reaction, e.g. 6, 12, 24, 36 and 48 hours.

The results of this group are very interesting and may be summarised as follows: as before, the early exudate, e.g. 6 hours, consisted chiefly of small mononuclears with only a few larger cells. None of the smaller cells showed any trace of vital staining (fig. 90,92). In some, though not all, of the larger macrophages poorly stained granules

were present but only very occasionally were large heavily stained cells encountered. After 24 hours some of the latter had become very degenerate. In the further stages of the reaction, the condition above described persisted and vitally stained cells at no time formed a notable proportion of the exudate cells. In the later stages typical large macrophages were present as before but most of these were quite devoid of vitally stained granules (figs.91, 92). When tested with neutral red, such cells exhibited great capacity for supravital staining, showing that the lack of vital staining was not due to functional deficiency. Examination of the omentum, mesentery and organs of these animals showed that numerous vitally stained histiocytes were present.

The relative absence of vitally stained cells from the exudate is therefore not due to lack of such cells in the adjacent tissues. It would appear from these and previous experiments that the fully developed histiocytes of the omentum, mesentery and other peritoneal tissues do not take a very active part in the formation of a reactive cellular exudate both in stained and unstained animals. The influence inhibiting the migration of histiocytes into the serous sac is therefore not the presence of intracellular stained granules <u>per se</u> but must be correlated with the state of their functional development. The majority of

the large macrophages are developed from smaller cells which migrate rapidly into the serous sac and undergo progressive development there as long as the stimulus of the irritation is sufficient.

<u>The oxidase reaction of the reacting cells.</u>

In the mouse the polymorphonuclear leucocytes contain exceedingly fine granules which are difficult to stain with the Romanowsky dyes. To Goodpasture's peroxidase test they react positively, but often the whole cell is not filled as in the human subject, but contains only a small group of oxidase positive granules. The monocytes of the peripheral blood also react positively but in varying degree, and usually contain only a few scattered granules, while other cells of similar morphology react negatively. It seemed, therefore, improbable that the application of the oxidase test to the cells of peritoneal exudates would assist in determining whether any considerable proportion of the young mononuclears are derived from the circulating blood. In the early stages of the reaction 6 - 24 hours, about 10 per cent. of the young mononuclears show a few oxidase positive granules, while such are absent from other cells morphologically similar. The great majority of the larger macrophages are completely negative to the oxidase test, some do contain a few positive granules but it is questionable whether such granules are not merely ingested fragments of polymorphs.

<u>The rôle of the circulating monocytes in peritoneal exudates</u>.

In the foregoing investigations the evidence indicated that the small mononuclear cells which appear first in the stimulated peritoneum are of special importance since they appear to develop into the large macrophages which are so prominent later. No evidence was found that the latter are derived in any significant proportion from fully formed histiocytes in the local tissues. Two sources of origin of the small cells are possible (a) the local cellular accumulations constituting the adventitial sheaths, tâches laiteuses, etc., and (b) the mononuclears of the circulating blood. From the results previously observed to follow intravenous injection of india ink it seemed possible that this procedure might throw light on the problem. Since a considerable proportion of the circulating monocytes take up ink particles after intravenous injection, the absence of carbon from the peritoneal mononuclears would suggest that they do not arise to any considerable extent from circulating monocytes. It has also been pointed out that the capillary walls of the omentum and mesentery are more permeable to suspended particles than the capillaries elsewhere and that intravenous injection of ink is soon followed by deposition of carbon particles in the perivascular histiocytes of the adventitial sheaths and even in the

avascular tâches laiteuses. The complete absence of carbon particles from the mononuclear cells of the exudate in the later stages would therefore be confirmatory evidence that preformed histiocytes play no important part in the formation of the cells of the exudate. Accordingly, four mice were given by intravenous injection 0·3 c.c. per 20 gm. weight of a 20 per cent. dilution of Higgin's waterproof ink and immediately thereafter 1 c.c. of sterile broth was injected intraperitoneally. The animals were killed 24, 48, 72 and 96 hours after injection: in addition to the exudates obtained at autopsy samples were withdrawn at intervals from 6 - 24 hours.

At no stage of the reaction were carbon bearing cells numerous in the exudate. At any given stage several films often failed to show a single ink containing cell. Occasionally minute intracellular carbon particles were observed, sometimes in typical monocytes, sometimes in young macrophages, and in the later stages very occasionally in larger macrophages. It seems clear, therefore, that while a few blood monocytes may leave the circulation to appear in the inflammatory exudate, such cells do not form the most important source of origin of the peritoneal mononuclears. One must also consider the possibility that ink stained monocytes may be less capable of migration through capillary walls into the exudate; there is, however, no evidence

that this is so.

In spread preparations of the omentum and mesentery in these animals a considerable number of the perivascular histiocytes were found to contain carbon granules. The relative absence of such cells from the exudate in the later stages is confirmatory evidence that mobilisation of fully developed histiocytes does not constitute an important source of the macrophages found in the exudate.

McJunkin (1925) states that in experimental peritonitis the most important sources of monocytes and macrophages are the reticulo-endothelial cells of the lymphatic glands. In order to test this hypothesis India ink was injected into the subcutaneous tissue around the tail veins of 4 mice; as previously noted this leads to rapid flooding of the aortic and retroperitoneal glands with ink particles (fig. 37) and rapid mobilisation in the lymphatic sinuses and lymphoid tissue of macrophages which become demarcated by ingestion of ink granules. After 24 hours, peritoneal reaction was induced as before. In each case the peritoneal exudate examined 24 hours later was found to contain numerous macrophages in various stages of development, but no trace of carbon pigmentation was found in any of the cells of the exudate.

This experiment was repeated on 4 mice, using killed broth cultures of staphylococci as the peritoneal irritant.

The results were similar but the exudate was more abundant and richer in polymorphs. Numerous young macrophages were present in all stages of development, but no carbon-bearing cells were found in the examination of numerous films.
As already noted, in the supravitally stained films confusion was apt to arise owing to staining of ingested cocci with janus green; these could not always be clearly differentiated from mitochondria.

<u>The reaction following the intraperitoneal injection of colloidal dyes, e.g. diamine fast scarlet, isamine blue.</u>

In this investigation over 100 animals have received intraperitoneal injections of various dyes. Some received a single injection, to others repeated doses were administered. As before sometimes the exudate was examined repeatedly in the same animal, while others were killed at corresponding intervals. In every case the condition of the peritoneum was noted at death and films were prepared from the cavity by the methods already described. Spread preparations of the omentum and mesentery were made, and portions of the organs were fixed for histological examination. No attempt has been made to describe the findings in individual animals but the general results may be stated as follows.

When a highly colloidal dye is injected into the peritoneum, e.g. diamine fast scarlet, isamine blue, in 1 per cent. solution it induces a serous exudation which soon becomes cellular. Admixture of the dye solution with the serous fluid poured out results in the formation of innumerable soft flocculi, which at the end of two hours are numerous and generally small, but after six hours are more abundant and are often aggregated into larger masses.

These flocculi appear to be composed of a mixture of dye and proteins derived from the serous exudate. They are

not soluble in physiological saline or in distilled water, and after fixation by heat or by chemical fixatives they resist decolorisation in a manner similar to the intracellular granules and vacuoles of vitally stained cells, e.g. in the case of isamine blue, the dye colour is slowly discharged by weak alcohol, but resists prolonged washing in running water; with diamine fast scarlet the flocculi resist decolorisation by distilled water but the colour is rapidly discharged by alkalis, e.g. lithium carbonate, ammonia or sodium bicarbonate. At the earliest stage examined, i.e. 2 hours, the cellular content of the peritoneum is scarcely increased and the cells are of normal type, but a few appear rather larger than the macrophages usually found in a normal peritoneum. No evidence of dye ingestion can be found at this stage; although in fixed and stained preparations the cells are often diffusely coloured; this is not seen in fresh preparations of the living cells. At the end of 6 hours the cellular content of the exudate is definitely increased, the new cells being similar to those already present, i.e. chiefly mononuclears resembling lymphocytes, monocyte-like cells with reniform nuclei, and a number of larger macrophages, but all intermediate varieties between these cell types are met with. After supravital staining with neutral red some of the smaller cells are seen to possess the vacuolar structure characteristic of that in monocytes, the vacuoles being aggregated in the nuclear

indentation, but such cells are in the minority, and others morphologically similar show the neutral red vacuoles scattered irregularly throughout the cytoplasm. The latter are undoubtedly young macrophages. The larger macrophages originally present also appear to have increased in size, but at the 6 hour stage few show more than a trace of vital staining and some are degenerating and stain diffusely. In the stimulated macrophages at this stage the cytoplasm sometimes has become filled with vacuoles of varying size, most of which contain no dye, though on addition of neutral red they show very intense staining. This alteration would appear to represent a hypertrophy of the "segregation apparatus" of the cells and is a precursor of dye storage. The earliest vital staining of these "vacuoles" is best seen in fresh living cells without the addition of any supravital stain; in fixed and stained preparations faint degrees of vital staining are not apparent. By the end of 12 hours the flocculi in the peritoneum have become aggregated into larger clumps. Occasionally large masses of sticky precipitate form and become covered with cells as will be described later. Commonly one finds both in fresh and fixed preparations one or two young macrophages attached to each of the larger flocculi; these cells rapidly enlarge and vital staining appears in their cytoplasm coincidentally with progressive diminution in size of the individual flocculi. It is difficult to follow the mode of transference of the

stain from flocculus to cell. Although definite phagocytosis of gross particles may occur the process of vital staining seems to result mainly from the cells dissolving the flocculi and absorbing the products into their cytoplasm where they accumulate within the "segregation vacuoles". At first, e.g. 6 - 12 hours, the colouration of the vacuoles is uniform, but in the next 12 hours a separation occurs from the general constituents of the "vacuoles", and individual particles more intensely stained appear in the walls of the vacuoles or free in their interior (fig. 93). This is especially pronounced with diamine fast scarlet, but it has also been observed with other dyes, e.g. isamine blue, benzopurpurin.

In the presence of free dye in the fluid exudate the cytoplasm and nucleus of dead or severely damaged cells become diffusely stained. Such damaged cells may be observed at all stages, but are best seen within the first 24 hours, since at later stages free dye is no longer abundant. In supravitally stained preparations, damaged cells can be recognised in a similar way, and this affords the most delicate indication of loss of vitality in any given cell. In the first 12 hours after injection of dye, a considerable number of the desquamated mesothelial cells are diffusely stained and some of the larger macrophages show a similar appearance. As Sabin, Cunningham and Doan (1925) have pointed out, the large fully developed macro-

phages appear to be delicate cells as appears from their tendency to burst during the making of preparations. These damaged cells degenerate further and some are removed by phagocytosis. Many of the desquamated serosal cells, however, appear to undergo chromatolysis and progressive disintegration in the exudate fluid.

After 24 hours the peritoneum usually contains a small amount of free fluid, generally rather sticky, but the loose flocculi have almost disappeared. The exudate is rich in cells, the majority of which are mononuclear. Most of these now show vital staining in varying degree, some possessing a large number of vacuoles of irregular size in which are more deeply coloured granules, while others show the merest trace of dye storage. Many small lymphocyte-like cells free from vital stain are present together with similar cells in which the cytoplasm is slightly more abundant and shows commencing dye storage. All degrees of transition can be found, from typical small lymphocytes through large lymphocytes and monocyte-like forms to young macrophages with abundant dye deposits; the evidence clearly points to a close relationship between these cells.

It is probable that some of the monocytes and lymphocytes are derived from the circulating blood. As long as they remain in the blood stream, however, they do not take up the vital stain in visible amounts. At this stage the cellular exudate also contains many polymorphs which have

migrated from the blood stream.

The polymorphs in the mouse practically never show any trace of vital staining of specific granules, but in the rabbit and guinea-pig staining of the specific granules has been observed. This is of course a staining of pre-formed granules and must be distinguished from true vital staining where the dye accumulates in vacuoles and granules, the majority of which at least are newly formed as a result of the presence of dye in the environment of the cell. Such true vital staining or dye ingestion is found in a small proportion (1-2 per cent.) of the polymorphs in the exudate.

During the following 24 hours, i.e. from 24 - 48 hours after a single injection of dye into the normal peritoneum, the number of cells in the exudate increases, and the mono-nuclear elements undergo progressive development. From about 30 - 36 hours onwards large macrophages with massive irregular dye granules become increasingly prominent (fig.95). Small cells continue to enter the peritoneum but more slowly than at first so that, at any time, there may be found in the exudate cells in all stages between lymphocytes, mono-cytes and macrophages. From 48 - 72 hours after injection the migration of new cells decreases still more. By this time the majority of the mononuclears free in the fluid have developed into vitally stained macrophages; monocytes and young macrophage forms are less numerous than earlier and

are chiefly free from vital staining as the supply of available dye in the peritoneum appears to be exhausted owing to absorption and to storage in the vitally stained macrophages. After 72 hours the reaction is retrogressing, in that few new cells are arriving in the cavity, and the development of the vitally stained macrophages appears to have reached a maximum unless a further injection of dye be given. Among the fully formed macrophages no morphological or other differences indicating their origin can be detected, the cells originally derived from local histiocytes being indistinguishable from those arising from emigrated lymphocytes or monocytes. With diamine fast scarlet a striking feature at this time is the development of enormous vacuoles in the cells (fig. 93). These may be intensely dye stained, but are more often filled with an almost colourless fluid in which intensely coloured dye granules float freely or are attached to the walls. When neutral red is applied supra-vitally these enormous vacuoles rapidly become deeply stained, this is seen by the change in colour, even when already apparently filled with the acid dye previously administered. With isamine blue, the formation of such enormous vacuoles has not been observed; here the cytoplasm becomes packed with deeply coloured globular dye masses of all sizes, in which more deeply stained granules are present, but the inter-granular material in the isamine blue vacuoles never is colourless as it is with diamine fast scarlet.

If no further injection of dye be given the peritoneal reaction gradually subsides, and the large free vitally stained macrophages become fewer as the cells leave the cavity. The smaller less heavily stained cells persist longer, or perhaps new cells enter the cavity. After 120 hours there is very little free fluid and at the end of 144 hours the peritoneum is usually as dry as in the normal mouse, though of course the tissues are deeply stained. In the trace of fluid, cells of the smaller type normally present are found, and the larger of these usually exhibit a slight degree of vital staining: only a very few heavily stained cells can be recovered in the fluid later than 7 - 10 days after a single injection.

<u>The absorption of dyes (diamine fast scarlet, isamine blue) after intraperitoneal injection.</u>

While the cellular reaction is proceeding in the peritoneal cavity the dye is being slowly absorbed, probably in large part by way of the lymphatics of the diaphragm and mediastinum but perhaps also directly into the blood vessels (Cunningham, 1926). The reticulo-endothelial cells of the mediastinal glands through which the lymph passes before entering the circulating blood are thus stained, and a small amount of dye is then passed into the circulating blood from which it is removed by the cells of the reticulo-endothelial system generally. In this way generalised vital staining is gradually brought about. The amount absorbed after a single intraperitoneal injection is small, by far the greater part of the dye being fixed locally in the tissues, but by repeated inoculations at weekly intervals a high degree of generalised vital staining may be attained. Some dyes, e.g. benzopurpurin do not appear to be absorbed sufficiently to produce generalised staining; these substances produce at the site of inoculation an even more pronounced cellular reaction which persists for a longer time.

<u>Changes in the peritoneal tissues, mesenteries and omentum.</u>

After the intraperitoneal injection of dye the local tissues are bathed in the stain which, while provoking serous and cellular exudation, penetrates to the underlying histiocytes and leads to rapid vital staining of these cells. The unstained small round cells of the adventitial sheaths and tâches laiteuses are mobilised and form probably the chief source of the free mononuclears of the exudate. Those which migrate at once appear in the fluid as lymphocytic and monocytic forms, but examination of the tissues at intervals shows that some undergo differentiation <u>in situ</u> and develop into large dye storing cells which are soon indistinguishable from the preformed histiocytes. During the early stages mitotic figures are extremely scanty; there is thus no evidence that the small vitally stained cells are produced by division of the pre-existing histiocytes. Mitotic division of serosal cells is fairly common after 24 hours and is chiefly a reparative change, following desquamation after irritation (fig.104). Mitoses in the cells of the adventitial sheaths are more easily found from 18 hours onwards, but never appear to be very numerous.

It is noteworthy that mitosis is very uncommon in well developed macrophages; it would appear that in actively stained cells the capacity for multiplication is much reduced. It is extremely difficult to estimate the

amount of migration of cells from the blood, but in spread preparations I have sometimes observed mononuclears as well as polymorphs in process of migration through the capillary walls, and I therefore consider that lymphocytes and perhaps monocytes of haematogenous origin take part in furnishing cells to the exudate and probably serve to make good the loss of cells from the local storage depots in the adventitial sheaths and tâches laiteuses.

While the reaction is progressing in the peritoneum the local tissues, e.g. omentum and mesentery, are becoming steadily richer in vitally stained cells. As mentioned above, this is partly due to new formation _in situ_, but probably it is in part to be accounted for by the return of stained cells to the tissues. During the reaction many cells are found adhering to the surface of the mesentery and entangled in the meshes of the omentum; it is probable that a continuous interchange is going on, some of the earlier free macrophages returning to the tissues while new unstained cells continue to be liberated into the cavity. In the later stages when the reaction is subsiding the stained cells re-enter the tissues and accumulate in masses, sometimes along the adventitial sheaths but often in clusters in the intervening avascular portions (fig. 98). These cells are morphologically indistinguishable from the pre-formed histiocytes and it is not possible to recognise whether a heavily stained cell has remained _in situ_ during

the reaction or has returned to the tissues after a stage as a free macrophage. It is, however, clear that the number of vitally stained cells is much greater than in the case of animals which have been stained by intravenous or subcutaneous administration of the dyes.

Further evidence that the stained cells return to the tissues has been obtined as follows. Portions of the omentum and mesentery were removed post mortem at various stages after the intraperitoneal injection of dye, and were washed in saline to remove as far as possible the cells adhering to their surfaces. Spread preparations were then made as usual. By comparison of similarly prepared specimens of omentum and mesentery in the early stages with those of the later stages, it is found that the number of stained cells actually within the tissues increases. That this is not due entirely to development of stained cells in situ is shown by the greater number of such cells in the tissues coincidently with the disappearance of the free cells from the cavity at 96, hours, as compared with 48 hours, although during this further period there is practically no free dye in the peritoneum to lead to increased staining of cells in situ.

Changes in the peritoneal mesothelium following the injection of dyes.

It is convenient to draw attention here to the degrees of vital staining found in mesothelial cells *in situ*. After a single intraperitoneal injection of isamine blue staining of the serosal cells is often absent in fixed preparations and in fresh cells is limited to a few tiny granules near the nucleus. After repeated intraperitoneal injections of isamine blue a small crescent of fine stained granules may be formed, with diamine fast scarlet the granules are coarser and more abundant; they are also more scattered in the cytoplasm. After repeated intrperitoneal injections of trypan blue, however, dye deposits of much greater extent occur and large masses of small irregularly shaped granules develop in the serosal cells (fig.103). These are usually close to the nucleus and form the generally described perinuclear crescent (fig. 103). When such cells become free as a result of irritation, the crescent of granules is usually situated around the nucleus, but I have observed cells of undoubted mesothelial type in which the granules were more scattered in the cytoplasm and even extended into the outstretched processes. By comparison with the results of supravital staining in normal animals it is clear that such appearances represent a markedly stimulated condition of the cells. It is interesting to note that

after decolourisation of the animal occurs from lapse of time, the cells appear to retain for a period their enhanced capacity for dye storage. Thus I have observed that a single intravenous injection of a rapidly stored dye, e.g. lithium carmine, in a rabbit partly decolorised after repeated intraperitoneal injections of trypan blue is followed by a degree of carmine staining in the otherwise colourless mesothelial cells in excess of that obtained by similar injection given to an untreated animal. The macrophages also acquire this property of enhanced staining as a result of preliminary treatment.

As early as 2 hours after intraperitoneal injection of diamine fast scarlet or isamine blue, a few serosal cells can be recognised in the peritoneal fluid, and such cells can generally be found at all stages up to 96 hours. They usually occur singly or in small groups, the larger sheets sometimes found are probably traumatic in origin in collecting the exudate.

In the earlier stages, i.e. 6 - 24 hours, degenerative changes in the desquamated serosal cells are common; the nucleus and cytoplasm become diffusely stained indicating that cell death has occurred. Pyknotic changes in the nuclei are uncommon, most of the damaged serosal cells appearing to disintegrate by lysis, on the other hand pyknosis is frequently seen in polymorphs and mononuclears. From 24 hours onwards, mitoses can be found both in the

free and attached mesothelial cells more often indeed than in the macrophages (fig. 104). The daughter cells can generally be distinguished from macrophages by their nuclear configuration, basophilic cytoplasm and by the relatively scanty vital staining (fig. 104), but it has been observed that in the later stages some of the free proliferated serosal cells bear a strong resemblance to macrophages.

Proliferation of serosal cells is more marked on the omentum than on the mesentery or on the parietal peritoneum; and the omental mesothelium seems to be shed and gives rise to free cells more readily than elsewhere. In spread preparations of omentum rounded swollen mesothelial cells are frequently seen with unusually large accumulations of vitally stained granules, and occasionally even with an ingested polymorph in their cytoplasm. It is especially in such cases that difficulty is encountered in deciding whether the cells are actually in process of transformation into macrophages, and while I do not consider that they furnish any considerable proportion, I am not prepared to deny that transformation of serosal cells into macrophages may occur.

In the later stages of the reaction following diamine fast scarlet, i.e. 48 - 96 hours after a single large dose of dye or after several smaller doses, curious cellular formations are found. Free mesothelial cells appear to

attach themselves to masses of the sticky precipitate resulting from the mixture of dye solution and serous exudate (fig. 104), or to aggregates of heavily stained macrophages adhering to such masses. The serosal cells divide by mitosis and eventually form a complete surface layer covering the mass, which now presents a curious mulberry appearance. The mode of formation of these mulberry-like structures is illustrated in fig. 104 , and it is noteworthy that such altered serosal cells may show enhanced degrees of vital staining. The covering of these aggregates of precipitate and entangled macrophages by serosal cells appears to correspond to what Marchand (1921) observed following the introduction of lycopodium spores, or to the healing of the explant by growth of mesothelium or epithelium over the cut surface noted by Carleton (1925) in tissue cultures of lung.

Peritoneal reactions in animals vitally stained by previous intraperitoneal injection of dye.

It has been pointed out that if a peritoneal exudate be developed after vital staining induced by repeated subcutaneous injections of dye, the exudate contains only scanty stained cells unless there be a considerable amount of free dye available in the tissue fluids. It appeared that the majority of the large macrophages in the later stages of the reaction were cells developed from unstained precursors, and that the mature histiocytes of the local tissues formed only a small proportion of the exudate cells. Lest this result should be due to the numerical inferiority of fully developed histiocytes in the tissues in comparison with the immature cells of the adventitial sheaths, etc., experiments were performed using animals whose peritoneal tissues contained a very large number of heavily stained histiocytes as a result of previous repeated intraperitoneal injections of dye. This group consisted of 33 mice, which had received repeated intraperitoneal injections in 10 animals of trypan blue, in 10 of isamine blue, in 10 of diamine fast scarlet and in 3 of vital new red. In addition, 4 guinea-pigs received one or more intraperitoneal injections of diamine fast scarlet. After the last injection of dye, an interval was allowed sufficient to permit of the

return of the free vitally stained macrophages to the tissues as previously described.

Some animals then received broth as the peritoneal stimulant, others were given an injection of a suitable dye of contrasting colour, i.e. isamine blue stained animals received diamine fast scarlet, and <u>vice versa</u>; the resulting exudate was examined at intervals as before.

The following are illustrative protocols of the treatment received by individual animals in this group.

(a) A mouse received 5 intraperitoneal injections of 1 per cent. isamine blue at weekly intervals, and four weeks after the last injection was still intensely stained. A fragment of subcutaneous tissue was removed from the thigh under ether anaesthesia and in it the histiocytes were intensely stained. 1 c.c. of 1 per cent. solution of diamine fast scarlet was then injected intraperitoneally, and this was repeated after 48 hours. The resulting exudate was examined at intervals from 12 - 120 hours, when the animal was killed. The cells were of the usual type and showed progressive accumulation of the red dye. Blue stained cells were very scanty in the exudate at all stages, although such cells were numerous in the peritoneal tissues.

(b) A guinea-pig received two intraperitoneal injections of 10 c.c. of 1 per cent. diamine fast scarlet at an interval of 8 days. The exudate was examined and the presence of numerous vitally stained cells noted. Twenty-one days later, 1 c.c. of an 18 hour broth culture of <u>Staph. aureus</u> was injected intraperitoneally, and the resulting exudate withdrawn at intervals, the animal being killed after 96 hours when the peritonitis was almost resolved. In the exudate which, in the later stages, contained many large macrophages, vitally stained cells were extremely scanty, but they were present in large numbers in the omentum and mesentery especially in the adventitial sheaths.

The findings of the previous experiments were here

confirmed; histiocytes already heavily vitally stained take little part in the formation of a subsequent cellular exudate, and appear free in the peritoneum only in small numbers (fig.106). The exudate produced by the second stimulus is, however, almost as abundant and cellular as the first, and is of similar general character. In the early stages there is an abundant outpouring of lymphocytes and monocytes which develop rapidly into young macrophages as previously described (fig. 106). If the agent used to produce the second reaction be a dye of different colour from that employed in the preliminary staining, e.g. diamine fast scarlet following after isamine blue, it is found that the newly formed macrophages stain vitally with the usual rapidity, and we have thus the phenomenon of free cells vitally stained red while the cells throughout the body are stained blue. In the peritoneal tissues of such animals in addition to many histiocytes laden with the first administered dye there are many cells which have also taken up the second dye, but others contain only the latter (fig.107). It is clear therefore that the relative absence of stained cells in the exudate is not due to lack of such cells in the tissues, but must result from an inhibition of their migratory powers. Nevertheless such cells are still able to absorb and store further quantities of dye reaching them _in situ_, and they cannot thus be

considered as functionally paralysed. Even after repeated injection of highly colloidal dye, the peritoneal tissues continue to exhibit many small round cells of lymphocyte- and monocyte-type, no matter how great the number of vitally stained histiocytes present. As previously described, mitosis in fully formed histiocytes is rare, the great majority of the dividing cells found in spread preparations or in sections are either of mesothelial type or are small unstained cells. It would thus appear that in the small round cells of the adventitial sheaths and _tâches laiteuses_ there is a practically inexhaustible supply of cells from which new macrophages are developed as need arises.

DISCUSSION AND SUMMARY.

In the foregoing experiments in mice and guinea pigs the characters of the cellular response in the peritoneum have been studied following the introduction of various mild irritants such as sterile broth and various highly colloidal dyes. The reaction following the intraperitoneal injection of dyes such as diamine fast scarlet and isamine blue is slower in development and lasts longer than that following sterile broth, but the changes are essentially similar, and the outstanding feature of both is the high proportion of mononuclear cells in the exudate.

The attempt has been made by means of vital and supravital staining to trace the origin and development of the large mononuclear cells which are so prominent in the later stages of a resolving peritonitis. By the study of very early stages it has been shown that the first cells to increase in the peritoneum are small mononuclears resembling lymphocytes and monocytes. Supravital staining with neutral red and janus green has shown that these cells cannot be sharply divided as Sabin Doan and Cunningham (1925) and also Witts (1928) claim, but that these are merely functuonal variations of the same cell type; this is in agreement with the views of Maximow (1927, 1928). Many

workers have noted the presence of lymphocytes and monocytes in the early stages of the reaction and have called attention to the relatively late appearance of the fully developed macrophages. Since the latter cells are indistinguishable from the histiocytes of the adjacent tissues especially the omentum and mesentery it has generally been considered that the late appearance of the macrophages in the exudate was due to the slow mobilisation and migration of the histiocytes (Pappenheim 1913, Pappenheim and Fukushi 1914, Cunningham 1922). Evidence has been presented to show that cells indistinguishable from lymphocytes when free in an inflammatory exudate may undergo a series of progressive changes culminating in their transformation into macrophages, and that during these changes they may pass through a stage in which they present the morphological appearance and supravital staining reactions of monocytes. The experiments here recorded have shown clearly that fully formed histiocytes even when present in enormous numbers in the omentum and mesenteries play a relatively unimportant part in furnishing cells to the exudate. The late appearance of the typical large macrophages is due to the length of time required for the development of these cells from their smaller progenitors. The large cells are merely the smaller lymphocytes monocytes and immature histiocytes which have undergone a striking functional hypertrophy.

The small cells which appear at first do not show vital staining to any significant extent, but as they develop in the later stages of the reaction they acquire the capacity to stain. The intensity of their coloration depends on the amount of free dye available to them. When free dye is present in the blood and tissue fluids as a result of intravenous or subcutaneous injection staining of the exudate cells is not usually very intense owing to the small amount of dye passing into the fluid exudate, but when the dye is administered intraperitoneally very intense staining of the free cells occurs. The lack of vital staining in the typical small lympnocytes appears to be due to the fact that at least several hours contact with the dye solution is required before the formation of 'vacuoles' and the accumulation of dye within them is sufficiently marked to be clearly visible. With the more highly colloidal dyes used this lag period is about 5 - 6 hours; by the end of this time, however, the lymphocytes which are in process of differentiating into monocytes or macrophages have already begun to alter their morphology, the cytoplasm becoming more abundant and the nuclear texture less dense. Maximow (1902) pointed out how extremely rapidly lymphocytes begin to develop when extravasated into the subcutaneous tissues; recently (1928) he has

confirmed that similar changes occur in cultures of blood leucocytes _in vitro_, the lymphocytes showing increase of cytoplasm with marked accumulation of neutral red granules and nuclear indentation within six hours after explantation. On account of its rapidity the method of supravital staining with neutral red is here of great service in demonstrating that intervening stages during which the tiny segregation apparatus of lymphocytes is undergoing hypertrophy, but has not yet effected a sufficient storage of vital stain to be clearly visible. In the larger cells of the early stages, a similar hypertrophy of the segregation apparatus has been observed before storage of the vital stain became visible.

The origin of the small round cells - lymphocytes, monocytes and immature histiocytes - which appear so rapidly in the peritoneum is believed to be chiefly from the cells of the adventitial sheaths and _tâches laiteuses_, especially of the omentum and mesenteries. As previously pointed out these cellular collections are composed chiefly of the above types of immature cells, while the number of fully developed histiocytes capable of active dye storage is relatively small in normal animals. It has been shown, however, that the number of vitally stained cells is greatly increased in conditions of local stimulation following the intraperitoneal injection of dye, as a result both of development _in situ_

and from the return of stained cells from the exudate into the tissues. As regards the part played by emigration of mononuclear cells from the blood, no evidence has been obtained to show that the majority of the exudate cells are so derived; it is probable that some mononuclear cells come from the blood in inflammatory conditions generally. This source, however, is like to be of less importance in the peritoneum the tissues of which are so richly supplied with cells capable of emigration and development than in other situation where such collections of adventitial cells are lacking. Since mitotic figures are conspicuously absent in the early stages of the reaction both in the free cells and in the adjacent tissues, it is clear that the small cells are not produced by division of the larger histiocytes. The persistence of lymphocytes in the later stages of the reaction cannot be adduced as evidence against the progressive development above described. Maximow (1928) has also shown that in cultures of blood cells *in vitro* a proportion of the lymphocytes fail to develop and persist in their original state; such cells could not be distinguished either by their supravital or fixed staining reactions from those which subsequently underwent transformation into polyblasts.

When the stimulus producing the reaction is exhausted the majority of the free cells return to the tissues where

they may assume the resting form after digesting or otherwise disposing of the materials which they took up when in the free state. It is probable that the long duration of the reaction following the injection of a dye such as diamine fast scarlet is due to the difficulty in absorbing the flocculated colloid. The persistence of large vitally stained cells in the tissues appears also to be related to the difficulty in disposing of the practically inert dye granules, since such substances do not appear to be broken up to any extent by cellular metabolism. Such cells are therefore easily recognised in the tissues for long periods. When a further peritoneal reaction be induced in such an animal it has been shown that the cells previously active and still recognisable by their content of vital dye take relatively little part in the new exudation, the large macrophages of which are again formed by growth and differentiation of smaller unstained cells. This process appears to be capable of almost indefinite repetition, and the supply of small undifferentiated cells seems to be nearly inexhaustible. It must be emphasised, however, that while the vitally stained histiocytes are not prone to migrate into the exudate nevertheless they are not devoid of capacity for taking up further quantities of foreign substances reaching them _in situ_. The storage capacity of individual cells is, to judge by the size which intracellular dye

deposits may attain, very great and in addition the number of active dye storing cells in the tissues is very rapidly increased when required.

As regards the part played by serosal cells in inflammatory conditions, the evidence indicates that these do not give rise to any considerable proportion of the macrophages, though such transformations may occasionally occur. Schott (1909) considered that the peritoneal phagocytes were derived in large part from the cells of the omentum, especially the serosal cells, but that lymphoid and other connective tissue elements also took part. As I have already pointed out the changes in the omentum are especially difficult to analyse, since the serosal cells in this situation undergo more pronounced morphological alterations following stimulation than they do elsewhere. While I have not obtained evidence that serosal cells are specially concerned in the formation of macrophages, it is clear that they may undergo various reactive changes under pathological conditions and may become converted into tall columnar cells or even into a squamous type following repeated mild stimuli (Cunningham 1924) or even a single acute stimulus (Young 1928). Marchand (1921) described the formation of syncytial giant cells around lycopodium spores introduced into the peritoneum; the serosal cells became loosened, proliferated and covered

the spores which had become attached to the surface of the peritoneum by fibrin. It is interesting that Marchand also describes the occasional transformation of serosal cells into free macrophages. In the human subject I have observed similar changes in inflammatory conditions and transudations into the peritoneum; the serosal cells become loosened, swollen and form syncytial masses (fig.109) which may cover over and enclose small foreign bodies or portions of fibrin. In the experiments recorded above this tendency is exemplified by the formation of small mulberry-like masses of cells due to the growth of a surface layer of mesothelium over aggregations of precipitated dye and entangled macrophages.

VI. The influence of vital staining and of reticulo-endothelial blockade on the resistance of mice to pneumococcus infection.

The effect on resistance of vital staining of the reticulo-endothelial system as a whole.

The effect of vital staining of the reticulo-endothelial cells at the site of inoculation--the peritoneal cavity.

The effect on resistance of blockade of the intravascular elements of the reticulo-endothelial system alone or combined with splenectomy, nephrectomy or other procedures.

The capacity for vital staining in moribund animals.

Discussion.

Summary and conclusions.

In the defence of the animal body against infection by micro-organisms two factors have long been recognised as being of special significance, viz. the cellular defensive mechanism, and the formation and action of humoral anti-bodies. The former was first emphasized by Metchnikoff and he long maintained that the cellular reactions constituted the decisive factor in the establishment of immunity. Others, however, considered that the humoral factors were of paramount importance. Recently evidence has been brought forward to suggest that the reticulo-endothelial system is specially concerned in both connections, but as yet there is no general agreement as to how far such conclusions are justified.

Experimental work has been carried on along two main lines: (a) to determine in which organs antibodies appear first and in greatest concentration after injection of antigen into the circulation or body cavities; (b) to determine the effect either on resistance to infection, or on antibody formation of removal of organs, e.g. splenectomy, or destruction of specialised tissues, e.g. the haemopoietic system and glands by irradiation or by specific poisons such as benzol. The most recent development along the second line has been the attempt to paralyse the functions of reticulo-endothelial cells by taking advantage of their phagocytic power. The idea has gained wide acceptance that,

by filling these cells with relatively inert particulate matter such as india ink, oxide of iron, or vital dyes, their capacity for further functional activities may be seriously reduced. It is of interest to note that Bardach in 1889 utilised this method in studying the natural immunity of dogs to anthrax, and found that intravenous injections of charcoal suspensions greatly increased susceptibility to the infection. This method has recently been termed "reticulo-endothelial blockade;" it has usually been combined with splenectomy for the purpose of removing the most accessible depot of reticulo-endothelial cells.

The present work is concerned with the influence of the so-called reticulo-endothelial blockade on the resistance of mice to pneumococcus infection. In previous communications the morphology and distribution of the reticulo-endothelial system were described, and it was pointed out that the constituent cells fell into two main groups, (1) the reticulo-endothelial cells of the organs, e.g. liver, spleen, lymphatic glands and bone marrow, which are in direct contact with the blood and lymph stream; and (2) the extravascular histiocytes. The importance of both groups of cells in the establishment of immunity to pneumococcus and other infections has been emphasized by various writers (Kyes 1916 , Singer and Adler 1924 [12], Neufeld and Meyer 1924).

In view of these results a series of experiments with pneumococcus infection in mice has been carried out and the attempt made to assess the effects of (a) vital staining of the reticulo-endothelial system as a whole; (b) vital staining of reticulo-endothelial cells at the site of inoculation; (c) blockade of the intravascular elements of the reticulo-endothelial system either alone or combined with splenectomy.

<u>The effect on resistance of vital staining of the reticulo-endothelial system as a whole.</u>

A strain of pneumococci preserved in the dried spleen of a mouse dead of pneumococcal septicaemia was passed several times through mice until 0.5 cc. of a 1 - 10,000 dilution of heart blood produced a fatal infection in normal animals on intraperitoneal injection.

Two series of mice were previously subjected to intra-vitam staining by subcutaneous administration to avoid peritoneal reaction to the dyes. To one set of fifteen mice five injections of isamine blue were administered at intervals of about a week. To another set of eleven mice three injections of trypan blue were given. These animals together with a number of untreated controls were injected intraperitoneally with appropriate dilutions of infected heart blood prepared in 0.1 per cent. gelatin in distilled water, in order to avoid possible deleterious action of saline solution. The following are representative examples of treatment received by individual animals.

A mouse received subcutaneous injections of 1:150 isamine blue, 1 c.c. per 20 gm. body weight, on the 1st., 11th., 24th., 33rd. and 40th. days of the experiment during which time the weight of the animal increased from 16.0 gm. to 19.0 gm. On 48th. day the animal received 0.5 c.c. of a 1:1,000 dilution of heart blood from a mouse dead of pneumococcal septicaemia: death occurred within 48 hours.

TABLE I.

Each animal received an intraperitoneal injection of 0·5 c.c. of a dilution of infected heart blood from a mouse recently dead of pneumococcal septicaemia. The approximate time of death after inoculation is given in hours; S = survival.

Dilutions of infected heart blood.	TIME OF DEATH after inoculation.		
	Isamine blue stained.	Trypan blue stained.	Controls.
1 : 500	48 48	48	
1 : 1000	48 48	48 48	48
1 : 10000	S S	S	S
1 : 25000	48 S	72 S	S
1 : 50000	S S	S S	S
1 : 100000	S S	S S	120
1 : 250000	S S	S	
1 : 500000	S		

In all the animals dying acutely, microscopic examination of the heart blood and peritoneal fluid revealed abundant pneumococci. The surviving animals were killed at intervals from 30 to 90 days later, and both cultural and microscopic examination was made of the heart blood and peritoneum.

ANALYSIS OF TABLE I.

Condition of animals.	Critical range of infecting doses.				Totals.
	1:10000	1:25000	1:50000	1:100000	
Stained	0/3	2/4	0/4	0/4	2/15
Unstained	0/1	0/1	0/1	1/1	1/4

The denominator indicates the number of animals receiving each inoculum, the numerator shews the number of these which died acutely with pneumococcal septicaemia.

A mouse received subcutaneous injections of trypan blue, 1:200, 1 c.c. per 20 gm. body weight, on the 1st., 4th. and 8th. days of the experiment, during which time the weight of the animal remained stationary at about 16·5 gm. On the 17th. day of the experiment the animal received an intra-peritoneal injection of 0·5 c.c. of a 1:1,000 dilution of infected heart blood; death occurred within 48 hours.

The results are shown in TABLE I; the analysis brings out the significance of the figures. It is noteworthy that in the unstained animals the doses 1:1000 and 1:100000 proved lethal, whereas survival occurred after inoculation with the intermediate doses, 1:10000, 1:25000, 1:50000. This result, which is met with repeatedly throughout the experiments, indicates that there is a range of dosage lying between the certainly fatal and the certainly non-fatal which may or may not prove lethal according as the individual animal is more or less susceptible. In the course of this work the endeavour has been made to employ this critical range, as it may be termed, since differences in behaviour of animals receiving such inoculations might be expected to afford the most delicate evidence of the influence of experimental procedures which aim at altering susceptibility. Thus in the present experiments the behaviour of the stained animals suggests that they do not differ significantly from the unstained controls in their resistance to the infection. Among those animals which received sublethal inoculations and which failed to die of acute septicaemia it was surprising to find how long after the original inoculation organisms similar

in morphology and staining reactions to pneumococci could be found in smears of the heart blood and peritoneum in certain cases, although attempts to grow such organisms failed. Similar chronic infections were noted by Browning and Gulbransen (1923)[2].

From this experiment it was concluded that a moderate degree of vital staining had no appreciable influence on the resistance of mice to pneumococcus infection. It was realised that in these animals the vital staining was not carried to anything approaching the maximum degree possible with the dyes employed, but histological controls showed that the reticulo-endothelial cells of the liver, lymphatic glands, marrow and spleen all contained a considerable amount of dye, while the local subcutaneous histiocytes at the site of injection were very intensely stained. Moreover, an interval of seven days had elapsed between the last injection of dye and the inoculation of the animals with the pneumococci. Nevertheless, the experiment serves to demonstrate that simple dye storage is not effective in lowering or raising the immunity of mice to acute pneumococcal infection.

The effect of vital staining of the reticulo-endothelial cells at the site of inoculation - the peritoneal cavity.

Gay and his co-workers (1923) have claimed that the presence of a greatly increased number of macrophages at the site of inoculation, e.g. as a result of the injection of broth, increases the resistance of rabbits to strepto-coccal empyema. It was therefore decided to investigate the influence of such a reaction in relation to pneumococcal infection in mice.

In the previous communication (p. 288) I have described the characters of the cellular reactions produced by intraperitoneal injection of sterile concentrated broth and by dyes of highly colloidal type. In view of the statement that the protective action of clasmatocytes is inhibited when the cells are laden with dye (Gay, Linton and Clark, 1926) three series of animals were employed, (a) normal controls and (b) mice injected intraperitoneally with concentrated broth and (c) animals injected intraperitoneally with 1 per cent. solution of diamine fast scarlet. It had previously been ascertained that diamine fast scarlet produces an abundant cellular exudate and that after 72 hours the majority of the free macrophages and the histiocytes in the local peritoneal tissues are heavily laden with dye granules. By the intraperitoneal injection of concentrated meat infusion broth a cellular exudate is produced which

TABLE II.

The animals were injected intraperitoneally with a standard inoculum of 0·5 c.c. of infected heart blood, 18 hours after injection of broth and 72 hours after injection of dye.
The approximate time of death after inoculation is shown in hours; S = survival.

Dilution of infected heart blood.	TIME OF DEATH after inoculation.		
	Broth injected.	Dye injected.	Controls
1 : 1000	48 48	96	48
1 : 10000	S S	S S	S
1 : 25000	S S	S S	48
1 : 50000	S S	S S	S
1 : 100000	S	S S	S
1 : 250000		S	

In all animals dying acutely microscopic examination showed numerous pneumococci in the heart blood and peritoneal fluid.

ANALYSIS OF TABLE II.

Condition of animals.	Critical range of infecting doses.			
	1:1000	1:10000	1:25000	Totals.
Broth injected	2/2	0/2	0/2	2/6
Dye injected	1/1	0/2	0/2	1/5
Controls	1/1	0/2	1/1	2/4

after 18 hours is especially rich in young macrophages. It was hoped that by comparing the course of intraperitoneal pneumococcal infection in parallel series of animals injected with broth or dye information would be obtained as to the influence of the local cellular reaction in combating the infection. It appeared that the presence of a richly cellular exudate composed chiefly of macrophages might protect the animals against infection; on the other hand, macrophages heavily laden with dye might suffer some inhibition of their activities and so the resistance of the animals might be lowered. Accordingly two series of ten mice were prepared by intraperitoneal injection, one set receiving concentrated broth and the other set 1 per cent. diamine fast scarlet. After 18 hours and 72 hours respectively these animals together with five normal controls were inoculated intraperitoneally with varying dilutions of heart blood of a mouse dead of pneumococcal septicaemia. The virulence of the strain of pneumococci used in this case was such that 0·5 c.c. of 1 - 10,000 dilution of heart blood had invariably proved fatal in previous passages, and 1 - 100,000 led occasionally to an acute fatal septicaemia. The result of the experiment is shown in TABLE II.

Although the virulence of the inoculum is lower than the results of previous passages had led one to expect it is

TABLE III.

The animals were inoculated intraperitoneally with 0·5 c.c. of infected heart blood, 18 hours after injection of broth and 72 hours after injection of dye respectively. The time of death after inoculation is shown in hours.

Dilution of infected heart blood.	TIME OF DEATH after inoculation.		
	Broth injected.	Dye injected.	Controls.
1 : 1000	28 33	28 33	28
1 : 10000	48 48	48 48	28
1 : 25000	33 46	32 48	33
1 : 50000	28 29	48 48	33
1 : 100000	33 33	33 48	30
1 : 250000	44 48	48	24

In all animals dying acutely microscopic examination showed numerous pneumococci in the heart blood and peritoneal fluid.

apparent that no decisive effect has been produced by the injection either of broth or dye. The animals are neither more nor less susceptible to the infection; it is clear that the range of infecting doses covers the critical zone in which the individuality of the animals plays an important part in determining the outcome.

In view of the falling off in virulence of the organisms the experiment was repeated. The same strain of pneumococci was again obtained by passage from the spleen of a mouse dead acutely of the infection, and the virulence after repeated passage was such that 0·5 c.c of 1 - 50,000 of heart blood was uniformly fatal. Two series of 12 mice were prepared as before by intraperitoneal injection of concentrated broth and of diamine fast scarlet; these, together with 6 controls, were inoculated intraperitoneally after the same intervals with varying dilutions of heart blood of a mouse dead of pneumococcal septicaemia. One dye-injected mouse did not appear to be well and was discarded, leaving 12 broth-injected, 11 dye-injected and 6 control mice. The result of the experiment is shown in TABLE III.

On this occasion the virulence of the infecting organism has been so augmented, that both series of inoculated animals died acutely of pneumococcal septicaemia. The actual duration of survival was noted as accurately as possible in

every case and it was observed that the periods, while short, bore no relation to the dose of pneumococci or to the previous treatment of the animal. Thus the first death was the control animal with the lowest dosage, viz. 0·5 c.c. of 1 - 250,000 at 24 hours, followed by seven animals distributed over all three groups between 24 and 28 hours, 10 animals between 28 and 33 hours and the 11 remaining animals between 33 and 48 hours. Although the dosage does not actually fall within the critical range in this experiment, it nevertheless seems to demonstrate clearly that no striking degree of protection has been afforded to any of the animals by means of the experimental stimulation of the peritoneal cavity.

The effect on resistance of blockade of the intravascular elements of the reticulo-endothelial system alone or combined with splenectomy or nephrectomy or other procedures.

A further series of experiments was undertaken to determine the influence on infection of the so-called "blockade" of the reticulo-endothelial system alone or when combined with splenectomy or other operative measure. In the literature of this subject, it is generally stated that "blockade" by the intravenous injection of dyes or suspensoid preparations alone has little or no depressing effect, either on the resistance of animals to infection (Wright 1927), or on their capacity to produce the more readily demonstrable antibodies such as agglutinins or precipitins (Bieling 1923-24; Stewart and Parker 1926; Ross 1926). Mere removal of the spleen, which is regarded as a highly developed part of the reticulo-endothelial system in mammals, is said to produce a slight depression of these processes (Pfeiffer and Marx 1898: Standenath 1923). It has frequently been stated, however, that the combination of splenectomy with intravenous injection of dyes or saccharated oxide of iron has a depressing effect on the capacity of the treated animals to produce antibodies (Siegmund, 1922: Bieling and Isaac 1921 1922[1,2,3]: Gay and Clark, 1924), and these procedures are also said to diminish the resistance of the treated animals to infection

(Neufeld and Meyer, 1924; Singer and Adler, 1924).[1,2]
It must be pointed out, however, that such conclusions have frequently been founded on scanty data, e.g. the number of animals used has not always been large enough to allow for the variations due to the individuality of different animals. Further, in the majority of the recorded experiments the effect of operative procedures other than splenectomy, or of the intravenous injection of substances not stored in the reticulo-endothelial system does not appear to have been investigated. Results of a directly opposite nature have also been recorded. Standenath (1923) stated that blockade alone exercised a stimulating effect on precipitin formation and when combined with splenectomy might abolish the depressing effect of the latter procedure. Fränkel and Grünenberg (1924) and Rosenthal, Moses and Petzal (1924) also failed to detect any diminution of antibody formation in splenectomised and blockaded animals and the latter state in addition that these procedures may actually increase the output of immune antibodies such as agglutinins and precipitins.

In view of the conflicting results reported, it was considered of interest to study the effect of such procedures on the resistance of mice to pneumococcus infection. It is of course essential to control adequately the experimental methods designed to affect the reticulo-endothelial system by carrying out others which might be expected to exercise

a similar general effect although lacking selective action on those cells. Accordingly parallel series of animals were prepared, the general plan of the experiments was as follows. The operation was performed on mice under ether anaesthesia with the usual aseptic precautions and about 30 hours later the animals were inoculated with varying doses of pooled heart blood of mice dead of pneumococcal septicaemia, the inoculum being derived from 3 mice in order to compensate as far as possible for the variations in virulence due to the influence of the host. In one series of animals splenectomy was performed, in others unilateral nephrectomy was chosen as a suitable control operation of comparable severity. These operated animals together with a similar number of normal mice were injected intravenously with a suitable dose of 10 per cent. solution of saccharated oxide of iron 6 - 8 hours before inoculation. The same number of splenectomised and nephrectomised mice were also injected with 10 per cent. solution of saccharose as an example of a relatively non-toxic substance not stored in the cells of the reticulo-endothelial system. Accordingly the influence of the following conditions on resistance to infection was studied: - removal of spleen alone: removal of kidney alone: removal of spleen + injection of saccharated oxide of iron: removal of kidney + injection of saccharated oxide of iron: removal of kidney + injection of sugar: injection of saccharated oxide of iron: injection of saccharose: injection of glucose.

TABLE IV.

Operation was performed about 30 hours before inoculation; 0·3 c.c. of 10 per cent solution of saccharated oxide of iron per 20 gm. weight was injected intravenously 6 - 8 hours before intraperitoneal inoculation with infected heart blood. The time of death is given in hours; S = survival.

Dilution of infected heart blood.	TIME OF DEATH after inoculation.			
	Splenectomy + Sacch. ox. of iron.	Nephrectomy + sacch. ox. of iron.	Sacch. ox. of iron.	Controls.
1 : 100	12	17	12 12	18
1 : 500	17	23	36	18 36
1 : 1000	23 36	20 17	14 14	86 S
1 : 5000	20 36	23 42	12 18	192 (1) S
1 : 10000	23 36 (2)	1½ (3) 72	12 12	65 S
1 : 25000	72 84	18 S	18 20	S S
1 : 50000	36	20	36	S
Analysis: ratio of deaths to inoculated.	12/12	11/12	12/12	5/12

In all animals dying acutely microscopic examination showed numerous pneumococci in the heart blood and peritoneal fluid, and an abundant growth of pneumococci was obtained on culture of the heart blood.

(1) The peritoneum contained much fewer organisms than usual, but there was a bilateral fibrinous pleurisy and pericarditis.
(2) This animal was distinctly jaundiced.
(3) Death was due to haemorrhage into the peritoneum, probably post-operative.

In these experiments all the mice were from the same stock, and as far as possible animals of the same age, size and weight were used, the majority being about 3 months old and weighing about 20 gm.

Attempts were made to assess the virulence of the organisms before infecting the main series of animals, but it was found that the lethal dose in any one passage bore no constant relation to that of the succeeding passage. Further the length of survival in animals dying acutely appeared within a wide range of doses to depend more on individual variations than on the actual number of organisms introduced, for example in one passage an animal receiving 0·5 c.c. of 1:10000 died in 40 hours whereas that receiving 0·5 c.c.of 1:100 of the same heart blood survived for 120 hours.

<u>SERIES I.</u> Eleven splenectomised and eleven nephrectomised mice together with twelve normal mice were injected intravenously 24 hours after operation with 10 per cent. solution of saccharated oxide of iron, the dose being 0·3 c.c. per 20 gm. body weight; 6 - 8 hours later these animals together with 12 untreated controls were inoculated with varying doses of pooled heart blood from three mice recently dead of pneumococcal septicaemia. The result is shown in TABLE IV.

In addition two mice which had survived inoculation three months previously and three mice splenectomised three months before each received an intravenous injection of saccharated oxide of iron in the usual dose. These animals together with three which has survived inoculation during earlier passages within the previous week were inoculated with the same range of dilutions of the same infected heart blood as the main series. All of these animals succumbed within 96 hours to acute septicaemia.

This experiment appears to show that the preliminary treatment has knocked out the resistance of the mice to the infection, for only one of the treated animals survived, whereas 5 out of 12 of the normal controls survived. Since the factor common to all three groups of treated animals was the injection of saccharated oxide of iron, the experiment was repeated separating the factors of blockade and operation.

SERIES II. In the second experiment performed 14 days later five groups each of 10 mice were treated as follows under the same conditions as before: intravenous injection of saccharated oxide of iron: intravenous injection of saccharose: nephrectomy: nephrectomy *plus* intravenous injection of saccharated oxide of iron: nephrectomy *plus* intravenous injection of saccharose. In addition two

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TABLE V.

As before the animals were inoculated intraperitoneally 30 hours after operation: 10 per cent solution of saccharose and 10 per cent saccharated oxide of iron were administered intravenously 6 - 8 hours before inoculation, the doses being 0·3 c.c. per 20 gm. weight. S = survival.

Dilution of infected heart blood.	TIME OF DEATH in hours after inoculation.					
	Reinoculated controls.	Nephrectomy.	Nephrectomy + sacch. ox. iron.	Nephrectomy + saccharose.	Saccharated oxide of iron.	Saccharose.
1 : 500		36 40	27 36	36 36	36 36	23 25
1 : 1000	36	25 36	18 24	48 60	25 48	23 40
1 : 5000	25 84 (1) (2)	36 42	36 40	42 S	25 25	26 36
1 : 10000	26	36 36	22 36	23 36	18 36	46 108 (2)
1 : 25000	20 36	36 42	24 40	23 36	23 44	36 36
1 : 50000	36	48 S	36 36	25 42	36 S	25 S
Analysis: ratio of deaths to total inoculated.	7/7	11/12	12/12	11/12	11/12	11/12

(1) = Nephrectomised and blockaded animal from series I.

The two normal controls inoculated respectively with 1 : 10000 and 1 : 25000 died in 42 and 25 hours.

(2) In these animals the peritoneal cavity was almost free from exudate but there was a bilateral fibrinous pleurisy and pericarditis.

normal controls and the six surviving controls from the first series were reinoculated, the latter receiving the same dilutions as before. The treated mouse surviving from the first series was also reinoculated. The result is shown in TABLE V.

The virulence of the infecting organisms has increased in this experiment, but it is clear that none of the methods of preliminary treatment has had a decisive effect on the resistance of the animals. It is noteworthy that the duration of survival after inoculation bore no relation to the size of the infecting doses, for the earliest deaths occurred with 1:10000 (18, 22, and 23 hours) while animals receiving 1:500 lived for 36 - 40 hours.

SERIES III. With a view to bringing into greater prominence the influence of operation and of blockade, the experiment was repeated 4 days later with 5 doses of infected heart blood ranging from 1:1000 to 1:300000. Groups each of ten mice were submitted to the following treatment and constituted the third series: nephrectomy: splenectomy: injection of saccharated oxide of iron: injection of glucose: untreated - normal controls. On this occasion the animals were inoculated about 24 hours after operation and 4 - 6 hours after intravenous injection. The result is shown in TABLE VI.

TABLE VI.

The animals were inoculated intraperitoneally about 24 hours after operation. Saccharated oxide of iron and glucose in 10 per cent solution were administered in the same doses as previously about 4 - 6 hours before inoculation. S = survival.

Dilution of infected heart blood.	TIME OF DEATH in hours after inoculation.				
	Splenectomy.	Nephrectomy.	Sacch. oxide of iron.	Glucose.	Normal controls
1 : 1000	42 42	28 28	28 52	30 31	64 68
1 : 10000	46 S	29 29	23 52	18 (2) 30	33 30
1 : 50000	29 42	32 42	32 S	32 52	31 42
1 : 150000	S S	33 42	42 S	32 42	30 S
1 : 300000	S - (1)	42 S	48 78	52 64	S S
Analysis: ratio of deaths to total inoculated.	5/9	9/10	8/10	10/10	7/10

(1) One splenectomised mouse was not inoculated owing to post-operative haemorrhage.

(2) Purpuric spots on paws.

In all animals dying acutely films from the heart blood and peritoneum and cultures from the heart blood showed the presence of abundant pneumococci.

Again, no evidence of orderly progression of the infection is seen within the individual groups, and it is evident that none of the preliminary procedures has produced any decisive effect on resistance. Indeed so far as the actual results go, splenectomy would appear to increase resistance, and intravenous injection of glucose to diminish it, which is absurd. The virulence of the infecting organism is very high, for the heart blood used to prepare the dilutions contained only a small number of cocci as shown in films and culture. Nevertheless, although 0·5 c.c. of 1 - 300,000 of the infected heart blood was fatal in 50 per cent. of the mice, others survived 1 - 10,000. This fact indicates that the doses employed were within the critical range in which the individuality of the animal was the decisive factor in determining whether or not a fatal septicaemia would result, and such conditions offered a favourable opportunity to study the influence on resistance produced by operation or by intravenous injection of foreign material.

It is interesting to note that survival after the minute doses of infected heart blood led to no appreciable immunity to subsequent reinoculation. The untreated surviving animals, together with several others which had received sublethal doses during passage were reinoculated in

subsequent experiments, usually with the same dilution of heart blood as they had previously survived. In every case a fatal septicaemia ensued. There is no evidence that these mice were hypersensitive, as the general level of virulence had also increased. The only possible exception to this was a mouse which survived subcutaneous inoculation with 0·5 c.c. of infected heart blood during passage. This animal was reinoculated intraperitoneally 7 days later along with the first series, receiving 0·5 c.c. of 1:500 without ill effect. 14 days later this animal was injected intravenously with saccharated oxide of iron in the usual dose, and was reinoculated along with the second series, again receiving 0·5 c.c. of 1:500 intraperitoneally. Death occurred in 25 hours from pneumococcal septicaemia.

<u>The capacity for vital staining in moribund animals</u>.

In the present experiments a few observations on the storage capacity of the reticulo-endothelial system were made on control mice dying of pneumococcal infection. Three such animals when moribund were injected intravenously with the usual dose of saccharated oxide of iron at periods which proved to be from 2 to 6 hours before death. The tissues of these mice were examined microscopically together with those of non-infected animals killed at corresponding intervals after similar injections of iron. In each case no noteworthy variation in the distribution of iron or in the degree of intracellular storage was observed. Accordingly it must be concluded that the reticulo-endothelial cells in mice dying of pneumococcal septicaemia show no significant lack of capacity to deal with particulate matter introduced into the circulation.

D I S C U S S I O N.

Many attempts have been made to estimate the part played by the reticulo-endothelial system in antibody formation by interfering with the cells of the system, splenectomy and the so-called blockade being the methods selected. In the group of protozoal infections numerous studies have been made on the effect of therapeutic agents in infected animals subjected to splenectomy and blockade. Jungeblut (1927) and Kritschewski (1927) summarising their experiences with various chemotherapeutic substances state that the majority, e.g. the salvarsan group, Bayer 205, etc. act much less powerfully in animals previously treated in this way, and they conclude therefore that the reticulo-endothelial system plays an essential part in activating such substances in the body. Comparatively few experiments have been recorded, however, in which the influence of these procedures on resistance to acute bacterial infections has been investigated, although Bardach (1889) had observed that the natural resistance of dogs to anthrax was abolished by splenectomy. He showed further that the resistance of normal dogs could be destroyed by intravenous injection of a large amount of a suspension of charcoal powder; in these animals the phagocytes of the organs were loaded with carbon particles while the organisms were extracellular. Bardach

therefore concluded that the resistance of dogs to anthrax depended on the capacity of the phagocytes to ingest and destroy the anthrax bacilli.

Neufeld and Meyer (1924) found that splenectomised mice which had received saccharated oxide of iron intravenously failed to be protected against pneumococcal infection by preliminary vaccination with killed cultures of pneumococci. While the number of animals employed in each group was very small the results of the experiments appear definite, and the authors concluded that the reticulo-endothelial system was of especial importance in the establishment of immunity. Neufeld and Meyer recorded that whereas the serum of mice immunised against pneumococci failed to protect normal mice against infection, this property was conferred on the serum of immunised animals by injection of manganese chloride. They attributed this to a secretory action on the part of the reticulo-endothelial cells following stimulation by the manganese salt, which resulted in the throwing off into the blood of formed antibodies which were previously attached to the cells.

Singer and Adler (1924) [1,2] showed that in rabbits actively immune to pneumococcus infection blockade of the reticulo-endothelial system with ink temporarily lowered the immunity, which they therefore considered to be essentially

dependent on the activity of reticulo-endothelial cells.

Meersohn (1928) observed that splenectomised mice were more susceptible to streptococcal infection than normal controls but that their resistance was not appreciably diminished further by intravenous injection of oxide of iron. The surviving blockaded and splenectomised animals, however, showed a much higher proportion of positive blood cultures than the normal controls or those only splenectomised, a significant indication of reduced power to eradicate infection.

Bass (1925) recorded experiments similar to those of Singer and Adler, but with streptococci; he also concluded that the activity of the reticulo-endothelial system was the essential factor in immunity against this organism.

Gay and Morrison (1923) have approached the problem from another side, and have claimed that rabbits may be protected against intrapleural streptococcal infection by mobilising the histiocytes at the site of inoculation by means of previous local injection of broth. It is difficult, however, to understand the mechanism by which protection is brought about. The fatal dose of cocci was frequently less than 1000 - in the later experiments as low as 15 - in the form of a dilution of a 24 hour broth culture which was therefore probably not in the active phase of growth (Wright,1927), yet it is stated that over 4,000,000 clasmatocytes were required in the pleura to protect the animals. The excess

of cells required to destroy this small number of bacteria is striking.

Clark (1929) performed similar experiments using type I pneumococci, and found that the presence of abundant clasmatocytes in the pleura did not protect rabbits against death from pneumococcal empyema; the addition of immune serum, however, brought about protection although administered in doses insufficient *per se* to save normal animals.

Nakahara (1925) found that mice were protected against intraperitoneal infection with multiple lethal doses of staphylococci or pneumococci by previous intraperitoneal injection of olive oil, and he attributed the beneficial influence of the treatment to the presence of stimulated macrophages in the peritoneum.

In all these experiments it has been observed that the inflammatory exudates which protect *in vivo* do not possess a sufficiently destructive action on the organisms *in vitro* to explain the protection afforded (see also Gay and Clark, 1926)[2]

Wright (1927) also stated that there exists a mechanism for the destruction of organisms *in vivo* which has no parallel in *in vitro* experiments and which is independent of demonstrable antibodies of the commonly recognised types. Wright added further that procedures designed to throw out of action the reticulo-endothelial system had no influence

on the rate of clearing of the blood stream of pneumococci and attributes this to the presence of several mutually compensatory mechanisms which are together involved in the clearing process. It must be noted, however, that the doses of ink and dye used to produce "blockade" were relatively small.

Besredka (1923[1,2] 1924) has claimed that the injection into the skin, or even the mere application to its surface, of broth filtrates of old cultures of staphylococci and other organisms protects the skin against infection by the corresponding organisms, and to such filtrates he has applied the name "antivirus." Others have shown however that the protective effect is not specific, but can also be obtained by the application of broth alone; and they attribute the protective action to the increased number of histiocytes in the area of skin injected or sodden by the wet dressings (Gratia 1923; Mallory and Marble 1925; Miller 1927; Freedlander and Toomey 1928.)

Hach, Borodaj and Melnyk (1927 - 28) have found in rabbits dying of acute staphylococcal septicaemia following intravenous injection of virulent cultures that the number of organisms in the spleen progressively diminishes while that in the skin undergoes continued increase until the death of the animal. Hach and Melnyk (1927 - 28) subsequently

found that if areas of skin were prepared by extensive intradermic injection of sterile broth the number of organisms recoverable from such sites was only one hundredth of that found in untreated animals, and they considered that the difference was probably due to the protective action of the histiocytes mobilised in the skin as a result of the introduction of broth. The degree of immunisation however was in no case sufficient to afford complete protection against a lethal dose of the infecting organism.

Ledingham's results (1927) in vaccinia are specially interesting. He observed that the cutaneous response of rabbits to simultaneous inoculation of india ink and vaccinia virus was greatly modified; further, vaccination of areas of skin previously injected with india ink resulted in failures to take. This resistance was purely local and was strictly confined to the ink spots in the skin; it appeared to depend on local mobilisation of histiocytes at the site of injection. Ledingham also states that similar preliminary treatment modified the skin response of rabbits to intracutaneous inoculation with erysipelatous streptococci. In guinea pigs protection was similarly afforded against subcutaneous inoculation with diptheria toxin but not against B. diphtheriae or B. anthracis.

Other methods of conferring protection whose rationale is more obscure are exemplified by the work of Susman (1927) who found that brain extracts had a favourable influence on pneumococcal infection in mice, 51 per cent. of the animals treated by subcutaneous injection of the extract either before or after inoculation surviving after all normal controls had died. Walbum (1926)[1,2] has shown that the salts of certain metals especially manganese administered in suitable doses enable animals to withstand without harm lethal doses of various toxins. Mice could also be protected by small doses of caesium chloride against an otherwise fatal infection with ratin bacilli. Treatment of ratin infected mice with vaccine or manganese salts separately was ineffective whereas the combined administration of both saved a large percentage of the animals. Similarly the administration of antiserum along with suitable doses of manganese salts protected animals against various toxins in doses which were without protective effect when administered separately. In all these experiments a striking feature is the very narrow range of doses of metal salt which confer protection; the optimum dose in one infection or intoxication is, however, not necessarily the most effective in any other. The mode of action of these substances is quite unknown, and the findings cannot as yet be

correlated with other knowledge of immunity processes.

It is clear therefore that many procedures may exert a profound influence on immunity, both in regard to resistance to acute infection and in the formation of immune antibodies. Such other factors may have been neglected in interpreting the results of experimental procedures.

My own work consists of three main experiments in which attempts have been made to assess the effects on resistance to pneumococcal infection of (a) vital staining of the reticulo-endothelial system as a whole; (b) vital staining of the reticulo-endothelial cells at the site of inoculation; (c) blockade of the intravascular elements of the reticulo-endothelial system alone or combined with splenectomy or nephrectomy. In surveying the results it may be stated that control observations and examination of the tissues of the individual animals have shown that the preliminary treatment of the reticulo-endothelial system in each case succeeded in its immediate object. In the first experiment the tissues of all the dyed animals exhibited marked generalised vital staining. In the second experiment the introduction of broth and of dye respectively into the peritoneum produced in each case an abundant cellular exudate, the former resulting chiefly in young active macrophages and polymorphs; the latter chiefly large fully developed and intensely

vitally stained macrophages. In the third experiment the injection of oxide of iron or of sugar did not upset the general health of the animals; the operative procedures alone did not prove lethal, and no deaths occurred in the control mice from Bartonella infection, which appeared to be absent from the stock in use at that time. These experiments have all been adequately controlled. In this group the procedures designed to influence the active cells of the reticulo-endothelial system have been paralleled by others which might be expected to exercise a similar general effect on the animals' vitality without affecting specifically the reticulo-endothelial cells. It would thus appear that conditions have been established in which it should be possible to detect readily any <u>significant</u> alteration in the behaviour of the treated animals towards infection. Also the various controls described above would have enabled the effects of general factors to be separated from those due to specific alterations in the activity of the cells of the reticulo-endothelial system. Since these procedures failed to produce any decisive effect on the resistance of the treated animals to pneumococcal infection, it remains to be determined whether this indifference implies that the elements of the reticulo-endothelial system are not specially concerned in infection and resistance or whether the lack of effect is due to the presence of adequate compensatory

mechanisms. It must also be borne in mind that neither the factors designed to stimulate resistance nor those to depress it had any noteworthy effect, and it is therefore probable that resistance is a condition dependent on a large number of different factors, one or more of which can be disturbed without seriously impairing the efficacy of the remainder, or exalting the resistance of the organism as a whole. As regards the part played by the reticulo-endothelial system, histological evidence appears to show that the capacity of these cells for compensatory hypertrophy and regeneration is very great. Accordingly no significant abrogation of the functions of the "system" as a whole is likely to be attained by the means at present at disposal without at the same time producing such profound general depression of vitality that the part played by the reticulo-endothelial system *per se* is obscured.

It may be of importance that in my experiments the inoculum consisted of a dilution of heart blood from animals recently dead of an acute infection, whereas most workers have used 24 hour cultures. Felton and Dougherty (1924) state that pneumococci in the active phase of growth possess a higher virulence for mice than when in the latent phase. Wright (1927) has considered the effect of this latent phase in detail; it appears from his work that any prolongation of

the period of latency after introduction into the animal body may improve the animal's chance of survival. In the present work the infecting doses of pneumococci have been suspended in a medium likely to be innocuous to the organisms and have been injected with the minimum delay. It is therefore probable that the organisms were in all cases actively growing when introduced into the peritoneum.

As regards the interpretation of results in investigations of this nature it is essential to bear in mind that there are great variations in individual susceptibility among normal mice of apparently the same age, size and weight, so that with a series of doses one does not get uniformly fatal or non-fatal results unless the differences between the doses are very great. Accordingly a critical range of doses has been selected within which such individual variations are met with. If interference with the reticulo-endothelial system exercised any decisive effect on resistance, it is to be expected that such individual variations would no longer be apparent, or at least that they would not be found within the same range of doses in normal controls and in treated animals. Since this variability in resistance is manifested in the same wide range of doses in both treated and untreated animals, the various experimental procedures have clearly failed to exercise any decisive effect on resistance, though of course

the method may have failed to detect <u>minor</u> variations experimentally induced. These statements apply also to analogous methods used by other investigators.

My observations on the functional capacity of the reticulo-endothelial system during pneumococcal infection have yielded results different from those of Paschkis (1924) and Singer (1925). The former states that in rats injected with streptococcal vaccine the reticulo-endothelial cells of the spleen fail to store lithium carmine injected subsequently, and he attributes this to "blocking" or poisoning of the cells following the ingestion of cocci. Singer (1925) observed that during the course of anthrax infection in rabbits the reticulo-endothelial cells of liver and spleen lose their capacity for taking up congo red injected intravenously and he considers that there is in this infection evidence of a specific poisoning of the reticulo-endothelial system. In later communications he states (1926, 1926 -27) that the most striking histological evidence of damage is the loss of chromaffin staining in the suprarenals, and that changes in the reticulo-endothelial cells are slight, consisting chiefly of fatty degeneration in the early stages of infection; such changes were usually absent at death. The evidence in favour of specific damage to the reticulo-endothelial system does not appear to be very well founded.

The present observations show that the functional activity of the intravascular reticulo-endothelial cells during pneumococcal infection is not significantly altered according to the criterion of phagocytic capacity.

SUMMARY AND CONCLUSIONS.

Mice subjected to moderate degrees of intra-vitam staining either by the slow method (isamine blue) or by the rapid method (trypan blue) show no noteworthy difference from normal mice in their resistance to pneumococcal infection.

The presence in the peritoneum of a cellular exudate rich in large vitally stained macrophages does not protect mice from intraperitoneal pneumococcal infection nor is the resistance lowered under these conditions. Young active unstained macrophages also fail to confer protection. Stimulation of local macrophages at the site of inoculation thus appears to be without definite influence on this infection in mice.

'Blockade' of the reticulo-endothelial system alone or combined with splenectomy or other operative measures also fails to modify the course of pneumococcal infection in mice, and it has been clearly shown that such procedures do not produce any significant alteration of resistance. The variations in survival met with in animals so treated do not differ from natural variations in untreated animals due to individual susceptibility.

It cannot be inferred from such experiments that the reticulo-endothelial system plays no special part in immunity processes. Rather one must conclude either that the

experimental disturbances have been adequately compensated, or that the method employed, viz. the estimation of resistance to organisms which rapidly multiply and invade to produce a general infection, is not suitable for the detection of such minor variations in resistance as may be produced by operative measures combined with blockade of the reticulo-endothelial system.

The cells of the reticulo-endothelial system of mice dying of pneumococcal septicaemia remove saccharated oxide of iron from the circulating blood and store it intracellularly as readily as those of normal mice. There is therefore no significant loss of functional capacity as judged by phagocytosis in the intravascular elements of the reticulo-endothelial system even at the height of the infection.

VII. The application of intravitam staining to pathological investigations.

The histological identification of cells by means of intravitam staining.

The so-called reticulo-endothelial blockade.

The estimation of the functional capacity of the reticulo-endothelial system *intra vitam.*

A comprehensive description of the methods and general results of intravitam and supravital staining has been given in part I of this study, and a detailed account of the distribution of the vitally stained cells has been presented in part II. It has been shown that we can by this means demonstrate the entire group of cells which are at the time exhibiting the physiological capacities of macrophages and which may thus be conveniently grouped together under the title "reticulo-endothelial system". But since vital stains *per se* produce a marked reaction when introduced locally, the administration of such dyes by the subcutaneous or intraperitoneal routes gives an erroneous impression of the number of histiocytes present in the tissues at the site of injection and, owing to slowness of absorption, may fail to demonstrate adequately those in distant parts. It follows therefore that in order to obtain a complete demonstration of the cells normally comprising the system but without provoking the formation of new cells it is necessary to administer by the intravenous route repeated doses of a fairly rapidly diffusible dye such as trypan blue or vital new red, and to examine the tissues within a relatively short time, e.g. 24 hours after the last injection of dye.

As regards the experimental application of methods of intravitam staining it may be stated from a study of the literature that investigations have been directed chiefly along two lines : (1) the histological identification of cells in

inflammatory and other reactive states by vital and supravital staining, and (2) attempts to detect differences in the behaviour of animals after the so-called reticulo-endothelial blockade, with a view to estimating the part played by the reticulo-endothelial system. Examples of such applications of the methods have been given in parts IV, V, and VI of this study. It is proposed here to formulate critically the conclusions which have been reached in my experimental work regarding the conditions under which these methods may be applied to the study of pathological problems.

From many recent descriptions of vital staining and of the reticulo-endothelial system it might appear that to study the participation of these cells in inflammatory conditions and other reactive states it would be sufficient to administer one or two doses of a rapidly diffusible dye, e.g. trypan blue, during the experiment. If then the reacting cells were found to be vitally stained they must be histiocytes or their immediate derivatives and could neither be mononuclear leucocytes from the circulating blood nor be derived from other cells which do not normally store vital dyes. This premise is, however, quite unsound, for as has been pointed out, unstained cells may come to exhibit a previously latent capacity for vital staining under the influence of altered environmental conditions. It

may be definitely accepted that under such circumstances vital staining of a reacting cell is merely an indication that it is in a certain condition of functional activity at the time of examination, but does not permit any conclusion in regard to its former state or its immediate origin.

It must also be remembered that soluble vital stains require to circulate in the body fluids for a period of at least several hours before intracellular dye deposits become visible (Kagan, 1927). During this lag period unstained cells may migrate into such a situation that the circulating dyestuff is no longer available to them, and thus appear unstained; such cells may then if the dye be later made available to them, rapidly exercise their hitherto latent capacity and store the vital stain actively.

The histological identification of cells by means of intravitam staining.

As regards the use of intravitam staining as a means of studying the origin of certain reacting cells in inflammatory exudates the following general statements can be made in the light of the experience gained in the foregoing investigations, as they form the necessary basis of interpretation in such experiments.

I. If an animal receive a first dose of a dye of group (a), e.g. trypan blue, during the production of an inflammatory exudate, no true vitally stained cells can be expected until at least several hours have elapsed. The vitally stained cells, however, will include both the pre-formed and the newly formed histiocytes, which cannot by this means be distinguished from one another. Failure to find vitally stained cells after a sufficient period may be due to several causes; the cells may possess no capacity to stain vitally, or they may be in such a situation that the injected dyestuff does not reach them.

II. By repeated injections of suitable dyes, e.g. trypan blue or isamine blue, the cells of the reticulo-endothelial system including the histiocytes in all parts can be selectively stained. After a sufficient interval the amount of free dye in the blood and tissue fluids will be so reduced that it is insufficient to produce visible staining of any cells which have just acquired the capacity to store the vital dyes, but the cells originally vitally stained can still be clearly recognised. If at this stage an inflammatory exudate be produced, the presence of vital staining in any considerable proportion of the cells of the exudate permits the conclusion that they are derived from cells vitally stained prior to the formation of the exudate. For this type of experiment soluble dyes of the less diffusible type, e.g. isamine blue, are preferable owing to their greater

resistance to decolorisation.

III. By repeating the above procedure II combined with intravenous administration of a rapidly diffusible contrasting dye, e.g. lithium carmine or vital new red given under the conditions specified in I, the information obtainable regarding the origin of the cells of an inflammatory exudate is extended. The presence of only the second dye in the exudate cells permits the conclusion that they have arisen de novo from unstained precursors, and have then acquired the capacity to stain vitally owing to their altered functional activity. Many of the preformed histiocytes, however, will be found to contain the second dye in addition to that first administered, but some containing only the first dye will also be found.

IV. The relation of the circulating monocytes to the reacting cells of the exudate may be ascertained by repeated intravenous administration at suitable intervals of the maximum tolerated dose of india ink or saccharated oxide of iron. Since a considerable proportion of the circulating monocytes take up these substances, the absence of the injected suspensoid from the cells of the exudate would suggest that the latter do not arise in any noteworthy proportion from circulating monocytes. This method of experimentation is, however, not altogether satisfactory because in certain situations, e.g. bone-marrow,

omentum and mesentery, the capillary endothelium appears to be more permeable than elsewhere, and the injected suspensoid may be found in cells of histiocyte type in the neighbourhood of the vessels. Desquamated rĕticulo-endothelial cells — the blood histiocytes of Kiyono — are also found in the blood of internal organs, but can generally be distinguished from monocytes by their larger size and much heavier content of ink, etc.

In planning pathological experiments in which it is desired to use intravitam staining as a means of identifying cells of the reticulo-endothelial system, the above described conditions must be satisfied before conclusions as to the nature of vitally stained reacting cells can be *justified*. That is to say, if when an inflammatory state is established it is desired to test whether the reacting cells possess the properties of histiocytes, it is necessary to ensure that an adequate quantity of free dye will be present in the tissue fluids available to them for a sufficiently long period to permit of visible staining at the time of examination (procedure I). If on the other hand, the *object of the* experiment is the identification of only the pre-existing histiocytes, it is essential that vital staining be carried out previously so that no free dye — or at least only a negligible amount — is present in the body fluids during the experiment, lest unstained cells begin to take up the dye

under the influence of altered conditions and thus be confused with previously stained cells (procedure II). If such newly formed histiocytes are stained by a second dye of different colour as in procedure III, it must be remembered that the histiocytes previously stained by procedure II may be capable of taking up the second dye also; but it is important to note that the capacity of such cells for rapid mobilisation and migration especially into serous cavities may be interfered with by intense vital staining (see p. 328).

To assess the rôle of the blood monocytes is more difficult, and it must be emphasized that only a certain proportion of these cells can be demarcated by vital staining as in procedure IV, the actual number varying considerably in different individuals under apparently identical conditions. Since these cells disappear from the peripheral blood fairly rapidly it is necessary to administer the suspensoid intravenously at intervals which are calculated to produce the greatest number of vitally stained monocytes in the blood at the time when the experimental cellular reaction is taking place. As mentioned above, this method of investigating the participation of monocytes is not above criticism and the results obtained by its use require to be carefully controlled by other experiments in which the rôle of the preformed histiocytes can

be assessed.

The so-called reticulo-endothelial blockade.

The capacity of reticulo-endothelial cells to ingest and store relatively enormous amounts of more or less inert substances such as india ink or oxide of iron, has led to the idea that these cells may be thrown out of action by saturating them with such substances — the so-called reticulo-endothelial blockade. This procedure first employed by Lepehne (1918) has generally been combined with splenectomy, for the purpose of removing the most accessible depôt of reticulo-endothelial cells. Attempts have been made to ascertain the capacity of the treated animals to form antibodies, e.g. precipitins, agglutinins, etc.; to determine the resistance of such animals to acute infections either bacterial or protozoal; and to compare in 'blockaded' and control infected animals the effects of chemo-therapeutic agents which apparently depend on co-operation with the natural processes of the body for their full effect, e.g. drugs of the salvarsan group.

The method most commonly employed to produce the reticulo-endothelial blockade has been the intravenous injection of finely divided particulate matter, e.g. colloidal silver (Lepehne, 1918), india ink (Standenath, 1923; Jungeblut and

Berlot, 1926; Singer and Adler, 1924) and especially saccharated oxide of iron have been used (Bieling and Isaac, 1921, 1922; Rosenthal and Spitzer, 1924; Kritschewski and Meersohn, 1926). Some writers have employed soluble dyes in place of suspensoids, e.g. trypan blue (Gay and Clark, 1924; Lewis and Loomis, 1926; Ross, 1926). It is necessary to consider what such procedures can achieve and what interpretations can justifiably be placed on the results.

It has been pointed out in the foregoing pages that finely divided particulate matter introduced intravenously produces vital staining of the reticulo-endothelial cells lining the blood vascular channels, but does not affect the extravascular histiocytes except in certain special situations. The reticulo-endothelial cells of lymphatic glands and the histiocytes of the areolar tissue in the cutis and stroma of organs are thus unaffected. Now Lewis (1929) has calculated that in the rat the number of histiocytes in the subcutaneous tissue alone is greater than the total number of leucocytes in the circulating blood. Since these cells are so active in storing easily demonstrated soluble foreign substances, this capacity cannot be disregarded in relation to bacterial and other toxins many of which also form semicolloidal solutions. The presence of such large numbers of unaffected active cells

belonging to the reticulo-endothelial system therefore invalidates the conclusion that the system as a whole can be thrown out of action by intravenous injection of insoluble suspensoids. It is also worthy of emphasis that the diverse elements comprising the reticulo-endothelial system do not exhibit uniform degrees of activity in dealing with substances of different physico-chemical properties. As has been shown earlier, the cells of the spleen remove and store particulate matter much more readily than soluble dyes; the Kupffer cells of the liver ingest india ink particles more rapidly after intravenous injection than do the histiocytes after subcutaneous injection and other examples have been given previously. Kagan (1927) has studied in detail the time of appearance of granular staining in the organs following subcutaneous or intravenous injection of trypan blue and concludes from the differences observed that the reticulo-endothelial cells are not equally active, the Kupffer cells and the histiocytes showing dye storage long before the cells of the spleen and bone marrow. That similar differences may exist in the defence of the body against bacterial invasion is suggested by the work of Singer (1926-27) who states that the local histiocytes at the site of cutaneous infection with anthrax are of less importance in preventing or delaying the development of anthrax septicaemia than are the cells of the liver, spleen and bone marrow, i.e. the intravascular

reticulo-endothelial cells.

Since even relatively inert substances such as india ink, saccharated oxide of iron, collargol, etc. are lethal in overdoses, the question arises as to what causes the toxic effect of excessive doses of such blockading agents. In the case of india ink and collargol the protective colloids used in the manufacture may play a part, and the sugar in the iron preparation may possibly be harmful when too much is given. It is at least clear that apart altogether from the risk of pulmonary embolism producing death by mechanical obstruction finely divided particulate suspensoids are tolerated only in limited amounts. I have observed, however, that the maximum single dose may be repeated safely after a short interval (½ - 1 hour) and that in some cases several such doses may be administered within 24 hours without ill effects. Histological observations show that this is not due entirely to the development of new cells since the active elements are by no means saturated by the first dose of suspensoid.

The use of soluble dyes to produce blockade cannot lead to results which are in any way more decisive, although storage in the extravascular histiocytes is attained. As already described, such dyes are excreted rapidly, and the maximum tolerated single dose produces intracellular dye deposits which are far short of the maximum storage capacity of the cells.

Further the dyes used as vital stains all possess a certain degree of toxicity, and the administration of maximal doses may produce a depressing effect on the vitality of the animals altogether apart from specific localisation of the dye in the cells of the reticulo-endothelial system. This poisoning effect is discussed more fully by Louros and Scheyer (1926) and by Paschkis (1929). Extensive loading of the cells can only be attained by repeated administration, but during this time the reticulo-endothelial system is undergoing fluctuations with destruction of some elements and formation of active new cells (see also Dieterich, 1927; Hesse, 1928), so that complete abrogation of function in all the active cells is practically impossible at any one time. Petroff (1923, 1924) has pointed out that after repeated intravenous injections of colloidal silver, approximately the same proportion of each successive dose is stored in the organs, and that previous multiple injections of india ink or trypan blue do not lead to any noteworthy difference in the amount of silver stored in each organ, as determined by chemical analysis. It would therefore appear that adequate compensatory mechanisms exist whereby the functional capacities of the organs as a whole are restored or even augmented following the storage of inert particles (see also Derman, 1928). While it may therefore be concluded that blockade of the reticulo-

endothelial system as a whole is impracticable, it is otherwise with individual cells. As I have shown in the preceding studies, histiocytes heavily laden with one substance possess a diminished capacity for absorbing other substances. Chalatow (1922) and Kusnetzowski (1923) have recorded similar observations, and the latter states that fatty substances injected into animals already vitally stained are taken up by and stored in histiocytes which show only traces of vital staining; the active cells thus appear to be newly developed in response to the local stimulus. Conversely when vital stains are administered to animals which have previously been injected with fatty substances, the histiocytes which have stored much lipoid fail to take up the dyes in any considerable amount. It appeared that the ingestion of one substance in large amount inhibited the storage of another substance of different nature. Migay and Petroff (1924) also note that colloidal iron and lithium carmine injected intravenously in rapid succession are generally deposited in different cells, and Petroff (1923, 1924) observed similar differences in the intracellular disposition of colloidal silver following injections of india ink and trypan blue.

Cannon, Baer, Sullivan and Webster (1929) have criticised most of the published work on reticulo-endothelial

blockade on the ground that the amount of inert material injected was insufficient to produce any real inhibition of cellular function. By employing enormous doses of india ink they were able to produce a definite depression of haemolysin formation without affecting the general health of the rabbits. The latter factor is one which cannot be overlooked, since it appears to be established that large doses of india ink and other finely divided suspensoids may bring about a severe anaemia from damage to the bone marrow, though it is not clear how far such changes may be due to the protective colloids used in the preparation of the suspensoids (Muller, 1926, 1927; Elvidge, 1926).

It may be said that in the hands of different workers the results after the so-called blockade have been extremely variable. Owing to the small number of animals used in many cases it has been overlooked that this irregularity may be due to natural variations in individual animals. Further, there has been no uniformity in the method adopted to produce blockade, and in most cases control histological observations have not been made to determine the condition of the cells of the reticulo-endothelial system at the conclusion of the experiments. In such experiments it is essential to control rigorously all the factors which may interfere with the normal state of the animals.

In a parallel series of animals, the effect of operative interference alone must be controlled by the infliction of an operation of similar severity, e.g. nephrectomy to control splenectomy; also the intravenous injection of "blockading" substances such as india ink or saccharated oxide of iron should be paralleled by the injection of corresponding amounts of substances which are known not to be stored in the reticulo-endothelial system, e.g. glucose or harmless crystalloids, e.g. sodium chloride. Observations must also be made to determine that the amount of inert suspensoid used is sufficient to produce a marked loading of the reticulo-endothelial cells.

In the experiments reported in part VI of this study I have shown that generalised and local vital staining, reticulo-endothelial blockade and various forms of operative interference failed to produce any decisive effect on the resistance of mice to acute pneumococcus infection. It appeared that interference with the normal state by any form of preliminary treatment tended to diminish the animal's chance of overcoming the infection, but the period of survival and the mortality rates of those in which "blockade" alone or "blockade" combined with splenectomy had been produced did not differ materially from those in the series with intact reticulo-

endothelial system. It appeared also that the storage capacity of the reticulo-endothelial cells in animals dying of septicaemia did not differ significantly from that of normal mice, as Louros and Schayer (1926) also observed in streptococcal infections.

It may therefore be stated that in the present state of knowledge regarding the results of intravitam staining no method yet devised is capable of bringing about selective abrogation of the functions of the reticulo-endothelial system as a whole for any considerable period. By the present methods, "blockade" even when combined with splenectomy is never complete and experiments along such lines have hitherto failed to yield decisive information regarding the relation of the reticulo-endothelial system to immunity processes and the defence of the body generally against organismal invasion. For further advances new methods will require to be devised and knowledge will have to be sought along lines of approach other than that of the so-called blockade of the reticulo-endothelial system as a whole.

The estimation of the functional capacity of the reticulo-endothelial system *intra vitam*.

Attempts have recently been made to estimate the

functional storage capacity of the reticulo-endothelial system by measuring the rate of disappearance of various substances introduced directly into the blood stream. Saxl and Donath (1925) and Adler and Reimann (1925) injected lipoid suspensions, congo red, neutral red and other substances intravenously in man and lower animals for this purpose. Nikolaef and Tichomiroff (1928) injected congo red into rabbits during immunisation, infection and anaphylactic shock and concluded that variations from the normal rate of disappearance of the dye were to be interpreted as the result of alterations in the absorptive capacity of the reticulo-endothelial cells. Singer (1925) in studies on anthrax infection also considered that delay in the disappearance of congo red from the blood after intravenous injection was evidence of diminished storage capacity on the part of the reticulo-endothelial system due to specific damage in this infection; he has also noted a similar delay in normal animals to which india ink had been administered. Roberts (1929) has shown that the rate of disappearance of soluble antigen (precipitinogen) from the circulation after intravenous injection is accelerated in blockaded animals, and that this is not due to any destructive property in the injected animal's serum. Leites and Raibow (1927) injected saccharated oxide of iron intravenously and measured the rate of disappearance from the circulation by estimating the total

iron content of the blood at short intervals. Later (1928) they attempted to assess the influence of the ductless glands on the activity of the reticulo-endothelial system by this method, and state that the storage capacity is diminished by adrenalin, unaffected by removal of the testes, parathyroids and thymus, and increased by injection of pituitrin, insulin and by thyroidectomy and ovariectomy.

The conclusions of the above writers do not seem to me to be justified. It is generally agreed that clinical methods of investigating the functional capacity of individual organs demonstrate only the grossest deviations from normal owing to the wide margin of reserve normally present. It is doubtful if the figures quoted in support of such conclusions are statistically significant. As regards the use of soluble dyes, many factors have to be considered other than that of the storage capacity of reticulo-endothelial cells. I have pointed out (p. 82) that there is always a certain rapid loss of dye partly owing to imbibition into structures which stain diffusely, e.g. elastic tissue, red blood corpuscles etc., and partly from the passage of dye into the tissue lymph. Any factor increasing the permeability of the general blood capillary endothelium will produce a rapid acceleration of the loss of dye from the circulation owing to more rapid passage into the tissue lymph. Trypan blue has indeed been employed by Ramsdell (1928) to detect

local variations in capillary permeability following sensitisation. Kusnetzowsky (1925) has also shown that local active hyperaemia experimentally induced leads to a marked intensification of vital staining in the adjacent histiocytes as compared with those in non-hyperaemic areas; this is much less pronounced in passive hyperaemia. Dyes such as neutral red are also unsuitable owing to their rapid reduction to colourless compounds; many of the basic dyes used as supravital stains are toxic and produce severe metabolic disturbances with hyperglycaemia (Zeckwer, 1926). In addition to storage in reticulo-endothelial cells such basic dyes are taken up by practically all parenchyma cells, e.g. liver, pancreas, and are rapidly excreted by the kidneys, stomach and bowel. It is clear that too many factors other than intracellular storage are involved for such tests to be satisfactory.

Finely particulate suspensions are similarly unsuited to this purpose, since their rate of deposition in the organs is also dependent on many factors other than intracellular storage. As already pointed out (p. 72) the rate of injection and the stability of the suspension play an important part in determining how long individual particles will persist in the circulating blood. The qualities of solutions of saccharated oxide of iron vary considerably with different samples, the optimum preparation forming an opalescent suspension of very fine particles which

cannot be readily separated even by the centrifuge. Acid solutions precipitate easily, are flocculated by the plasma and so produce death from embolism when injected intravenously, while alkaline solutions are transparent and free from discrete particles; such solutions are taken up much more slowly by the cells. The factors influencing the rate of deposition of saccharated oxide of iron have been investigated in detail by Boerner-Patzelt (1923, 1924) and by Pfeiffer and Standenath (1923). They have shown that the degree of dispersion of the particles plays an important part, and that this is altered by age on standing, by prolonged boiling, and above all by the reaction of the solution. It is therefore probable that such finely divided suspensoids may be susceptible to changes in the physico-chemical state of the blood plasma; thus the rate of deposition of any given sample of the suspensoid may vary considerably in pathological conditions irrespective of the phagocytic activity of the cells lining the capillaries. Accordingly it would appear that the fallacies which beset such methods are too numerous to permit of their use as a practical method of estimating the state of activity of the reticulo-endothelial system.

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I N T R A V I T A M

AND

S U P R A V I T A L

S T A I N I N G.

AN EXPERIMENTAL STUDY.

D.F. CAPPELL.

I L L U S T R A T I O N S.

I L L U S T R A T I O N S.

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FIG. 1. Subcutaneous tissue from thigh of normal mouse: supravital staining with neutral red and janus green, each 1·40,000 in buffered saline.

a = histiocytes with numerous large and small neutral red granules and small granular mitochondria grouped around the nucleus.

b = fibrocytes with scanty small neutral red granules and rod-like mitochondria scattered in the cytoplasm and extending into the outlying processes.

c = young histiocytes (polyblasts) with smaller and less numerous neutral red granules than the fully developed cells. The mitochondria are chiefly granular.

d = lymphocyte-like cells with very scanty neutral red granules of small size, and granular and short stout mitochondria.

d' = transition from lymphocyte to monocyte.

e = typical monocyte of Sabin with fine neutral red granules of more reddish pink colour, arranged in rosette formation.

e' = transition from monocyte to histiocyte.

Fresh preparation. ×900.

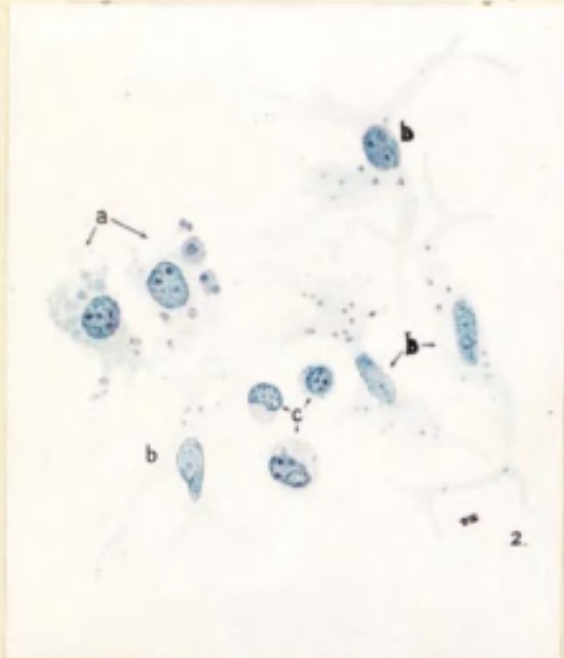


FIG. 2. Subcutaneous tissue of mouse stained by janus green 1-5000. By the use *of a more concentrated solution of the dye the cell nuclei and protoplasm* are demonstrated, but the mitochondria are unstained.

a = histiocytes of irregular outline with granular cytoplasm.

b = fibrocytes with long tapering processes and clear transparent cytoplasm.

c = young histiocytes (polyblasts) in various stages of development.

Fresh preparation. × 675.

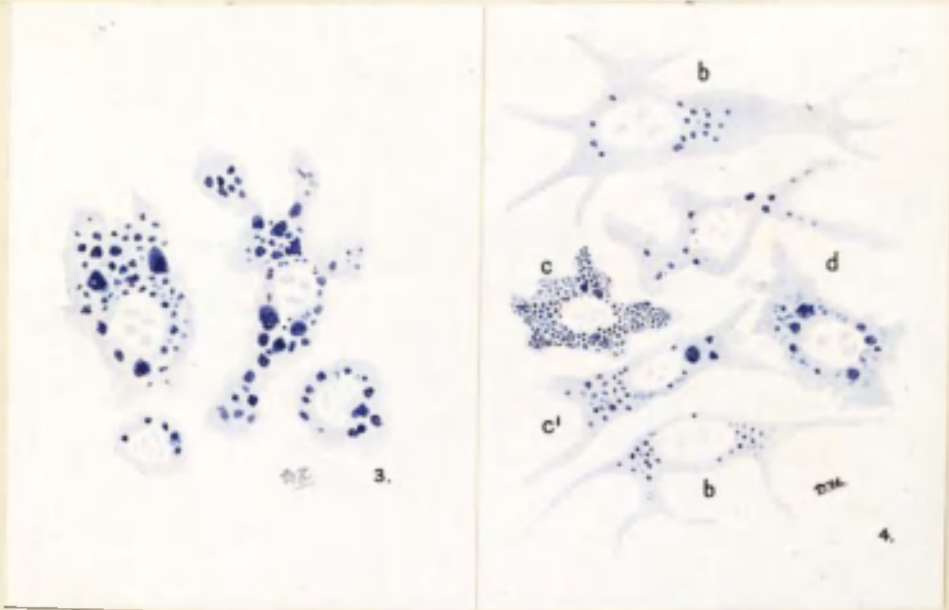


Fig. 3. Cells from the subcutaneous tissue of mouse after intraperitoneal injection of trypan blue 1-200.

histiocytes in various stages of development. The vitally stained granules are of varying size and shape, and often contain smaller more intensely stained particles.

Fresh preparation. ×900.

Fig. 4. Cells from the subcutaneous tissue of a mouse after repeated intraperitoneal injections of trypan blue illustrating the difficulties of classifying cells.

b = fibrocytes with scanty small dye granules.

c, c' = cells of indeterminate type, the morphology of the granules being at variance with the other characters of the cells.

d = cells with relatively little dye but otherwise resembling histiocytes.

Fresh preparation. ×900.

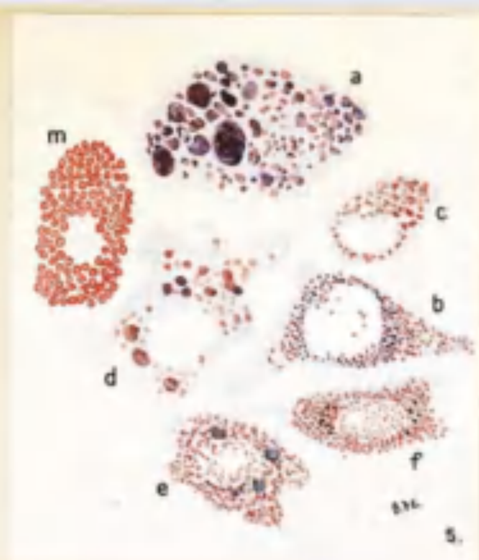


FIG. 5. Cells from subcutaneous tissue of same mouse as fig. 4 stained supravitally *with neutral red.*

a = histiocyte with all the trypan blue granules over-stained with neutral red.
b = fibrocyte with numerous fine trypan blue granules with neutral red over-staining, but there are also some small granules of each dye separately.
c = *young histiocyte (polyblast) with neutral red granules but no trypan blue.*
d = histiocyte with many well-formed neutral red granules ; only a few of the larger granules show the presence of trypan blue.
e = histiocyte with a few large trypan blue granules and many small neutral red granules. The former are scarcely tinged by the supravital stain.
f = fibrocyte containing neutral red granules similar to those of cell e, but with only a few tiny blue granules almost masked by the red dye.
m = tissue mast cell, with the uniform highly refractile granules stained with the neutral red.

Fresh preparation. ×900.

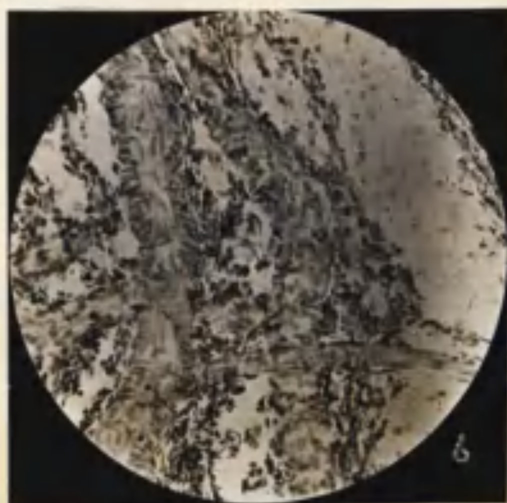


Fig. 6 Subcutaneous tissue of rabbit 48 hours after the seventh intravenous injection of trypan blue.
Vitally stained cells are especially numerous around the smaller blood vessels and nerves, where they form a large part of the adventitial cells.

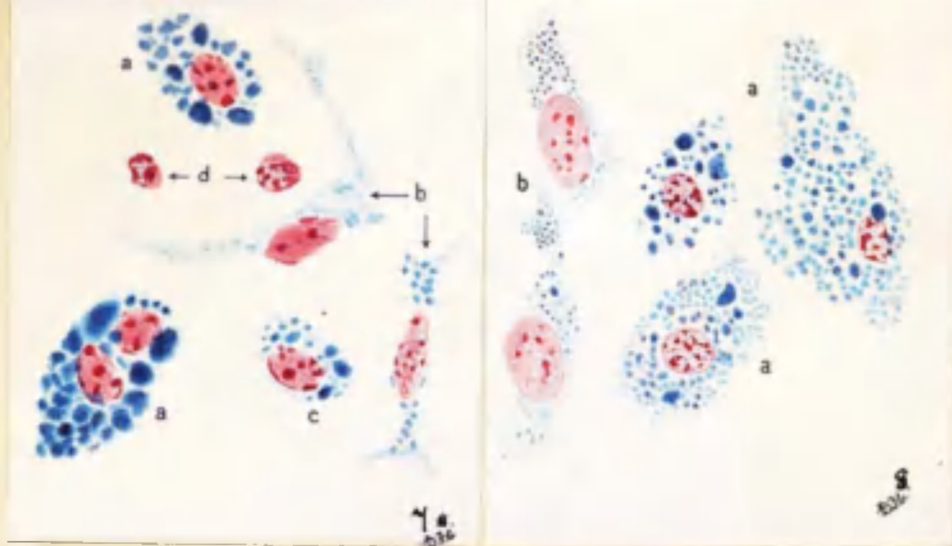


FIG. 7. Cells from the mesentery of a mouse after three intraperitoneal injections of isamine blue.

a = histiocytes with enormous globular deposits of vital stain.
b = fibrocytes with dye granules of varying size, but usually fine.
c = young histiocyte (polyblast).
d = lymphocyte-like cells.

Fixed in Bouin's fluid, counterstained carbol-fuchsin. × 900.

FIG. 8. Cells from the subcutaneous tissue of a mouse after three intraperitoneal injections of isamine blue (same animal as fig. 6).

a = histiocytes, with rather uniform vitally stained granules of smaller size than those in the cells of the injected area (fig. 6).
b = fibrocytes with very small granules. The full extent of the cytoplasmic processes is not seen in the fixed and counterstained preparations.

Fixed in Bouin's fluid, counterstained carbol-fuchsin. × 900.

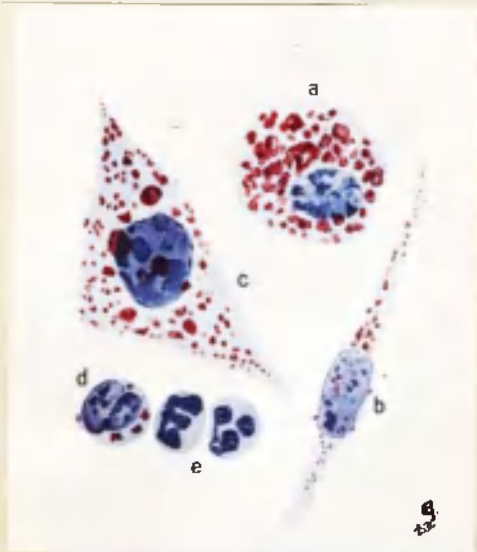


FIG. 9. Cells from the omentum of a rabbit after repeated intraperitoneal injections of diaminefast scarlet.

a = histiocyte with large coarse dye granules.
b = fibrocyte with typical fine granules.
c = cell of intermediate character, the size of the granules being characteristic of neither group.
d = young histiocyte (polyblast)
e = polymorphonuclear leucocytes showing absence of vital staining.

Fixed in corrosive sublimate, counterstained Harris's hæmatoxylin. × 1000.

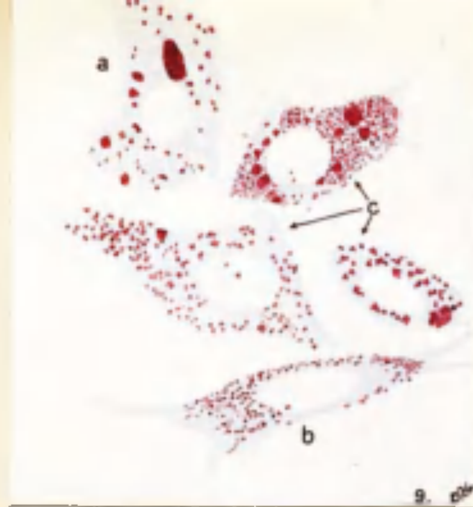


FIG. 10. Cells from the subcutaneous tissue of a mouse stained with vital new red, the last injection having been given four months previously. The cells are difficult to classify by the morphology of their vitally stained granules.

a = histiocyte.
b = fibrocyte.
c = cells of indeterminate character.

Fresh preparation. × 900.

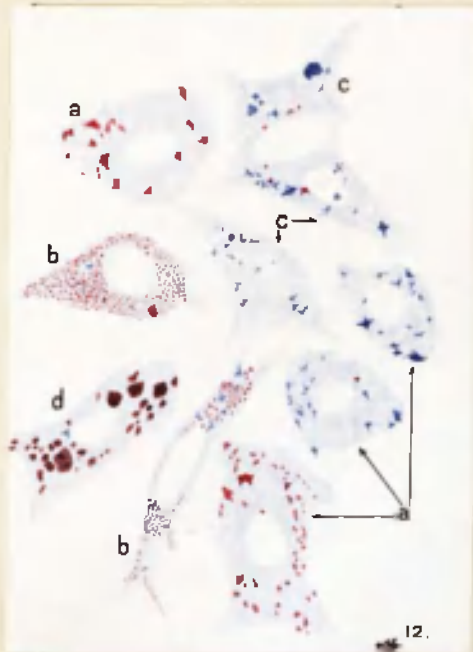


FIG. 11. Cells from the subcutaneous tissue of a guinea-pig which after staining with trypan blue six months previously, had received a series of small doses of lithium carmine four weeks before death.

Histiocytes and fibrocytes cannot be certainly distinguished by the character of their dye granules. Some cells contain only minute rhomboidal deposits of trypan blue, others contain only carmine. In one cell shown (d) the dyes administered separately have been combined giving rise to irregular brownish granules.

a = probably histiocytes.
b = probably fibrocytes.
c = cells of ill-defined character.
d = histiocyte with combined granules of both dyes.

Fresh preparation. × 900.

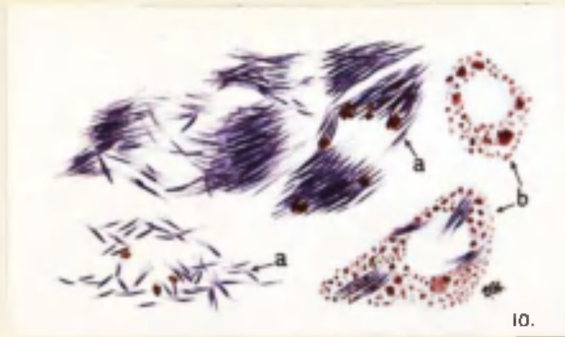


Fig. 12. Cells from subcutaneous tissue at the site of injection of dyestuff 2(*o*-hydroxy-*p*-dimethylamino-anil)-6-acetyl-amino quinoline methochloride.

a = histiocytes filled with dye crystals and granules of brownish colour possibly an impurity in the dye.

b = fibrocytes with less numerous dye crystals.

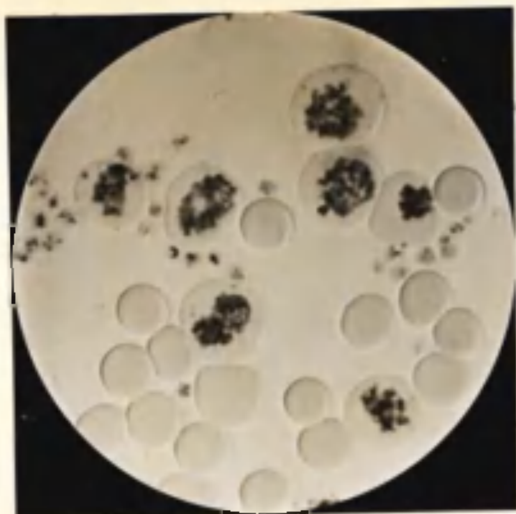
Mounted in balsam. × 900.

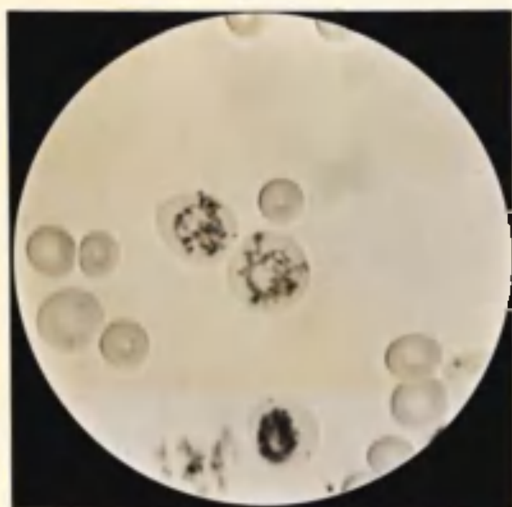
Fig. 13. Cells from marginal zone of site of injection of same dye as fig. 10, supravitally stained with neutral red 1-40,000.

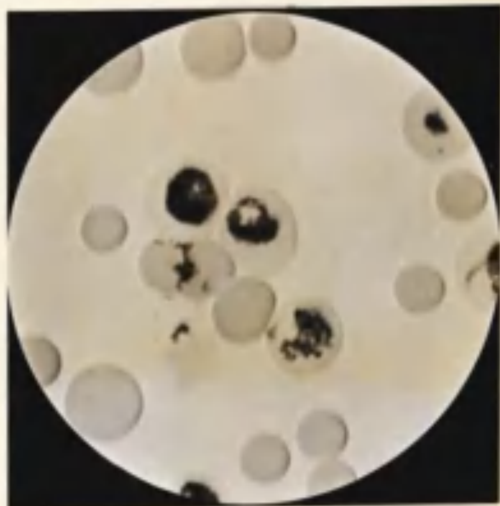
a = histiocytes with numerous neutral red granules, showing no special relation to the dye crystals.

b = fibrocytes with dye crystals and less abundant neutral red granules.

Fresh preparation. × 900.







Figs. 14, 15, 16.

Fresh blood films from a rabbit after injection of haemolytic serum, showing reticulocytes supravitally stained with Brilliant Cresyl Blue.
The immature red cells, many of which are of increased diameter exhibit a skein-like structure – the reticulum – when stained supravitally. Various forms of reticulated red cells are shown.

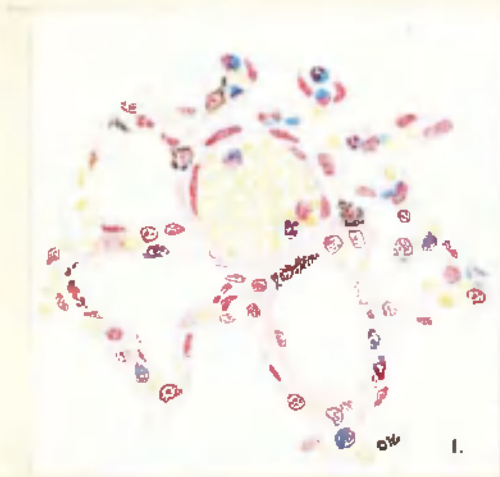


Fig. 17.—Lung of mouse 7 hours after intravenous injection of a large dose of saccharated oxide of iron. Iron-containing leucocytes, both polymorphonuclears and monocytes, are present in the capillaries in considerable numbers but the capillary endothelium and the alveolar epithelium show no trace of iron. Prussian blue reaction : carmalum. ×600.

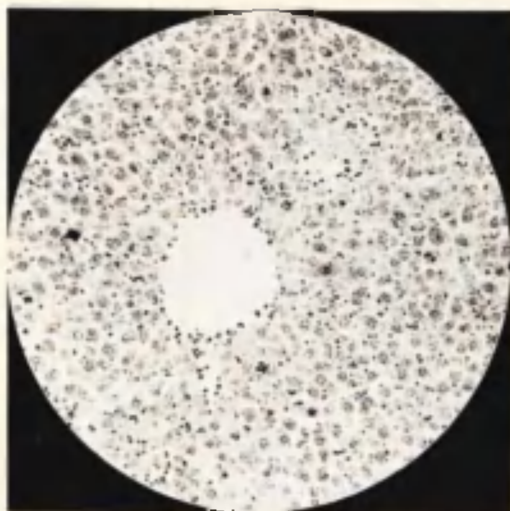


Fig.18 Liver of mouse one hour after intravenous injection of 0.3 c.c. of 10 per cent solution of saccharated oxide of iron. There is a marked accumulation of leucocytes in the smaller veins and in the sinusoids. This is largely responsible for the leucopenia in the peripheral blood.

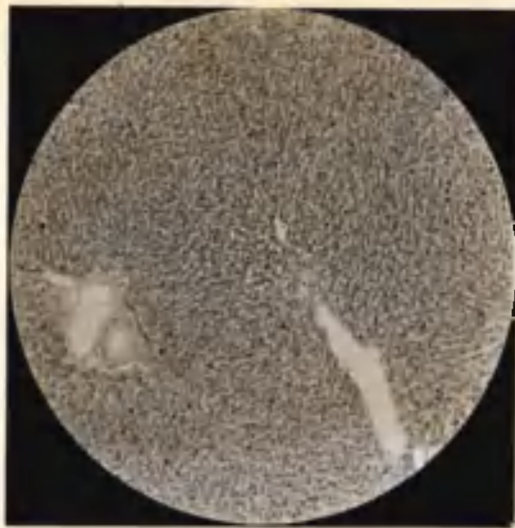


Fig.19 Liver of rabbit after seven intravenous injections of trypan blue. The Kupffer cells are swollen and are filled with coarse dye granules ; the hepatic parenchyma cells also contain less numerous and finer dye granules.

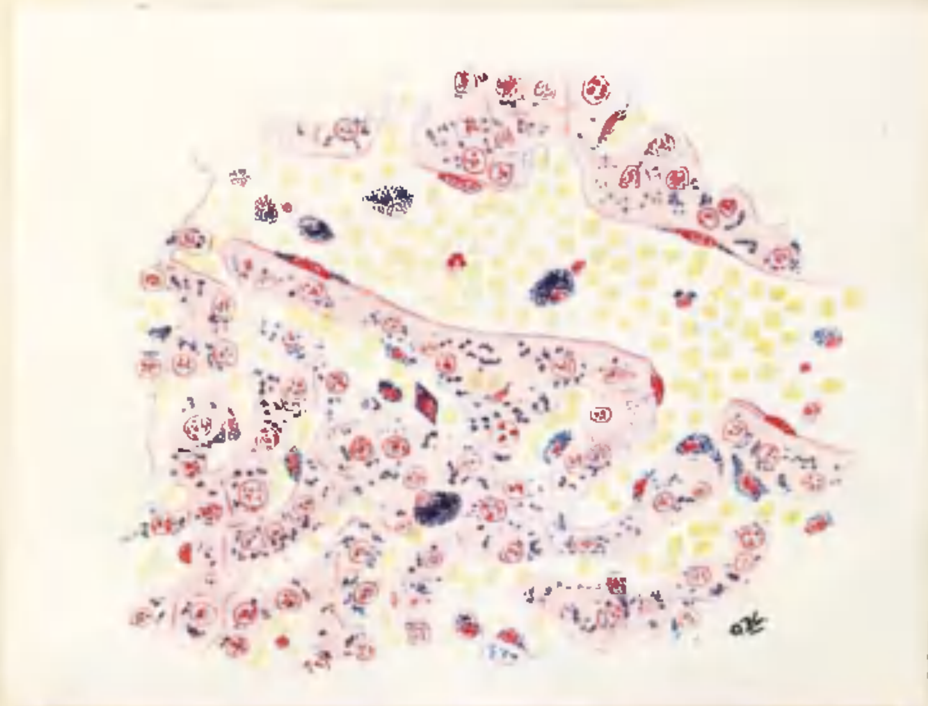
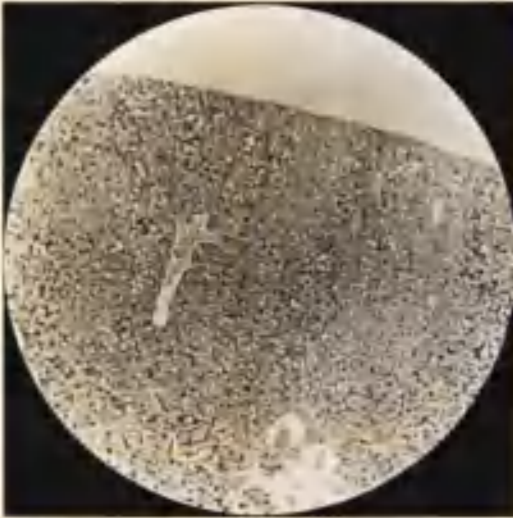


FIG. 20.—Liver of rabbit after seven intravenous injections of trypan blue. The Kupffer cells are greatly swollen and contain a large amount of dye; some have become free in the lumen of the sinusoids and have entered the central vein. The hepatic cells contain numerous small irregular vitally stained granules. ×500.



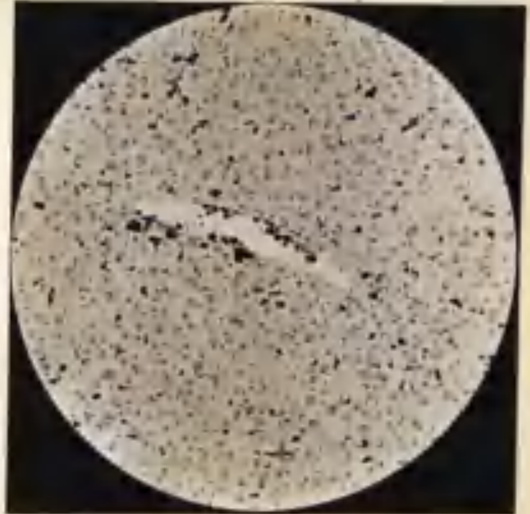


Fig.21 Liver of mouse ten minutes after intravenous injection of india ink. The sinusoids are almost outlined by carbon, and there is already considerable phagocytosis by the Kupffer cells.

Fig.22 Liver of mouse three weeks after the last of three intravenous injections of india ink. There is extensive migration of ink-laden Kupffer cells, some of which are seen around the central vein, while others are becoming free in the lumen.

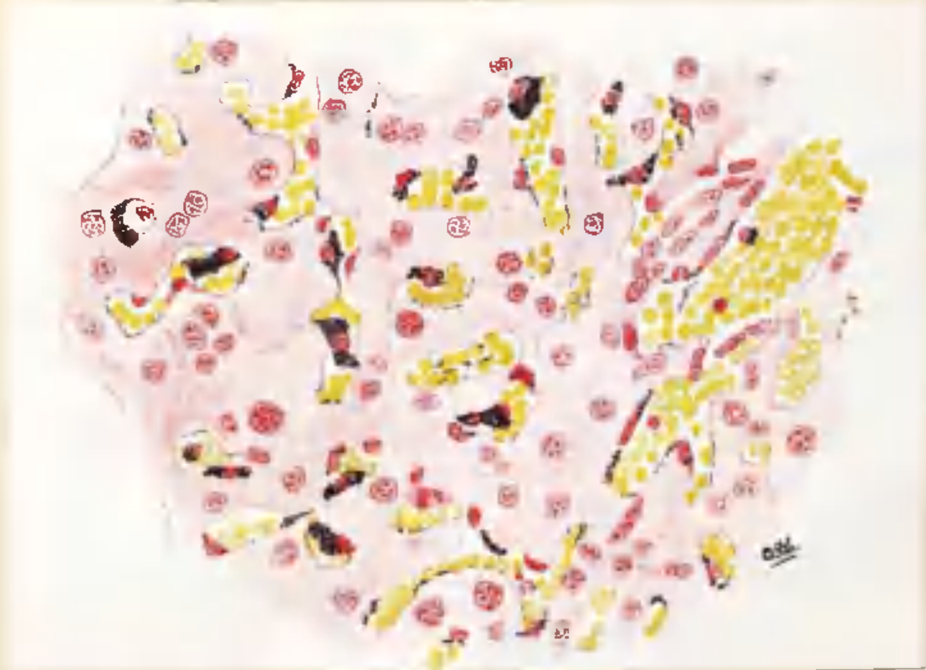
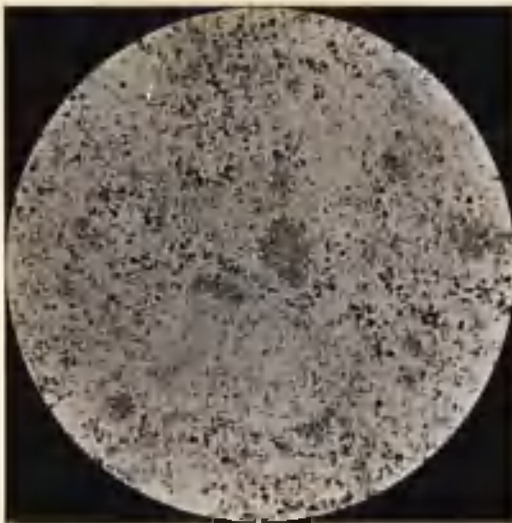


FIG. 23.—Liver of mouse 5 hours after a small intravenous injection of india ink. The Kupffer cells in the sinusoids and some of the flattened lining cells are filled with granules of aggregated ink particles. One ink-storing monocyte is seen in the central vein. ×500.



Spleen of guinea pig after eight intraperitoneal injections of trypan blue.

Fig.24 Accumulation of vitally stained cells in the interfollicular pulp.

Fig.25 Vitally stained cells in the central zone of a Malpighian body.

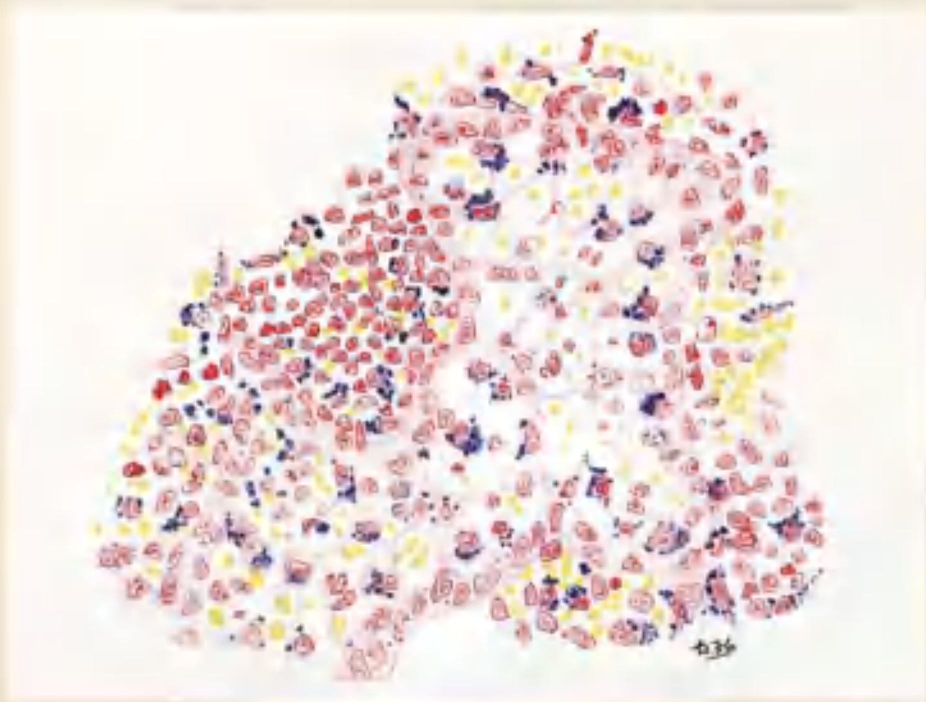
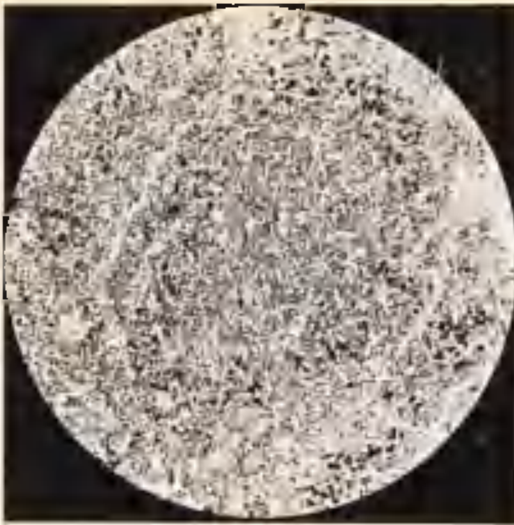


FIG.26.—Spleen of guinea-pig after eight intraperitoneal injections of trypan blue. A venous sinusoid is shown with numerous vitally stained floating cells attached to the wall by long processes. The mural endothelial cells contain very little dye. In the adjacent spleen pulp the reticulum cells and some of the free mononuclears of the pulp are vitally stained. Note the coloration of the specific granules of the polymorphs. ×500.



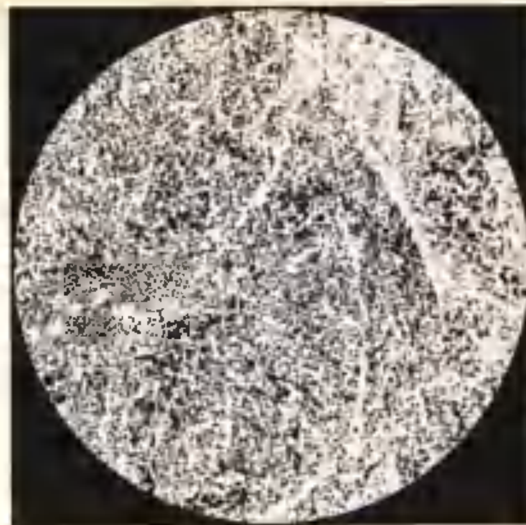


Fig.27 Spleen of mouse three hours after intravenous injection of a small dose of india ink. Carbon particles are most abundant in the pulp cells at the periphery of the Malpighian bodies.

Fig.28 Spleen pulp of same mouse showing a venous sinusoid on section; the endothelial lining cells are completely free from carbon granules.

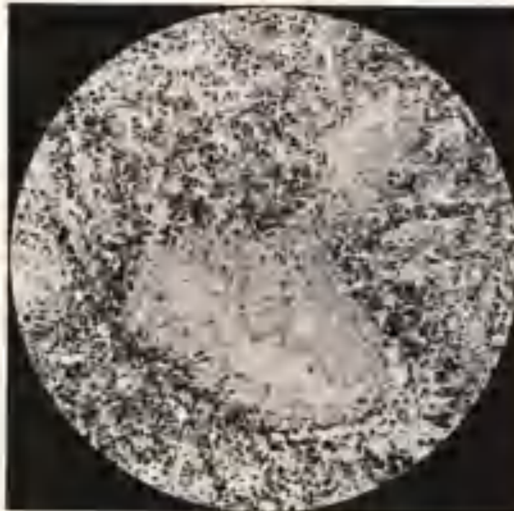
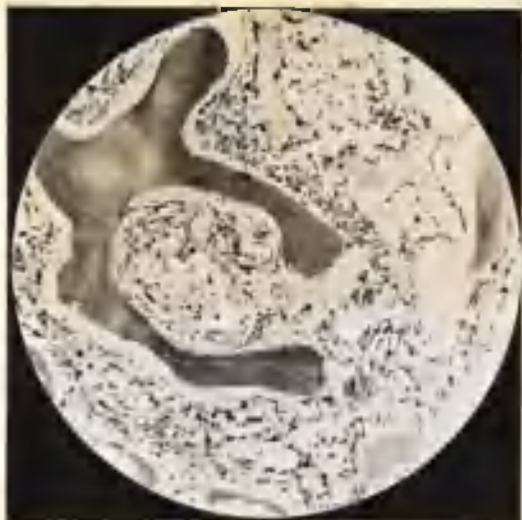
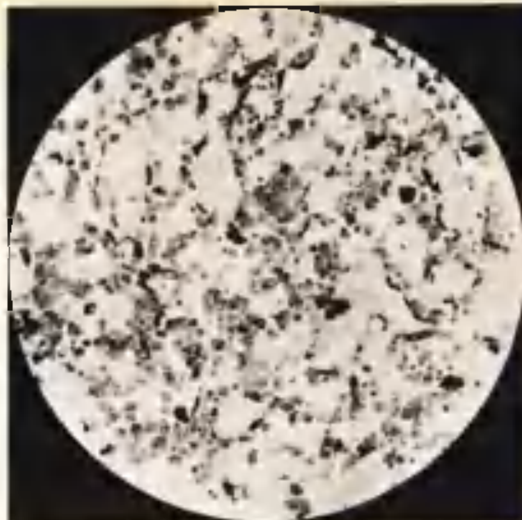


Fig. 29 Spleen of mouse six days after the last of three intravenous injections of saccharated oxide of iron, showing the marked intensification of iron-storage immediately around the Malpighian bodies.

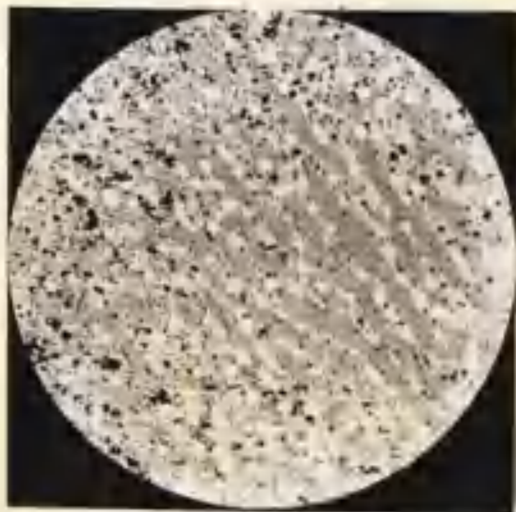


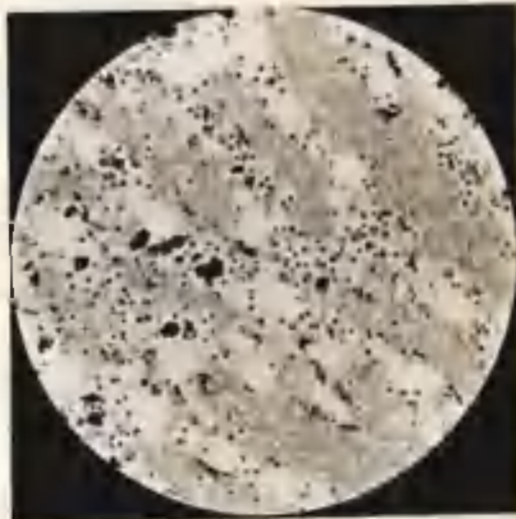


Bone marrow of rabbit 48 hours after intravenous injection of india ink, showing marked carbon storage in the endothelial cells of the sinusoidal capillaries.

Fig.30 A portion of the epiphysis.

Fig.31 A transverse section of the cellular marrow of the upper part of the femoral shaft.





Figs. 32
33
Bone marrow of rabbit ten days after intravenous injection of india ink. There is now much less carbon in the endothelial cells, but much more in the reticulum cells. In addition many free rounded histiocytes - probably derived from the sinusoidal endothelium - are present.

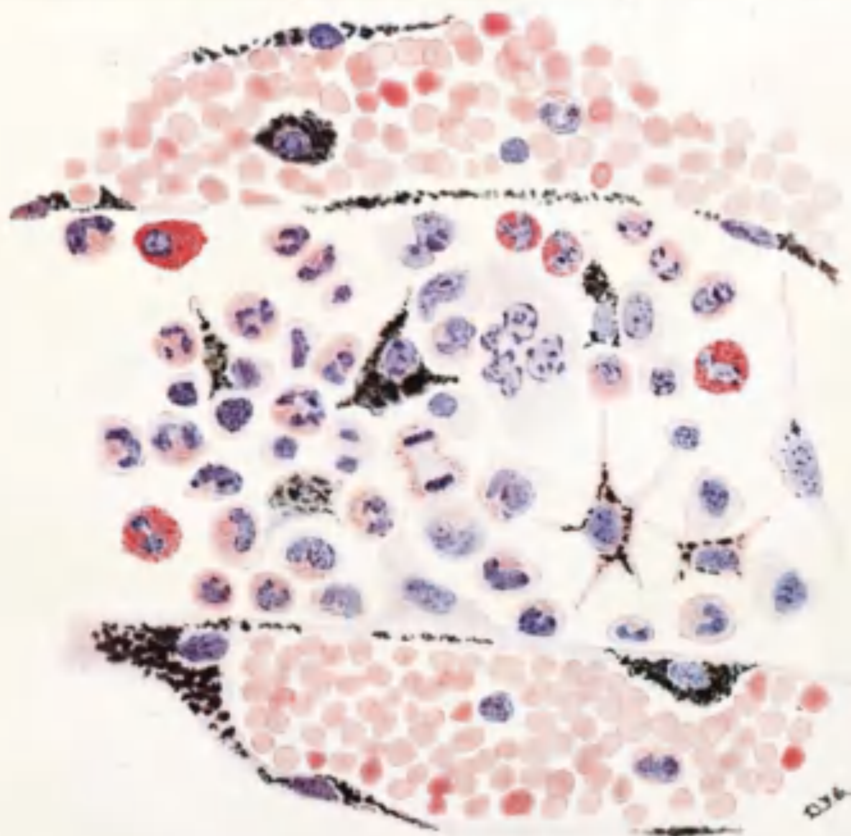


FIG. 34.—Bone-marrow of rabbit after four intravenous injections of india ink. The endothelial cells of the venous sinusoids and the stellate reticulum cells of the intersinusoidal leucoblastic tissue contain ink granules. A greatly swollen endothelial cell and a free blood histiocyte are shown. × 1000.

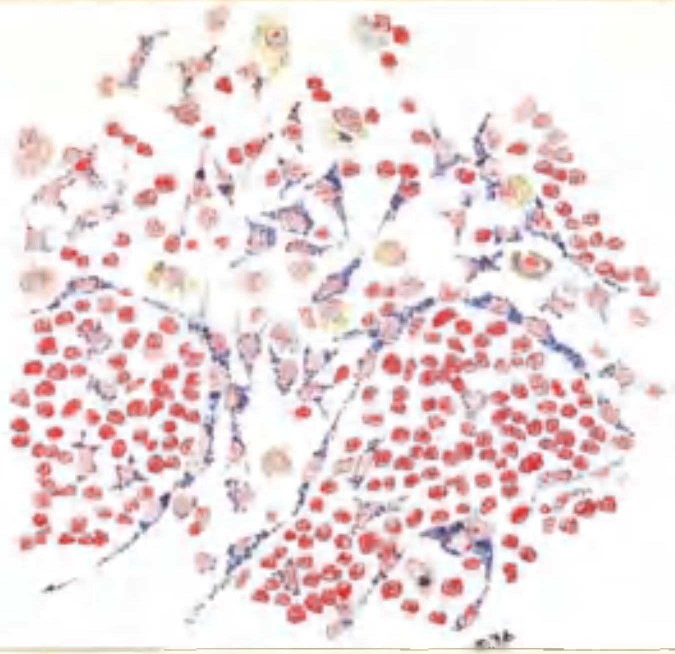


FIG. 35.—Lymphatic gland of mouse 96 hours after a subcutaneous injection of trypan blue. A portion of the peripheral sinus and lymph pathway to the medulla is shown. The endothelial cells lining the sinuses and the stellate floating cells which form a net across the sinus are all vitally stained. In the sinuses there are many free macrophages; some of these contain ingested debris and are only poorly stained by the vital dye. There are also many smaller unstained mononuclears in the sinus. The reticulum cells of the cortical lymph nodes are clearly demonstrated by their vitally stained granules. × 500.

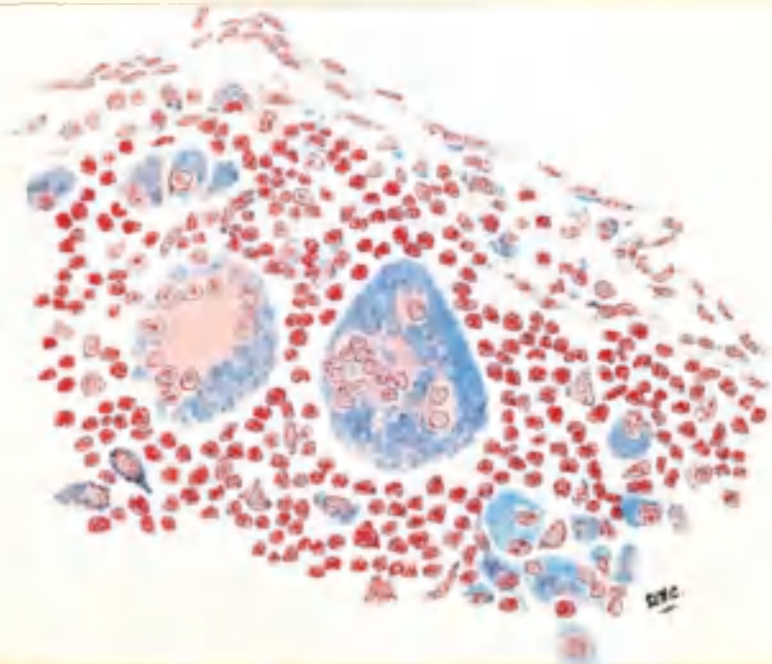


FIG. 36.—Lymphatic gland of mouse after repeated subcutaneous injections of isamine blue, the last of which had been given 4 weeks before death. The capsule of the gland and the peripheral sinus contain numerous vitally stained cells. In the adjacent cortical lymphoid tissue there are giant cells apparently formed by fusion of vitally stained cells. × 500.

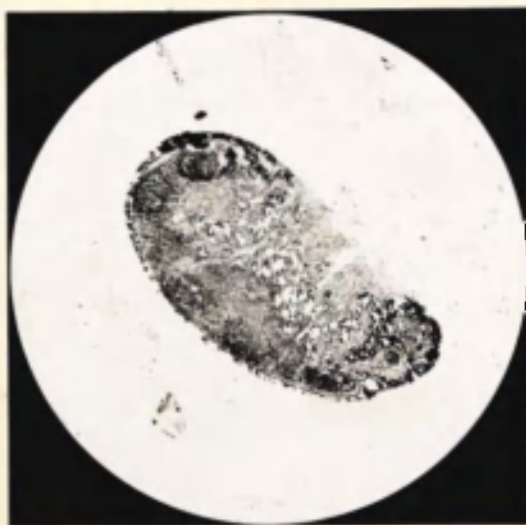


Fig.37 Lymphatic gland from the retroperitoneal region at the bifurcation of the aorta, after injection of india ink into the lymphatics of the tail. The peripheral sinuses are filled with carbon particles which are being actively taken up by the reticulo-endothelial cells.

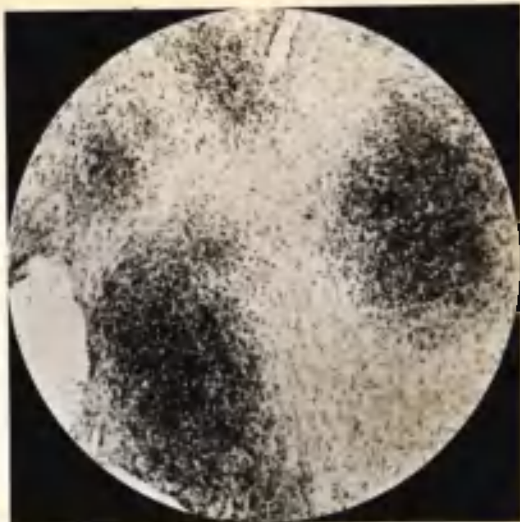


Fig.38 A thin portion of omentum from a normal unstained guinea pig.

Fig.39 A thin avascular portion of omentum from a rabbit stained by intravenous injections of india ink and trypan blue. Two avascular taches laiteuses are shown containing many vitally stained cells. Many of the cells have ingested both dye and carbon particles.

Fig.40 Low power view of mesentery of same animal showing accumulation of vitally stained cells in the perivascular adventitial sheaths.

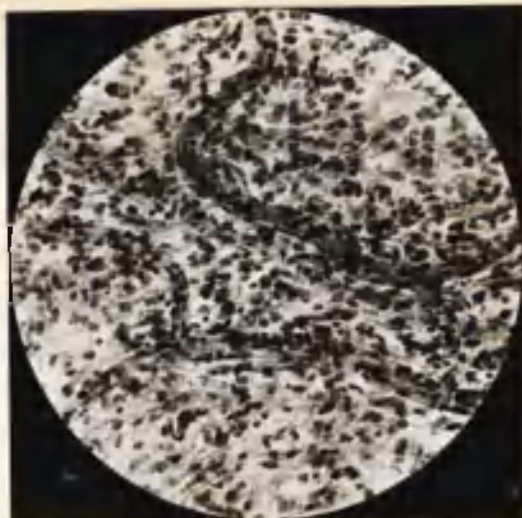


Fig.41 A portion of fig. at a higher magnification.

Fig.42 Omentum of rabbit stained by repeated intravenous injections of trypan blue. Numerous vitally stained histiocytes are present between the mesothelial layers, especially around the vessels, and are accompanied by many smaller unstained round cells.

FIG.43—Omentum of rabbit 48 hours after the seventh intravenous injection of trypan blue. A small vessel is shown with numerous vitally stained histiocytes around it and in the adjacent tissue. They are of irregular shape with blunt expanded processes—"trailer cells"—and there are some small round cells without vitally stained granules immediately beside them. The mesothelial cells show typical perinuclear crescents of small dye granules, and the fibrocytes also exhibit small deposits of the vital dye. × 600.

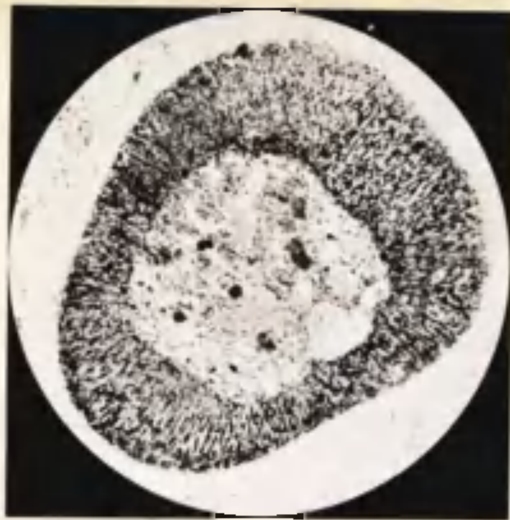


Fig. 44 Suprarenal gland of mouse five hours after intravenous injection of a large dose of india ink. There is a heavy deposit of carbon particles on the walls of the sinusoidal capillaries of the cortex; this is almost lacking in the medulla.

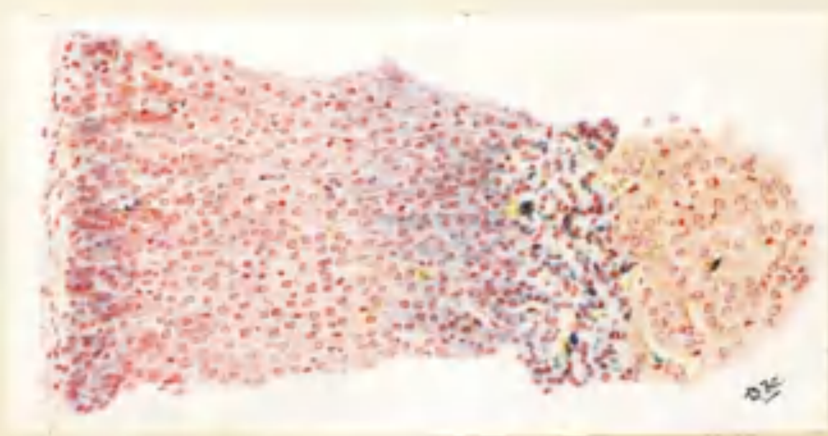


FIG. 45.—Suprarenal gland fifteen days after seventh injection. Traces of iron are present in the glomerular zone and in the inner third of the zona fasciculata, but iron storage is most pronounced in the inner part of the zona reticularis. A few iron containing endothelial cells are present both in cortex and medulla. × 225.

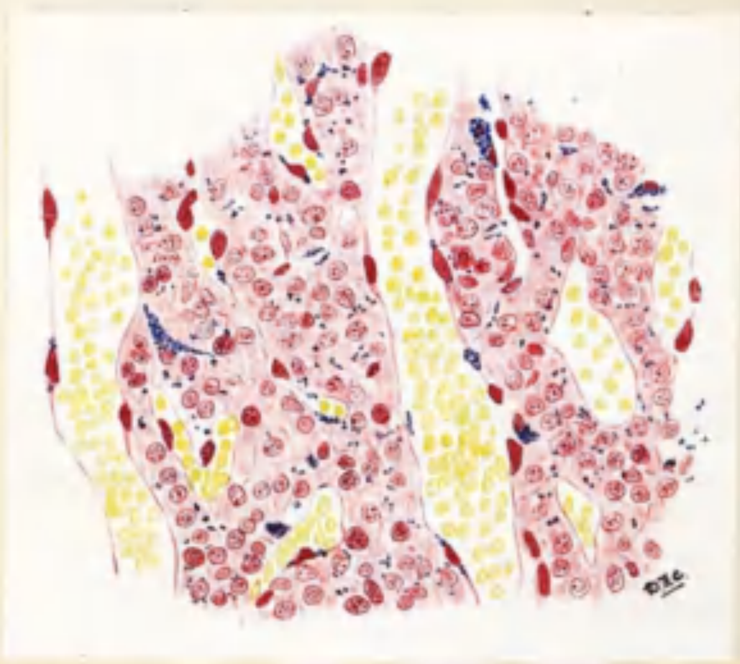


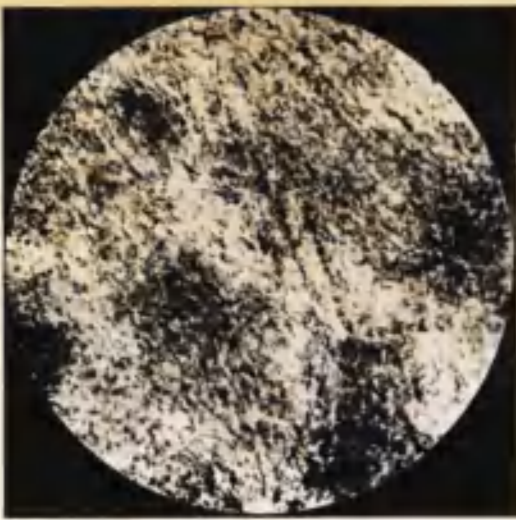
FIG. 46.—Pituitary gland of rabbit (anterior lobe) after seven intravenous injections of trypan blue. Some of the capillary endothelial cells contain small dye granules, but others are free. The large stellate cells with coarse vitally stained granules appear to lie not in the capillary walls, but just outside; they are thus *perivascular histiocytes. The pituitary gland* cells contain small vitally stained granules. ×500.

FIG. 41.—Pancreas of guinea-pig after eight intraperitoneal injections of trypan blue. The endothelial cells of the capillaries contain no dye granules, the numerous vitally stained cells being all situated just outside the walls, *i.e.*, perivascular histiocytes. The gland cells contain no dye granules. × 580.

Fig. 48 Kidney df mouse 48 hours after a second subcutaneous injection of trypanblue, showing intense vital staining of the renal epithelial cells in the proximal convoluted tubules.

Fig. 48.—Kidney of mouse 96 hours after a single subcutaneous injection of trypan blue. The glomerulus contains no dye granules, but the cells of the proximal convoluted tubules are deeply stained. ×240.

FIG. 52.—Skeletal muscle after repeated subcutaneous injections of isamine blue. The muscle substance is unstained. Numerous histiocytes are seen between the muscle bundles, especially in the neighbourhood of the smaller vessels. × 400.



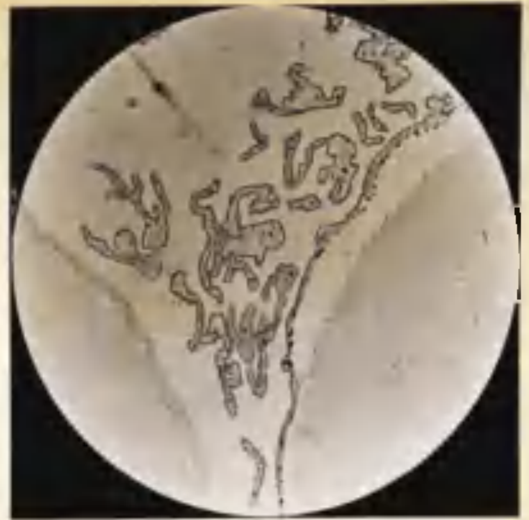


Fig.51 Dura mater of rabbit 48 hours after the seventh intravenous injection of trypan blue.. Vitally stained cells are most numerous along the course of the vessels and in small clusters in the intervening portions of the membrane .

Fig.52 Choroid plexus of same rabbit showing vital staining of the covering epithelium. A number of intensely stained histiocytes are present in the stroma.

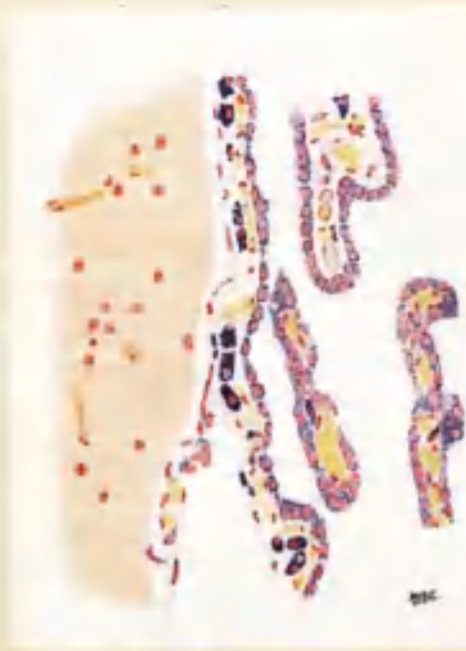
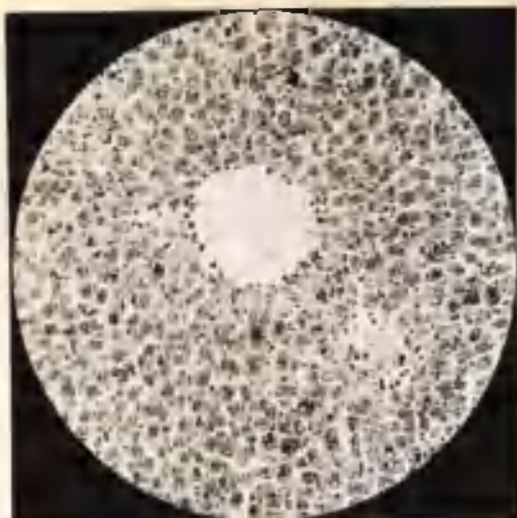


FIG. 52. — Choroid plexus of rabbit 48 hours after the seventh intravenous injection of trypan blue. The cubical lining cells contain numerous fine vitally stained granules. A few large perivascular histiocytes are seen in the stroma. The nervous tissue is unstained. ×240.



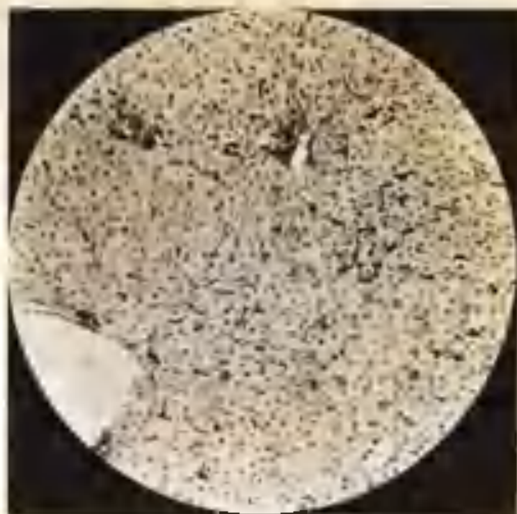
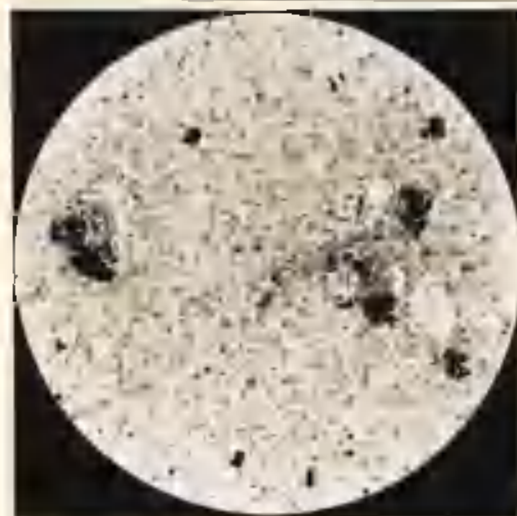


Fig.54 Liver of mouse one hour after intravenous injection of saccharated oxide of iron. There is marked accumulation of leucocytes in the small veins and sinusoids, and iron storage by the Kupffer cells is already pronounced.

Fig.55 Liver of mouse 14 days after a second injection of saccharated oxide of iron. Some of the Kupffer cells are greatly swollen and contain much intensely staining iron; iron-storing cells are commencing to accumulate in the stroma around the portal tracts, and regeneration of new Kupffer cells is in progress.



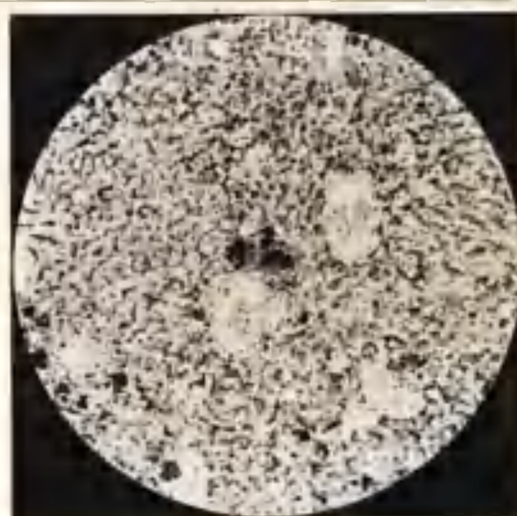


Fig.56 Liver of mouse 28 days after the last of 5 intravenous injections of saccharated oxide of iron. Marked accumulation of iron-storing phagocytes in the portal tracts and in small clumps in the sinusoids. Iron storage is commencing in the hepatic parenchyma cells.

Fig.57 Liver of mouse 8 months after last of 8 injections of iron. Atrophy of liver - probably senile - with abundant iron storage in the hepatic cells and bile duct epithelium. Many iron-storing cells are still present in the portal tracts.

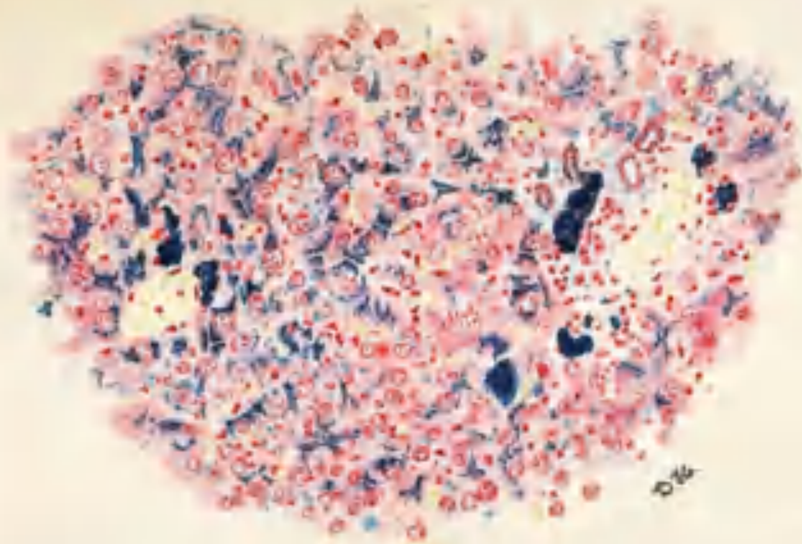


Fig.58 Liver of mouse six months after eigth intravenous injection of saccharated oxide of iron. The portal tracts contain many cells heavily laden with coarsely granular iron; the Kupffer cells have been regenerated and are now almost free from iron; the hepatic cells especially around the central vein show pronounced storage of iron , the granules being orientated along the bile canaliculi.

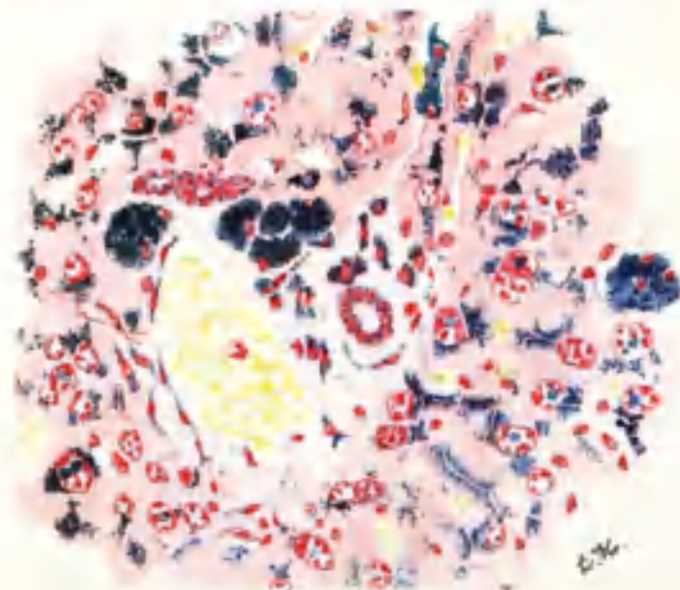


FIG.59.—Liver six months after eighth injection. The portal tracts contain numerous iron phagocytes and iron in granular form is abundant in the bile duct epithelium and in the liver cells, being heaped up along the bile canaliculi. Many of the nuclei contain iron-reacting bodies resembling plasmosomes. × 500.

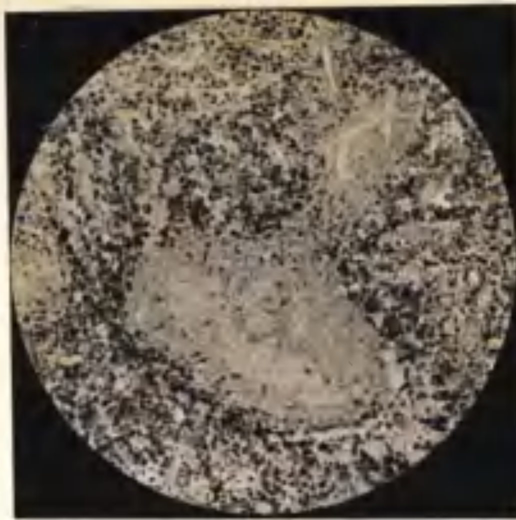


Fig.60 Spleen of mouse six days after the last of three intravenous injections of saccharated oxide of iron. Iron-storing cells are especially numerous in the pulp at the periphery of the Malpighian bodies, but the reticulum cells of the follicles also contain some iron.

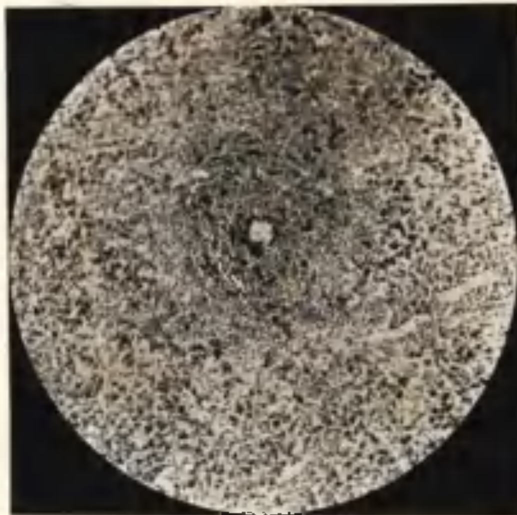


Fig.61 Spleen of mouse 14 months after the last of 4 intravenous injections of saccharated oxide of iron. Iron-storing cells are still numerous in the interfollicular pulp but are now most prominent within the Malpighian bodies especially around the central arteriole.

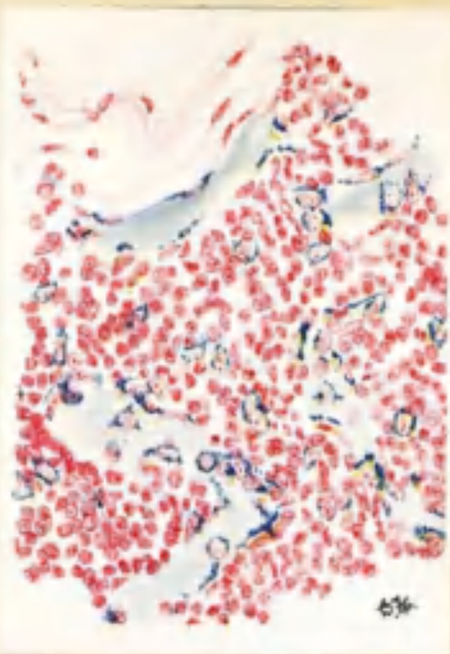


FIG. 62.—*Lymphatic gland from axilla six days after fifth injection.* The lymph in the peripheral sinuses gives a strong diffuse iron reaction, and there is iron storage in the reticulo-endothelial cells. × 500.

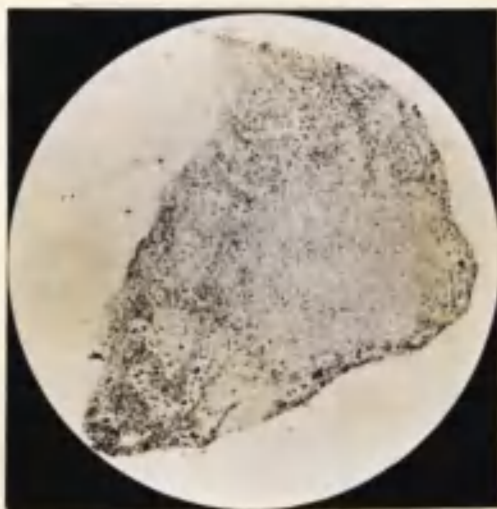


Fig.63 Lymphatic gland of mouse 8 months after the last of 8 intravenous injections of iron. The reticulo-endothelial cells throughout the gland are filled with iron in granular form.

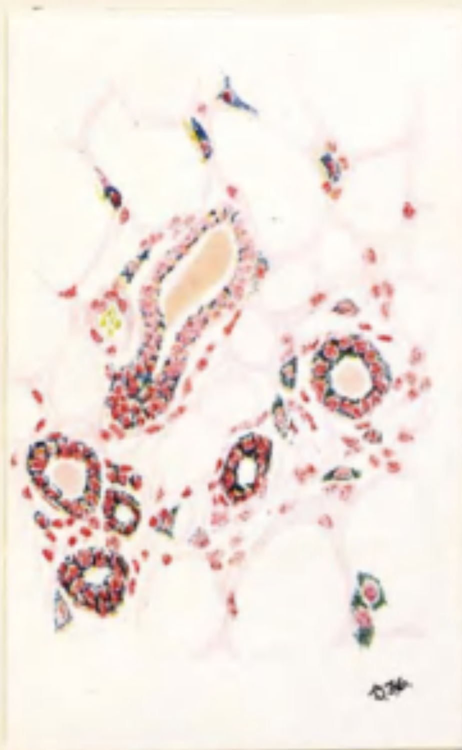


FIG. 44.—Mammary gland six days after fifth injection. Granular iron is abundant in the cells lining the *duct and related acini.* The adjacent histiocytes also contain much iron in coarsely granular form. × 500.

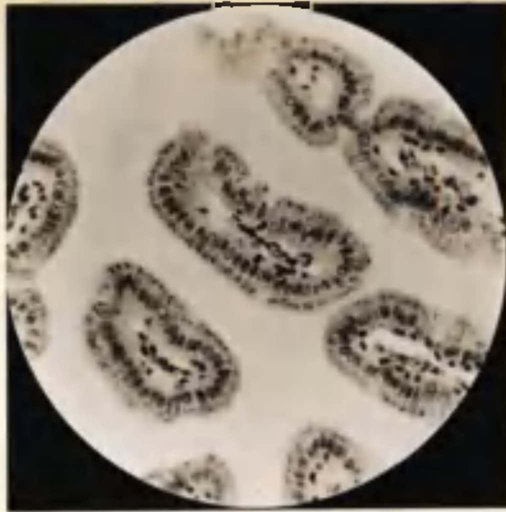


Fig.65 Duodenal villi of mouse 16 days after the third intravenous injection of saccharated oxide of iron. Numerous fine iron granules are present near the free margin of the epithelial cells covering the villi.

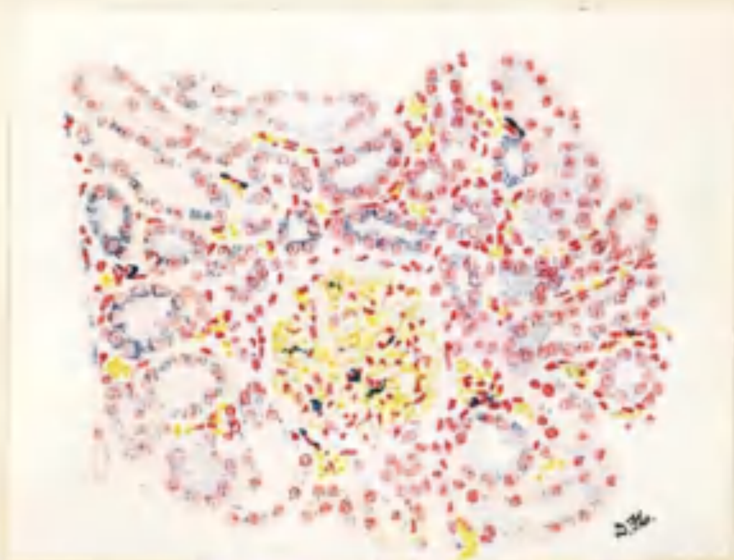


FIG.66—Kidney of rat ten days after eighth injection. Many of the tubules *contain finely granular iron, and a few cells with coarse granules are present* in the glomerular tufts and in the walls of the cortical capillaries. × 300.

FIG. 67.—Lung of mouse 7 hours after intravenous injection of a large dose of saccharated oxide of iron. Iron-containing leucocytes, both polymorphonuclears and monocytes, are present in the capillaries in considerable numbers but the capillary endothelium and the alveolar epithelium show no trace of iron. Prussian blue reaction : carmalum. × 600.

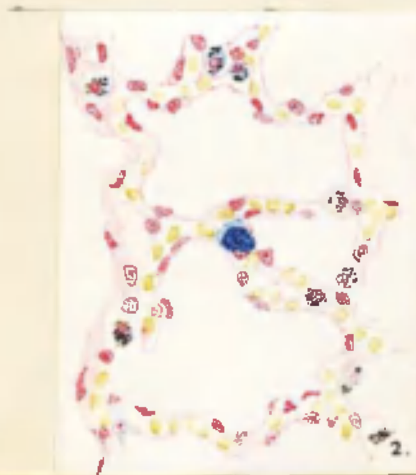


FIG. 68.—Lung of mouse 7 days after the last of three intravenous injections of saccharated oxide of iron administered at intervals of 24 hours. Vitally stained monocytes are no longer present in the capillaries ; a large iron-laden blood histiocyte is seen impacted in an interalveolar capillary. The cuboidal nucleated cells and the dust cells lying in contact with the alveolar walls show a faint diffuse iron reaction. Prussian blue reaction : carmalum. × 600.

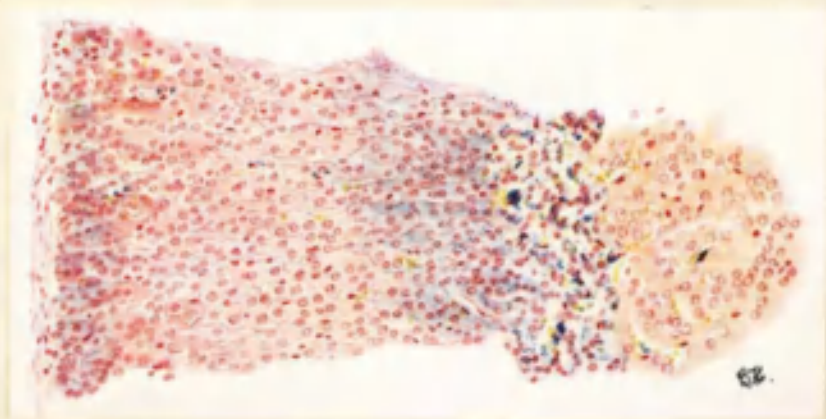


Fig. 49.—Suprarenal gland fifteen days after seventh injection. Traces of iron are present in the glomerular zone and in the inner third of the zona fasciculata, but iron storage is most pronounced in the inner part of the zona reticularis. A few iron containing endothelial cells are present both in cortex and medulla. × 225.

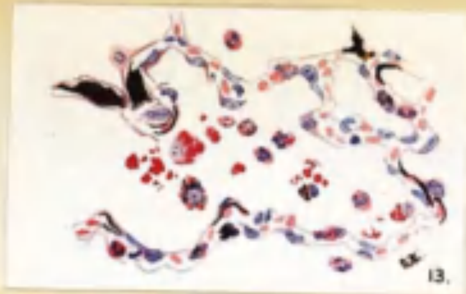


FIG. 10.—Lung of rabbit 5 hours after intravenous injection of india ink and intratracheal injection of carmine suspension. A few ink-storing monocytes are present in the capillaries; there is some precipitation of ink on the endothelial cells but no definite phagocytosis.

FIG. 11.—Lung of mouse 7 hours after intravenous injection of a large dose of saccharated oxide of iron. Iron-containing leucocytes, both polymorphonuclears and monocytes, are present in the capillaries in considerable numbers but the capillary endothelium and the alveolar epithelium show no trace of iron. Prussian blue reaction: carmalum. ×600.

FIG. 12.—Lung of mouse 7 days after the last of three intravenous injections of *saccharated oxide of iron administered* at intervals of 24 hours. Vitally stained monocytes are no longer present in the capillaries; a large iron-laden blood histiocyte is seen impacted in an interalveolar capillary. The cuboidal nucleated cells and the dust cells lying in contact with the alveolar walls show a faint diffuse iron reaction. Prussian blue reaction: *carmalum.* ×600.

Fig. 73. An interlobular septum is shown with many vitally stained histiocytes in the interstitial tissue;

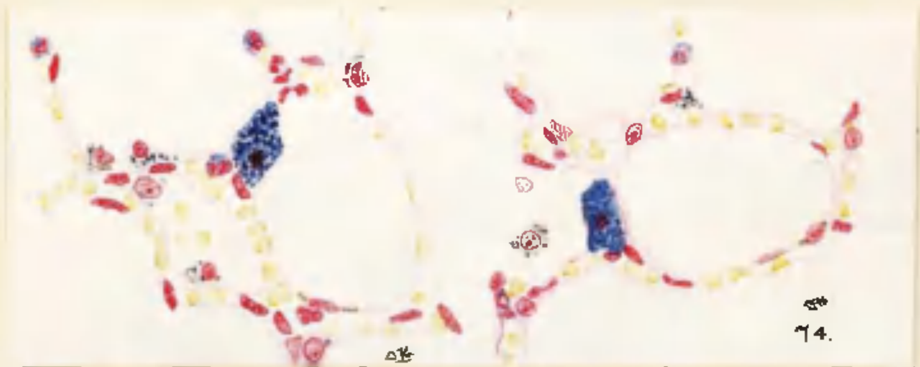


Fig. 74.—Lung of guinea-pig stained by repeated intraperitoneal injections of trypan blue, showing dye-laden blood histiocytes impacted in the capillaries. *The alveolar epithelium contains only traces of dye*, but the granules of the polymorphonuclear leucocytes are stained. Carmalum. ×600.

FIG. 15.—Lung of mouse vitally stained by repeated subcutaneous injections of trypan blue, killed 5 days after the first of a series of exposures to a smoky atmosphere producing experimental anthracosis. An interlobular septum is shown with many vitally stained histiocytes in the interstitial tissue; these take no part in the phagocytic reaction in the alveoli. Numerous fully formed alveolar phagocytes are present, and others are seen in process of development from the lining cells. There is still some extracellular soot on the alveolar walls. Carmalum. ×600.

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FIG. 76—Lung of guinea-pig 3 hours after an intratracheal injection of ink, showing the rapid mobilisation of phagocytes by desquamation of the flattened nucleated cells of the alveolar lining. Many of these show traces of trypan blue in addition to the ingested ink particles. Some of the cuboidal cells show increased swelling and vacuolation, but are not vitally stained. Carmalum. ×600.

FIG. 77—Lung of guinea-pig 30 hours after an intratracheal injection of ink. A larger quantity of ink than usual has penetrated into this alveolus, and the exudate consists of many large epithelial phagocytes, together with polymorphs and monocytes. The exudate cells show a slight degree of vital staining. Carmalum. ×600.

FIG. 73.—Lung of guinea-pig 1¾ hours after an intratracheal injection of ink. Two multinucleated cells are seen, apparently produced by detachment of small sheets of epithelium. They are still attached to the walls by delicate processes, and show fine stippling with ink granules and traces of vital staining. Individual flattened cells with ingested ink particles are also seen *in the process of detachment. Carmalum.* ×450.

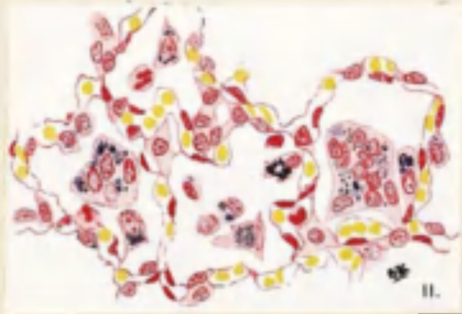


FIG. 74.—Lung of guinea-pig 96 hours after intratracheal injection of ink. Subpleural alveoli showing numerous vitally stained phagocytic cells and two multinucleated giant cells. Carmalum. ×450.

FIG. 80.—Lung of guinea-pig 1¾ hours after an intratracheal injection of india ink. The corner of an alveolus showing a cuboidal nucleated cell migrating through the lining into the air space. Carmalum. ×600.

FIG. 81.—Lung of guinea-pig 96 hours after an intratracheal injection of ink. Tangential section of corner of subpleural alveolus, showing three large ink-storing phagocytes which are also heavily vitally stained. Carmalum. ×600.

FIG. 92.—Lung of guinea-pig 30 hours after an intratracheal injection of ink. A larger quantity of ink than usual has penetrated into this alveolus, and the exudate consists of many large epithelial phagocytes, together with polymorphs and monocytes. The exudate cells show a slight degree of vital staining. Carmalum. ×600.

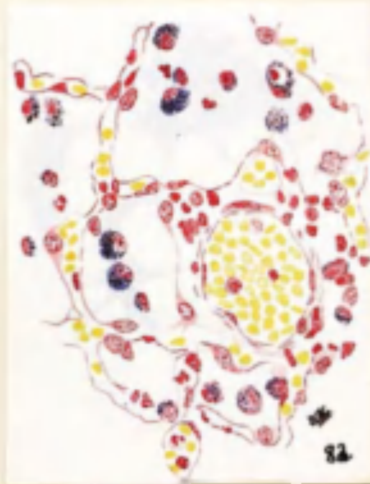


FIG. 83.—Lung of guinea-pig stained with vital new red; 24 hours after intratracheal injection of trypan blue. Many rounded free phagocytes of the usual type, deeply stained by trypan blue. A few polymorphs and monocytes are present and the latter show commencing dye storage. Two cuboidal cells are seen *in situ* showing vital staining of slight degree. Carmalum. ×450.

FIG. 84.—Lung of rabbit 5 hours after intravenous injection of india ink and intratracheal injection of carmine suspension. A few ink-storing monocytes are present in the capillaries; there is some precipitation of ink on the endothelial cells but no definite phagocytosis. In the alveoli large mononuclear phagocytes with ingested carmine granules but no ink; several polymorphs and an ink-storing monocyte are also present but the latter has *not* taken up any carmine. *Hæmatoxylin* and eosin. ×450.

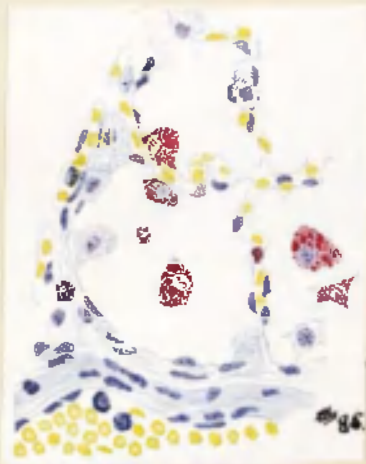


FIG. 85.—Lung of rabbit 30 hours after intravenous injection of saccharated oxide of iron and intratracheal injection of diaminefast scarlet. The alveoli contain several large dye-storing phagocytes; one dye-containing epithelial cell is seen *in situ*, but the other epithelial cells many of which are greatly swollen and vacuolated show no dye. None of these cells exhibits any trace of iron, but a few iron-bearing leucocytes are still present in the blood vessels. Hæmatoxylin. ×450.

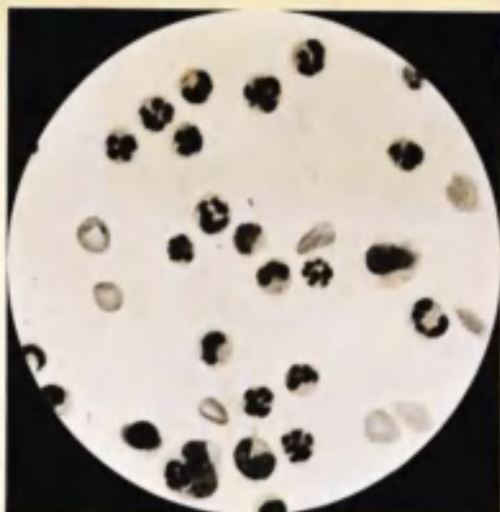


Fig.86 Peritoneal exudate of guinea pig 24 hours after injection of 1 c.c. of a 24 hour broth culture of Staphylococcus Aureus. Most of the exudate cells are polymorphonuclear leucocytes; a few young mononuclears —monocyte- and young macrophage-forms — are also seen.

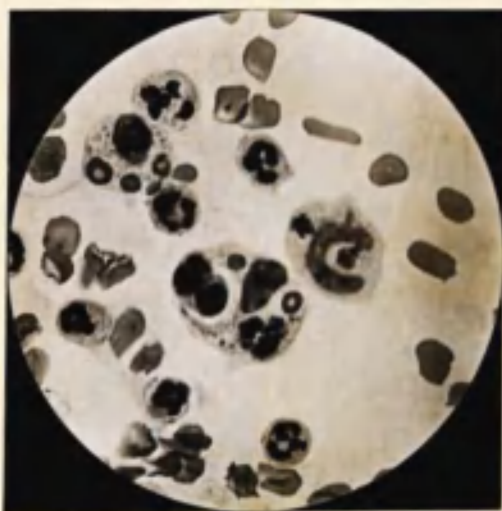


Fig.87 Peritoneal exudate of guinea pig 72 hours after similar injection of living staphylococci, showing resolution. Numerous highly phagocytic macrophages with ingested red cells and nuclear remains are shown.

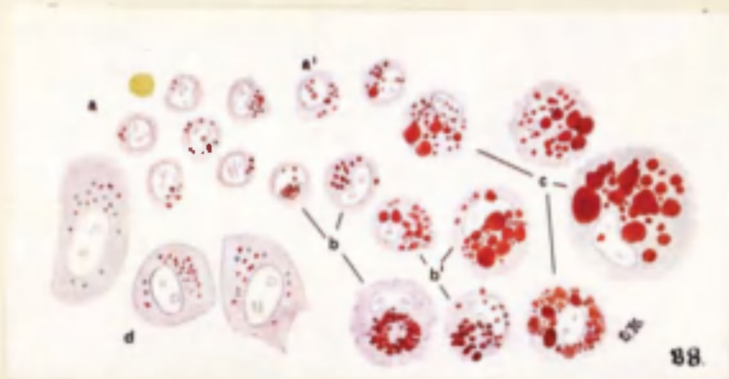


Fig. 88 *Cells from the peritoneal exudate of* a mouse 24 hours after injection of broth. Supravital staining with neutral red.

Stages in the formation of macrophages from lymphocytes.

(a) lymphocytes with only a few tiny neutral red granules.
(a') lymphocytes developing directly into macrophages.
(b) monocytes with definite "rosettes" of neutral red granules.
(b') monocytes developing into macrophages.
(c) macrophages with abundant neutral red granules irregularly arranged.
(d) desquamated serosal cells with scanty neutral red granules and a few highly refractile droplets.

Fresh preparation prepared by the dry dye film method; x 900.

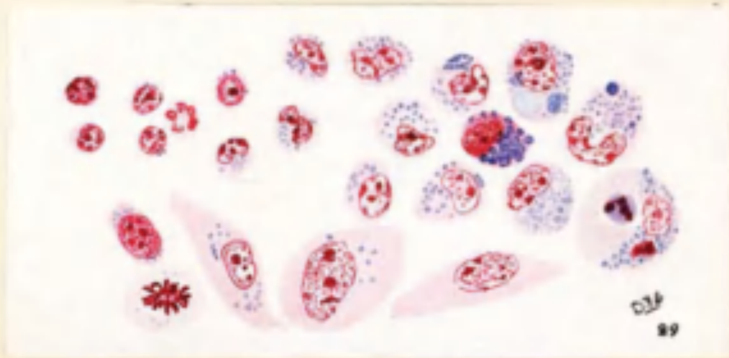
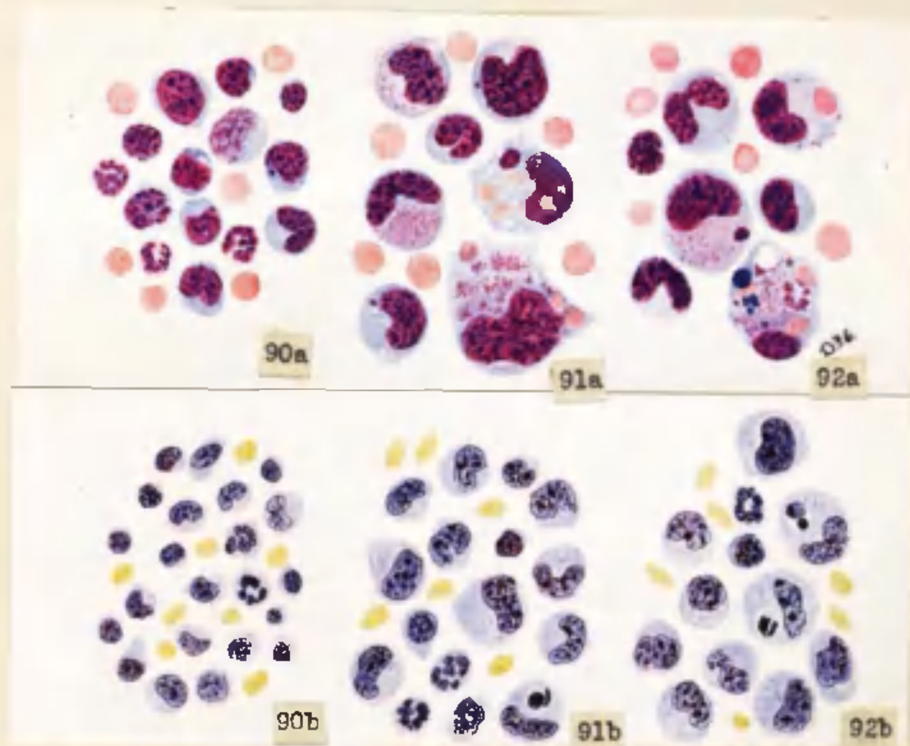


Fig.89 Cells from the peritoneal exudate of a mouse 26 hours after injection of broth and 24 hours after the last of three subcutaneous injections of trypan blue.

The cells are of similar types to those shown in fig. but their segregation apparatus is faintly coloured with the trypan blue.

Fixation in S U S A., counterstained with carbolfuchsin; x 900.



Cells from the peritoneal exudate produced by broth in a mouse 21 days after the last of 4 subcutaneous injections of vital new red.

a = air dried film stained by Leishman's method.
b = film fixed wet in corrosive sublimate, stained by alum-haematoxylin.

Fig.90 exudate after ten hours.

Fig.91 exudate after thirty hours.

Fig.92 exudate after fifty four hours. x 900

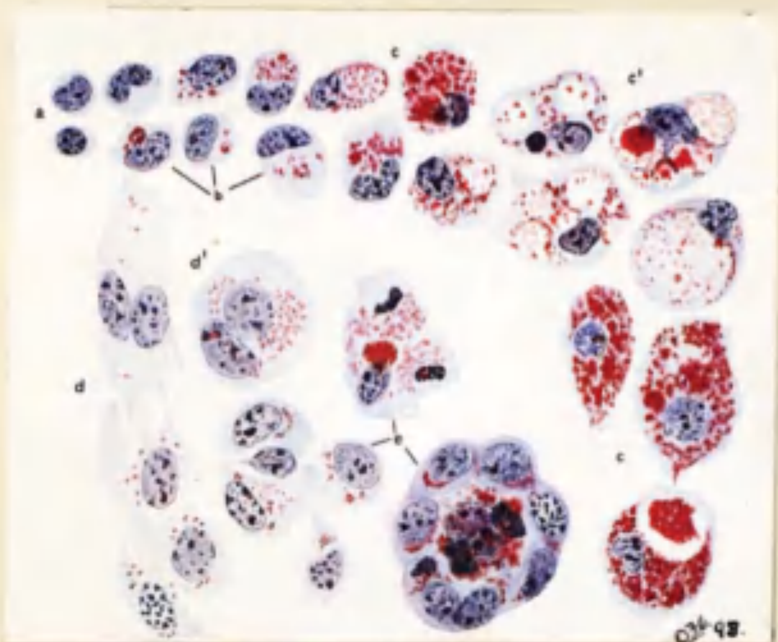
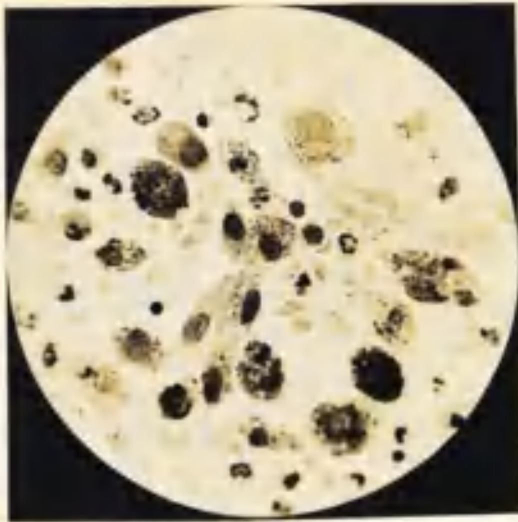


Fig. 93 Cells from the peritoneal exudate 48 hours after injection of diamine fast scarlet.

Formation of vitally stained macrophages by development of *small unstained cells.*

(a) lymphocytes devoid of stained granules.
(b) monocytes with stained granules arranged in rosettes.
(c) macrophages with very many stained granules almost filling the entire cytoplasm.
(c') macrophages with enormous vacuoles, some with masses of dye in their interior, others with the dye granules adhering to their walls.

Fixed in Bouin's fluid, counterstained with haematoxylin. x 900



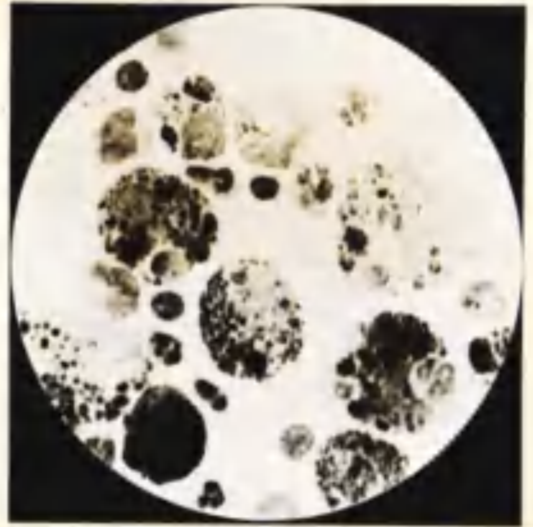
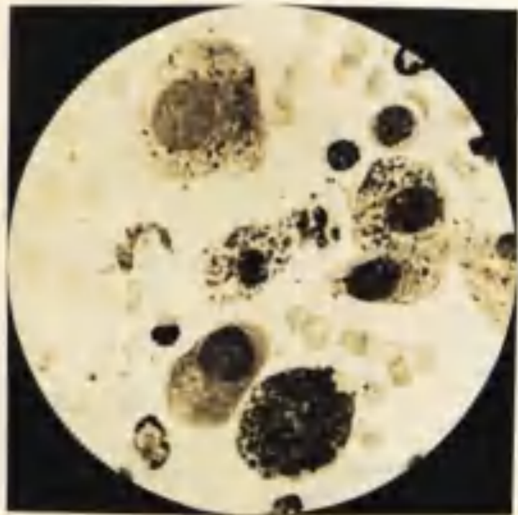


Fig.94 Peritoneal exudate of rabbit on the 8th day,after repeated intraperitoneal injections of diamine fast scarlet. A number of intensely stained macrophages and a few small unstained cells are seen together with a group of serosal cells with relatively scanty dye granules.

Fig.95 A group of intensely stained macrophages from the same exudate.



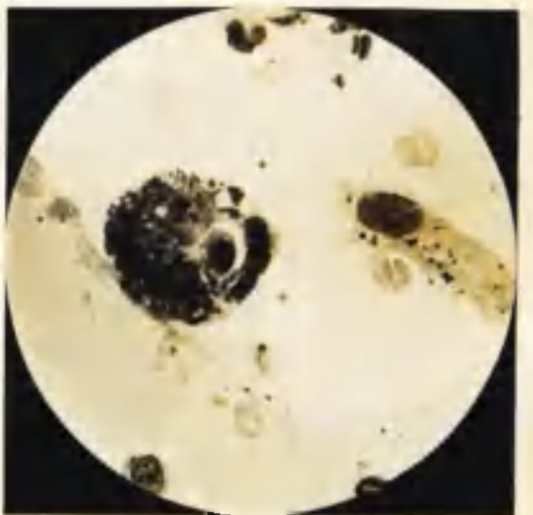


Fig.96 A portion of the field shown in fig.   at a higher magnification.

Fig.97 An enormous phagocytic vitally stained macrophage, and a desquamated serosal cell with scanty dye granules.

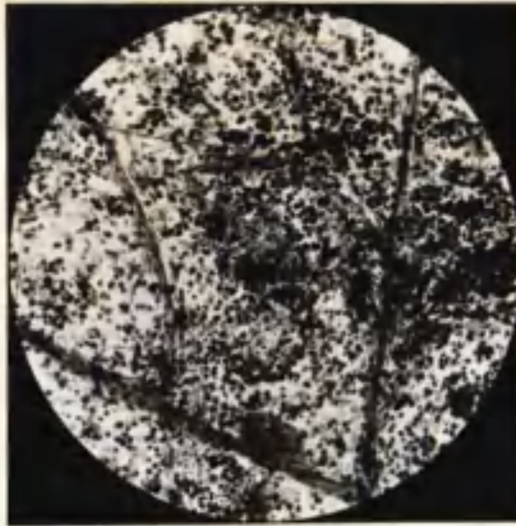


Fig.98 Omentum of rabbit after repeated intraperitoneal injections of diamine fast scarlet over a period of 8 days. Note the enormous accumulation of intensely stained macrophages together with many smaller unstained cells.

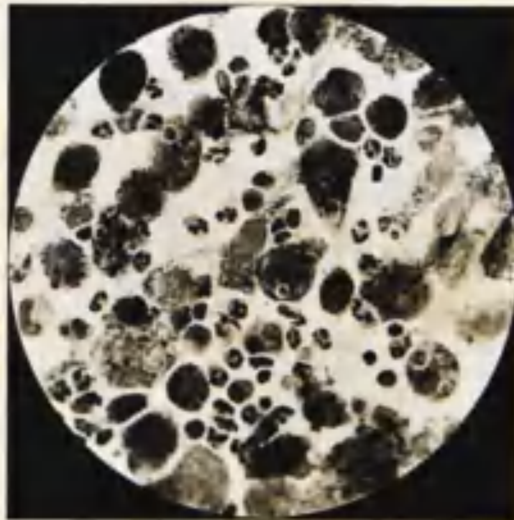
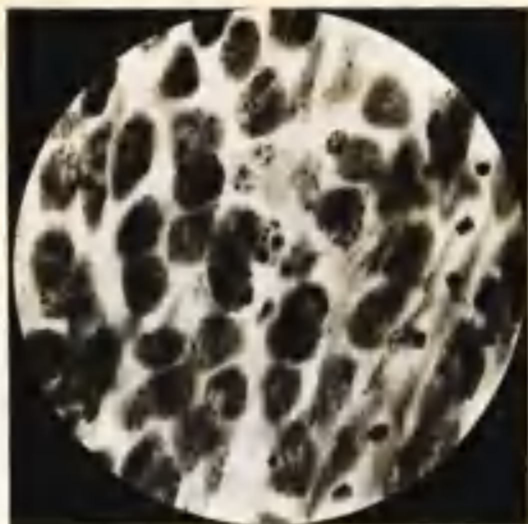


Fig.99 High power view of same omentum, showing various types of vitally stained cells. Polymorphs ; Macrophages with many coarse irregular stained granules; smaller mononuclear cells in process of development into macrophages; mesothelial cells with fine vitally stained granules forming perinuclear crescents; fibrocytes with similar granules more scattered in the cytoplasm and extending into the long processes.



Figs. 100
101
102

Mesentery of mouse after three intraperitoneal injections of isamine blue, the last being given 10 days previously. The mesentery contains an enormous number of intensely stained macrophages, which at the higher magnifications can be distinguished from the fibrocytes by the character of the granules. The mesothelial cells are not sharply in focus owing to the thickness of the spread preparation.

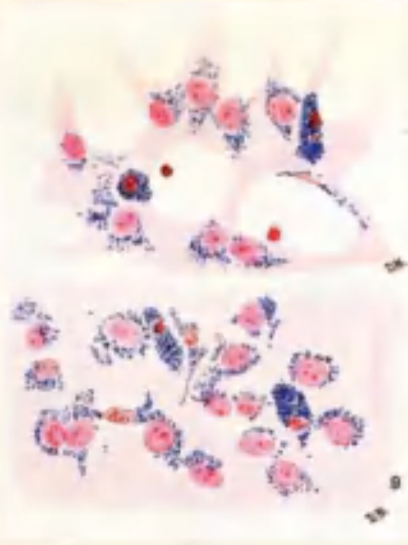


Fig.103 A thin fenestrated portion and a sheet of omentum from a guinea pig after six intraperitoneal injections of trypan blue. The serosal cells exhibit pronounced accumulation of dye granules generally in the form of a perinuclear crescent. A few histiocytes and fibrocytes are also shown; both contain numerous dye granules.

Fixed in formol-saline, counterstained with carmalum. x 400.

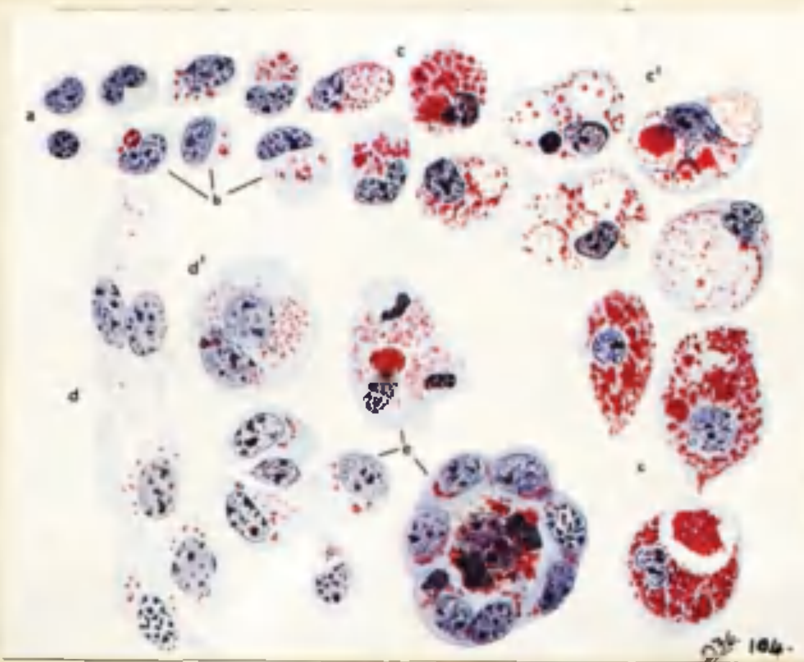


Fig.104 Serosal cells from peritoneal exudate 48 hours after injection of diamine fast scarlet.

(d) a small sheet of desquamated serosal cells, one of which is in mitosis.
(d') a binucleated serosal cell free in the exudate.
(e) the formation of small mulberry-like masses from the adhesion of serosal cells to masses of precipitated dye. Some of the mesothelial cells are more intensely stained than usual.

Fixed in Bouin's fluid, counterstained with haematoxylin. x 900

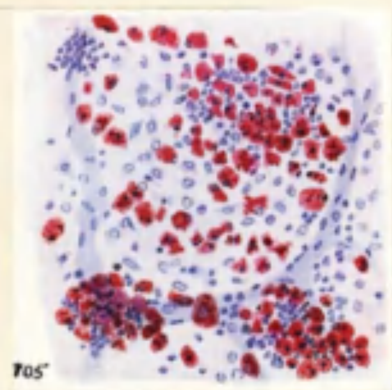


Fig.105 Omental spread of mouse 32 days after the last of 4 intraperitoneal injections of diamine fast scarlet. Numerous large vitally stained cells in clusters around the vessels and in the intervening avascular portions, accompanied by many small unstained cells. Broth was injected intraperitoneally 96 hours before death, and the exudate was examined at intervals (see fig.106).

Fixed in Bouin's fluid, counterstained with alum-haematoxylin.

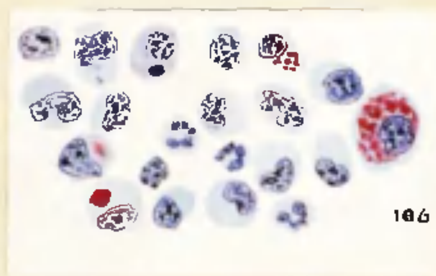


Fig.106 Cells from the peritoneal exudate produced in 36 hours by intraperitoneal injection of broth into a mouse previously stained by intraperitoneal injections of dye (see fig.105).
The majority of the cells are unstained mononuclears; only a few vitally stained cells are free in the exudate in spite of the large number of such cells in the tissues.

Fixed in Bouin's fluid, counterstained with alum-haematoxylin. x 900

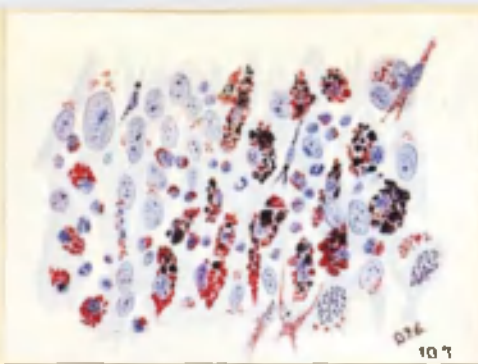


Fig.107 Omentum of mouse 48 hours after intraperitoneal injection of diamine fast scarlet, the animal having received 21 days previously an intraperitoneal injection of a dark blue sulphonated disazo styryl quinoline dye.

Numerous cells with granules of the first administered dye; some have stored also the second dye, and in addition there are many macrophages containing only the diamine fast scarlet. In the exudate produced by the latter blue-stained cells were practically absent at all stages.

Fixed in "S U S A.", counterstained with alum-haematoxylin. x 400.

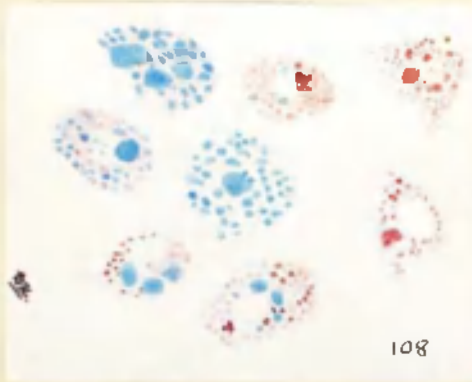


Fig. 108. Cells from the mesentery of a mouse which had received an intraperitoneal injection of isamine blue one month previously; this was followed three weeks later by an intraperitoneal injection of diaminefast scarlet. All the cells shown are histiocytes; some contain only isamine blue, others only diaminefast scarlet, while some contain both dyes in varying proportion.

Mounted in balsam. ×800.

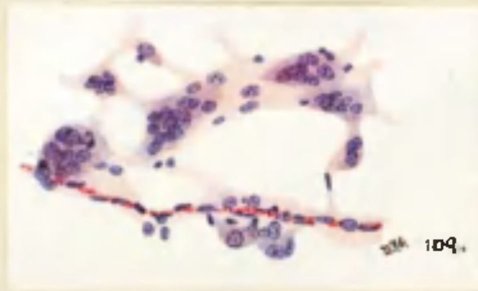


Fig.109 Omental net from a child with ascites following rheumatic cardiac disease. The mesothelial cells are greatly swollen and form syncytial masses on the omental trabeculae.

Fixed in Zenker-formol, stained with haematoxylin-eosin-azur.

Plate LXII

Plate 57.