On a New Method of Examining Normal and Diseased Tissues by Means of Intra-vitam *Staining.*

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(1) *History of Method.*

The older methods of vital staining, such as the methyl-blue stain for nerve fibres, neutral-red stains for glandular cells, and others, were limited in their use to the differentiation of specific tissues. Ribbert was the first to attempt a vital stain of the whole body by intravenous injections of carmine solutions. The results achieved by this method in normal (Ribbert, Schlecht) and pathological tissues (Pari) were not certain enough to permit of its general use in histological practice.

A new departure in the field of vital staining resulted from the attempts to cure trypanosome and other infectious diseases of the blood through the agency of aniline dyes, such as trypan-red and trypan-blue. Bouffard, of the Pasteur Institute, investigated the cellular conditions following upon the injection of trypan-blue. Without previous knowledge of his work I undertook an extensive study of normal mice and rats, injected with trypan-, isamin-, and pyrrhol-blue solutions. The substance of this research was published in 1909 in a paper, "Äussere und innere Sekretion des gesunden und kranken Organismus im Lichte der vitalen Färbung, Teil I."* Since then I have improved the method, searched for new staining media, and have finally applied the vital stain for purposes of comparative histological and pathological research.

(2) *Methods.*

A vital stain is achieved in an animal either by injecting the stain subcutaneously, intraperitoneally, or into the blood-vessels. Confining myself for the present to the two stains whose tissue reactions I have already published, I have now determined that in mice and rats trypan-blue acts equally well when injected under the skin as it does after introduction into the blood-vessels. But whereas 1 c.c. of a 1-per-cent. solution per 20 grm. of the animal's body-weight injected subcutaneously has no

* 'Beiträge zur Klin. Chirurgie,' H. Laupp, Tübingen. In this paper, as well as in a more recent one, published in the same journal in March, 1912, plates are to be found which illustrate histological appearances referred to in this paper.

ill effect on the animal, no more than 0·5 c.c. of the same solution should be used for intravascular work. In the latter case coloration sets in speedily, increases up to the second day after injection, but rapidly fades after the fourth day, in any case quicker than when gradual absorption of the staining fluid takes place through the lymphatic channels.

As for isamin-blue, the maximum dose for intravascular injection should not exceed 0·3 of a 1-per-cent. solution by a single drop, if its general toxic effect on the animal or the danger of widely-spread thrombosis is to be avoided. Where distinct and delicate granule staining is required the preference should be given to isamin-blue.

It is undoubtedly safest and best for histological study to inject the staining fluids subcutaneously. Injections of 1 c.c. of a 1-per-cent. solution per 20 grm. of the animal's body-weight may be repeated many times once a week. In some cases I have gone up to 15 injections, without doing any harm to the animal. Naturally, the vital coloration then becomes most intense and histological details very distinct. For bigger animals, such as the rabbit, dog, and ape, where larger and more frequent doses of the staining fluid are needed, intraperitoneal injections are preferable to subcutaneous, the dose being determined by the animal's weight. It is safe, as far as isamin- and trypan-blue are concerned, to abide by the standard, 1 c.c. of a 1-per-cent. solution per 20 grm. of the animal's weigh

Both isamin- and trypan-blue allow of fixation by means of a 10-per-cent. formalin solution (best applied from the beating heart of the anæsthetised animal), but it is only in tissues stained by trypan-blue that the ordinary processes of histological technique are applicable. Alcohol extracts isamin-blue also after lengthy fixation of tissues in formalin solution. But even after vital staining with trypan-blue it is advisable to carry along a small quantity of formalin in the dehydrating fluids. When these precautions are taken, durable histological specimens of vitally stained preparations may be obtained, which recall in every detail the conditions prevalent in life.

As a rule I use sections cut by the freezing microtome from specimens that have been fixed in a 10-per-cent. formalin solution for not less than 48 hours. The specimens are then transferred to a slide and allowed to dry until the section appears firmly fixed to the slide. Then the process of counter-staining, dehydration and mounting is rapidly performed. As to counter-staining, I have recently determined that, besides the alum-carmine stain I first advised, other methods likewise yield excellent results.

For studies of connective tissue reactions it is well to combine the vital stain with Pappenheim-Unna's pyrronin methyl-green solution, inasmuch as this combination permits of differentiating between the vitally-stained

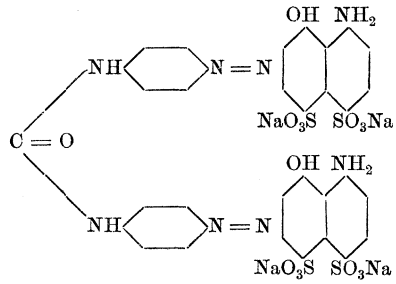
"pyrrhol cell," the basophile plasma cell and Ehrlich's "mast cell," distinguishable by the meta-chromatic orange colour which its granules display.

For purposes of leucocytic and lymphocytic analysis I have found the combination of the vital stain and Ehrlich's triacid mixture most useful. In this instance paraffin sections of tissues taken from animals which have been subjected to trypan-blue injections are necessary.

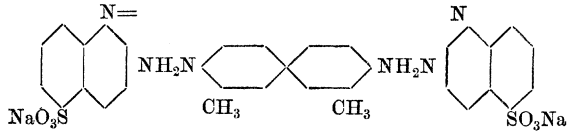
In my experiments on tuberculosis I have succeeded in retaining the vital stain even in sections which underwent the carbol-fuchsin staining and nitric acid differentiation for tubercle bacilli. Not only did we discover the bacilli, but we were able also to discriminate all the phases of disintegration, which the vitally-stained cell granules gradually underwent through the intracellular multiplication of the bacilli and their effect on cell structure. In this case also paraffin sections from trypan-blue animals are required.

The number of "vital stains" recently discovered by myself and by my pupil, Herr Schulemann, is rapidly increasing. Our chief object in the search for new stains is to discover the exact chemical radical which produces the vital reaction. We have as yet not come across a single "vital stain" which differs in its general behaviour towards the living organism from that of trypan- and isamin-blue. Hence, new proof has been furnished for my original contention, that in *intra-vitam* staining we are dealing with established chemical properties in the cell plasm and with an innate constitution of its structure. Amongst the new stains which are now engaging our attention I mention the following, appending a few remarks as to their application. All stains are procurable from Grübler and Co., Leipzig.

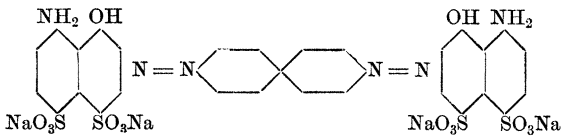
1. *Trypan-violet.*—It corresponds in all details of vital staining, application, fixation, etc., to trypan-blue. The formula is:—



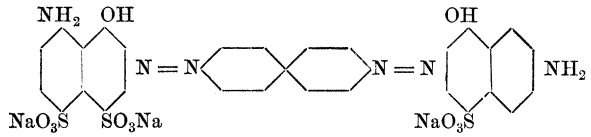
2. *Benzopurpurin B.*—



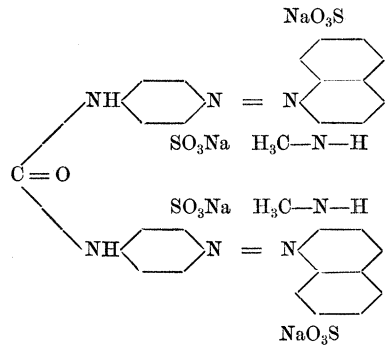
3. *Diamin-blue BB.*—It is an excellent *intra-vitam* stain. Herr Schulemann has found that 300 c.c. of a 1-per-cent. solution, injected into the peritoneal cavity of a dog weighing about 10 kgrm., produce within 24 hours a magnificent coloration of the animal. Its formula is as follows:—



4. *Diamin-black BH.*—A most reliable vital stain, of which the formula is as follows:—

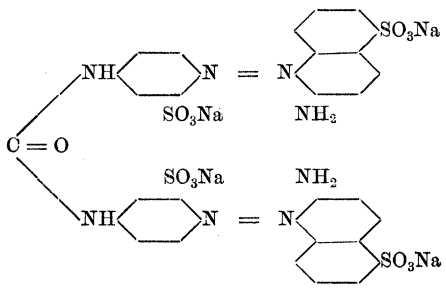


5. *Vital "Neu Rot."*—

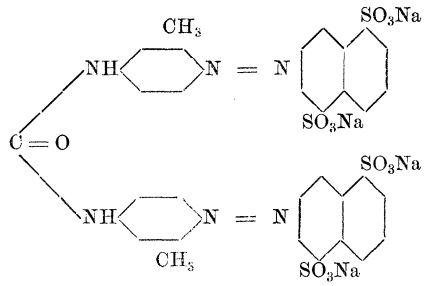


In mice 2 c.c. of a 1-per-cent. solution per 20 grm. of animal's body weight may be weekly injected. The effect is a brilliant scarlet stain of the animal. Unfortunately, this scarlet colour is transformed into a brownish red colour when the tissues are subject to formalin-fixation.

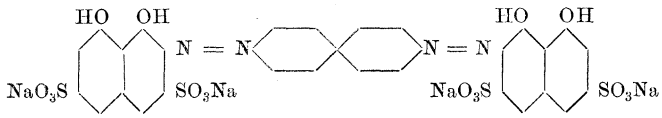
6. *Vital "Neu Orange."*—



7. *Vital "Neu Gelb."*—



8. *"Dianil Blau," R.*—



As to technique, I will merely add that a solution of these stains in Ringer's fluid is advisable for purposes of *intra-vitam* staining. It is well to begin experiments on animals whose toxic susceptibilities are not yet tested, by injecting a weaker dose than that which results from the formula for subcutaneous and intraperitoneal application—1 cm. of a 1-per-cent. solution for 20 grammes of animal's body weight.

Evidence gathered both in physiological and pathological fields seemed to point towards the fact that cell plasma, which accepts fat and lipoid stains, such as sudan, scarlet red, and Nile blue, also shows a marked tendency towards vital stains. But the exact relation between intracellular fat and lipoid substances, on the one hand, and bodies affected by the vital stain, on the other, was revealed by a careful analysis of the Pyrrhol cell (to be described in full below) during its transformation into the spindle cell of scar tissue and during the varied phases of its disintegration under the influence of bacillary necrosis. In both cases I discovered that when unimpaired, the pyrrhol cell accepts the vital stain only, whereas during the progress of its transformation or disintegration its affinity for the vital stain decreases and its powers of attracting the fat stains increase. Hence I am inclined to assume that, under normal conditions, fat or lipoid substances of the cell plasma unite with proteins and form loose compounds, liable to *intra-vitam* staining. Once this coherence is destroyed, the histo-chemical fat reaction becomes evident, whereas the vital stain is lost.

Similar relations between fat and albumen have already been demonstrated for the blood. Under normal circumstances, as long as fat is linked to albumen, its extraction by ether is rendered impossible by the "protective"

influence of the proteid "radical." But during the period of lactation, under the influence of starvation and phosphorus poisoning, a decomposition of "blood fat" results, and it becomes soluble in ether. That the vital stain is not directly due to the presence of fat or lipoid bodies may also be inferred from the fact that I have hitherto failed to discover a fat or lipoid solvent for any of our vital stains.

(3) *Normal Tissues.*

I quote the following general facts from my first paper :—

"Throughout the whole of the animal's tissues the stain is embodied in granules of specific cells. Although the stain circulates in the blood, no blood cell accepts it, nor has it any effect on the cells of the vascular coats. In the skin the stain is discovered in the granules of the fixed connective tissue cells of the cutis, but chiefly in free round cells belonging to the lower layer of the subcutis. Here the cells aggregate in great numbers, and especially in spots where an irritation or lesion of the skin is produced by artificial means or by pathological processes. These cells which belong to the type of the 'histiogenic migratory cell' are by no means confined to the skin ; they appear in every internal organ (with the one exception of the nervous system) and always in connection with interstitial fibrous tissue. We find them in muscles, tendons, in glands, but especially in serous membranes. The cells display besides marked chemotactic irritability and migratory powers phagocytic properties, which are most obvious in peritoneal lesions of every description. As the granules of their protoplasm eagerly absorb vital stains such as pyrrhol blue, I originally called them pyrrhol cells to emphasise their specific 'vital reaction' and to distinguish them from the various leucocytic and other migratory cells, which appear in the connective tissue."

In the course of my newest work I have discovered that the pyrrhol cell is to be found in great numbers in the subcutaneous tissue and the superficial muscular layers of the embryo towards the end of its development, and also under the skin and in superficial lymph glands of the new-born animal. Both in new-born and adult animals it is an inhabitant of the bone-marrow. It seems probable that the bone-marrow is concerned in the production of pyrrhol cells. My latest work, especially on the rat, has proved that the matrix of these cells is situated in the *tâches laiteuses* of the omentum and those of certain peritoneal ligaments, such as the ligamentum gastro-lienale and others. Only by means of the vital stain can we safely and surely distinguish the pyrrhol cell from its endothelial neighbour, the latter being refractory to the stain. The pyrrhol cell, when leaving the *tâche laiteuse*, spreads along the perivascular lymphatics into the wide area of the

peritoneum, also reaches the peritoneal cavity, where even in its vitally stained condition it becomes the "macrophage" *par excellence*, absorbing not alone dead material, such as carmine, dust, etc., but also living tubercle bacilli, manifesting a predilection for those of the avian type.

The importance of these cells will be fully illustrated by our inquiries into pathological conditions, such as tuberculosis and malignant growths.

By means of the *intra-vitam* stain we can differentiate the "Kupffer" star cell in the liver, the reticulum cell of lymph glands, spleen, and bone-marrow, the interstitial cell of the testicle, the follicular cell in the maturing Graafian follicle, the cortex cell of the suprarenal, the epithelial cells of the choroid plexus, the epithelial lining of the convoluted tubes of the kidney. Most striking is the appearance of the placenta and its behaviour in relation to the rest of the body. When pregnancy occurs in the vitally stained animal, the blue colour disappears from its skin and is concentrated in the uterus, the latter forming a centre of attraction for the vital stain, and actually dispossessing the remaining tissues of their blue. In the uterus we find the blue chiefly in the free cells of the decidua serotina, but also in the cells of the reflexa during the period of its existence.

In the first days of pregnancy, during which the development of the mouse and rat embryo is slow and its growth is solely dependent upon exuded maternal blood, I have recently found that peritoneal pyrrhol cells migrate into the uterine wall, penetrate into the primitive placenta and cast off vitally stained granules, which are snatched up by fœtal cells in the way of nutritive material. Once the placenta has attained its maturity we discover the vital stain in the "giant cells," which form the boundary line between the maternal and fœtal part of the placenta. We also find it in those fœtal cells which constitute the only barrier between the maternal blood spaces and the endothelial lined capillaries of the fœtus. Finally, the vital stain effects a most striking specific differentiation in the granulated cells of the vitelline membrane. Notwithstanding the fact that the yolk membrane is deeply stained throughout its whole extent, and the placental fluid shows a faint bluish colour, the embryo remains perfectly colourless, the placenta and its appendages thus forming a kind of protective barrier against the passage of the stain from the maternal into the fœtal organs.

As to the later stages of embryonic growth my new histo-chemical studies have proved that exactly the same fœtal cells of the placenta, which so vigorously absorb the vital stain, store also glycogen, fat, and hæmoglobin temporarily, ere these substances pass into the fœtal circulation. In the light of my new work the importance of vital staining for the purposes of embryological research will become more apparent.

(4) *Diseased Tissues* (*Mice and Rats*).

(*a*) Healing of wounds.
(*b*) Trichinosis.
(*c*) Experimental tuberculosis.
(*d*) Toxic degeneration of the liver.
(*e*) Malignant growths.

(*a*) In the healing of wounds produced in the skin, liver, and kidney, the pyrrhol cell appears on the scene after the initial emigration of leucocytes from the dilated blood-vessels has taken place. The extravasated leucocyte shows glycogen and fat granules in its protoplasm. The pyrrhol cell phagocytes such leucocytes or incorporates either glycogen or fat granules derived from leucocytic degeneration. Eventually it stretches into the spindle cell of the young connective tissue, losing consecutively both its affinity for the vital and fat stains. In skin wounds the pyrrhol cell is derived from the subcutaneous tissue, whereas in liver and kidney wounds I have been able by means of the vital stain to demonstrate the migration of pyrrhol cells from the serous coat of the injured organ or from the peritoneal cavity into the wounded area.

(*b*) The activity of the pyrrhol cell is most prominently displayed in the surroundings of parasites, such as the trichina and other worms. In the case of the trichina the "pole cells" of the muscular "spindles" accept the vital stain. It appears that in wandering towards the trichina the pyrrhol cell passes through lymphatic glands, whose marginal sinuses and lymphatic spaces in general abound with vitally stained cells of the pyrrhol type. These cells first spread into the interstitial muscular tissue and thence penetrate the sarcolemma, arranging themselves on the outer surface of the encapsuled parasite. Purely mechanical conditions are responsible for the typical arrangement of the pole cells at the extremities of the "trichina spindle."

(*c*) *Tuberculosis.*—I have established a fundamental difference in the distribution of avian and bovine bacilli of tuberculosis, when grafted into the peritoneal cavity of the mouse. Hitherto, in all cases of spontaneous tuberculosis in the mouse, the "avian" bacillus has been found. Koch had already drawn our attention to the chronic course of tuberculosis in the mouse. And yet, when the mouse is subjected to an injection with bovine or human tuberculosis, either through the blood-vessels or the peritoneal cavity, the disease runs a comparatively rapid course, in many cases assuming a form of bacillary septicæmia or miliary tuberculosis of the lung. In accordance with these facts I am able to show that after peritoneal injection of bovine material, besides rapidly caseating tuberculosis of the peritoneum, the chief

seat of trouble is the lung, whither the bacilli are carried by the blood stream after penetrating the portal vein and causing extensive tubercular thrombi throughout its larger branches. The liver and spleen contain merely microscopic lesions of inferior gravity, when compared with the large areas of tubercular necrosis in the lung. The pyrrhol cells of the peritoneum take no active part in this acute form of experimental tuberculosis.

An entirely different result follows the intraperitoneal injection of the avian bacillus. On macroscopic examination of the vitally stained animal, several weeks after inoculation of the virus, the peritoneum and intraperitoneal organs hardly show any trace of disease. All the more remarkable are the lesions revealed by the microscope. The omentum is full of blue patches, which to the naked eye wear the appearance of *tâches laiteuses*. By means of the specific stain for bacilli I was able to prove that these blue patches consisted entirely of pyrrhol cells, whose blue protoplasm was choked with myriads of bacilli. No trace of inflammation could be found in their immediate or more distant surroundings. Such aggregations of pyrrhol cells laden with bacilli were also discovered in the liver, spleen, mesenteric glands, and, in a smaller number, in the lung. In all these organs the tubercles had the vital stain and were thus easily distinguished by a low magnifying power. They lay in lymphatic spaces, in the liver surrounding the portal vessels, in the spleen arranged round the malpighian bodies. The blood-vessels were, with few exceptions in the liver, intact. No caseation occurred nor could small cell infiltration or giant cells be anywhere found in connection with these vitally stained tubercles of peritoneal origin.

A key to the whole process was afforded by the examination of animals at short intervals after the injection of the avian bacilli. The latter are quickly conveyed to the liver, where they are destroyed in great numbers by the Kupffer cells. Such as remain in the peritoneal cavity are phagocyted by the vitally stained pyrrhol cell. They multiply in those cells, which wander into the omentum, liver, spleen, and mesenteric glands, and eventually into the lung. As the bacilli increase, a most characteristic morphological change occurs in the cell. The granules of the protoplasm gradually disintegrate, the vital stain, which had originally been confined to the granule, now effects a diffuse stain of the whole cell. Eventually it can disappear entirely.

As this metamorphosis of the cell protoplasm proceeds, its biochemical reaction alters, inasmuch as in the place of a specific attraction for the vital stain, the protoplasm now shows an increased affinity for the fat stains. Fat first appears in the shape of tiny droplets. In the final stage, these droplets increase in size and eventually usurp the place of the cell body. And yet the cell continues to live, for even in this stage of excessive fat infiltration

the nucleus accepts the nuclear stain and shows no signs of degeneration. Naturally, in the end, the cell succumbs. But, after death, even fat disappears from its necrosed body, whose shape still remains visible as a ghost in the tubercles undergoing necrosis.

We have thus established a fundamental difference in the distribution of bovine and avian tuberculosis, when injected into the peritoneal cavity of the mouse. In the bovine variety, metastasis occurs along the blood stream through the thrombosed portal veins; in the avian variety the dissemination is effected by the lymphatics.

What prospects these experiments on the *intra-vitam* staining of tubercles hold out to chemo-therapy hardly need be mentioned, since we now know that the germ-bearing cell is selectively affected by the vital stain.

(*d*) Toxic degeneration of the liver was produced by various poisons, such as phosphorus, cumarin, cocaine, and, above all things, by a substance first prepared by Ehrlich, called icterogen. The latter is an arsenic compound of Ehrlich's 606 series. On injection of a centigramme of a 1/5000 solution the mouse develops severe jaundice, followed by miliary bland necrosis of liver cells. Similar to cumarin, icterogen induces thrombosis in the smaller interlobular portal vessels, which explains the consecutive necrosis of liver cells. I have included these experiments in this paper merely to show that, wherever non-inflammatory necrosis in the liver occurs, its organisation is attempted by vitally stained pyrrhol cells, which leave the peritoneal cavity, migrate along the liver lymphatics towards the seat of trouble, and eventually assist in the repair of the damage.

(*e*) *Malignant Growths.*—In no case, to my knowledge, does the aggregation of blue-stained pyrrhol cells assume such extraordinary dimensions as in the instance of malignant growths placed under the skin. They swarm around the growing tumour and penetrate it along the endless blood channels which furrow its lobules. In the interior of the growth most of these cells succumb. I am still engaged in an inquiry into the relations existing between the growing tumours and these cells. Not wishing to forsake the sure ground of established facts, I merely state that the appearance of these blue-stained cells on the field of tumour grafts may be regarded as a specific local reaction induced by the tumour cell. When exempt from inflammatory agents, the tumour attracts no other migratory cell, but only the blue-stained pyrrhol cell. I am inclined to believe that these cells are the bearers of nutritive material for the growth. My new chemo-therapeutic experiments on mouse tumours by means of agents which damage the liver, such as icterogen and "Jod-phenyl arsensaures Natrium," have brought to light another important fact in connection with pyrrhol cells. They absorb the degeneration

products of the liver cells, preferably the bile pigments, carrying them to the malignant growth. Hence the latter may wear both on its surface and in its interior a distinct yellow appearance, due to the aggregation of "jaundiced" pyrrhol cells. Since the tumour evidently suffers through the application of the above-mentioned substances, it does not seem improbable that the pyrrhol cell is also active in transporting substances to the tumour which impede, and in many cases stop, its growth.

Antelope and their Relation to Trypanosomiasis.

By Dr. H. L. Duke.

(Communicated by Sir J. R. Bradford, Sec. R.S. Received February 26,—Read March 28, 1912.)

[Plate 2.]

The flies on the Chagwe Lake shore are still capable of infecting monkeys with *Trypanosoma gambiense*. Four years and a-half have now elapsed since the Chagwe coast line was officially declared free of population, all villages destroyed within a zone of two miles bordering the lake, and their inhabitants removed inland. In spite of precautions, however, there is very little doubt that the islanders continued secretly to visit the mainland until they also were removed in 1909, and all the island villages destroyed. Since September, 1909, therefore, there have been no natives in the fly area except the recalcitrant few, who, at the risk of imprisonment, may from time to time return to their old haunts on the mainland or the islands. Instances of this have indeed occasionally occurred up to the time of writing. Apart from the captures made by the Government patrol, I have on several occasions had to report signs of recent native occupation within the prohibited area seen in the course of excursions from Mpumu.

In considering the question of the infectivity of the lake-shore flies, the possibility of infected natives being available as a food supply must still be considered, although this factor is apparently of small importance. These natives would most likely only venture forth at night time, and would be unlikely to approach the fly ground, which is visited almost daily by the boys from Kibanga. There remain the canoemen and fly-boys employed by the Laboratory, and in this connection it can only be said that, although