THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

I. General Features of Vital Staining with Litmus.

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The present paper and others to follow embody the results of attempts to determine the reaction prevailing within living mammalian tissues. The theme has long had attention from investigators of physiological processes; and within the past 20 years its importance has become increasingly manifest. Yet even now the study of it waits upon methods. For the work here to be reported indicators have been employed. The best of these is subject to error under body conditions; and the only justification for their use lies in the argument that inexact knowledge is better than none at all. This involves a point of view which not a few workers at the present day may be disposed to reject. Whatever the rights in the matter, the safest course in discussing the findings will be to lay stress rather upon observation than upon inference, to be content to record the relative reaction of individual tissues as compared with each other, and above all to avoid, wherever possible, the symbol pH as connoting an exactitude which could not obtain under the circumstances of the experiments.

Needless to say the conditions prevailing within living tissues are such as may well permit of the existence of marked differences of reaction in a narrow compass. The reactions of the intercellular fluid, of the lymph deriving therefrom, of the cell surfaces, of cell granules, of cytoplasm itself, and of the nucleus, have all to be separately reckoned with. Inside the confines of the cell there are, broadly speaking, three sorts of material, the reactions of which may, and almost certainly do, differ; namely, cytoplasm, nuclear material, and those substances which, as globules, granules, or more finely dis-

tributed matter, though in the cell are not of it. For the purposes of the present work litmus has been employed, a dyestuff which tends to be segregated in the granules of many cells, but which also, as the event has shown, becomes broadly distributed through certain tissues. In later papers the results of granular and diffuse staining with indicators of the phthalein series will be described.

Concerning the Previous Use of Indicators for Vital Staining.

Though the use of indicators to determine tissue reactions dates far back, it has found employment only sporadically, and for special ends. Some of the contributions possess noteworthy significance, as *e.g.* those dealing with the reaction of muscle under various conditions and the reaction within the secreting cells of certain organs; but they need not be reviewed here. Mention must be made, however, of certain recent observations which tend to show that the gastric acid is not formed within the cells of the mucosa,—as Claude Bernard deemed he had proved,—but only after the liberation from these cells of a precursor substance itself mildly alkaline;[1] for these observations have been taken to support the view that the reaction of the tissues is in general practically identical with that of the blood.[2]

Ever since Ehrlich's report on vital red in 1893 this indicator has been utilized to determine the reaction of cell granules. And largely in consequence of work with it there now exists the general impression that a faintly acid state is the rule about material which has been phagocyted by mammalian cells, as well as within the granules of macrophages.[3] The scope of observation has been limited owing to the circumstance that neutral red is suited to demonstrate only a very feeble acidity. Sodium alizarinate, which has also found frequent use, has a color range somewhat further to the alkaline side. In von Möllendorff's[3] comprehensive summary of the knowledge thus far acquired through vital staining one finds no mention of indicators with a notably acid range except litmus, and none of systematic attempts to determine the relative reaction of mammalian tissues during life.

For a full 50 years, since von Rustizky's abortive experiments of 1874,[4] tests for intracellular reactions have from time to time been made with litmus. In general, the lower organisms have been studied. Engelmann,[5] noting that litmus particles were turned red within paramecia, concluded that the protoplasm of

[1] Harvey, C. H., and Bensley, R. R., *Biol. Bull.*, 1912, xxiii, 225.

[2] Wilson, D. W., *Physiol. Rev.*, 1923, iii, 295.

[3] von Möllendorff, W., *Ergebn. Physiol.*, 1920, xviii, 141.

[4] von Rustizky, J., *Virchows Arch. path. Anat.*, 1874, lix, 202.

[5] Engelmann, Th. W., in Hermann L., Handbuch der Physiologie, 1879, i, pt. 1, 343.

these organisms is acid. Le Dantec,[6] Greenwood,[7] and others realized that the change in the indicator evidenced only the state of affairs within the digestive vacuole. Metchnikoff[8] used particulate litmus to study the reaction within the phagocytes of a wide variety of species, and developed a law according to which the degree of acidity about phagocyted material is less the higher up the organism in the animal kingdom. He had found that whereas protozoa and some of the cells of the lower metazoa are capable of altering ingested particles of blue litmus to red, the phagocytes of birds and mammals fail to do this though they change neutral red. He noted that when cells of the lower forms, containing red litmus, were injured by pressure upon the covers-slip, the indicator became blue again, and he attributed this to the alkalinity of the protoplasm; but le Dantec suggested that it is more probably due to the penetration of the surrounding fluid. Metchnikoff also noted red and blue particles of litmus within the same cell, and he believed this to indicate that certain parts of the cytoplasm only are capable of forming acid; whereas le Dantec held that the blue particles were merely the freshly ingested ones, as yet unaltered.

At the present day the view is generally accepted[9] that the acidity developing on occasion within the granules of mammalian cells is insufficient to turn blue litmus to red.

Recently Stieglitz[10] has employed neutral red, sodium alizarinate, and azolitmin, the principal constituent of litmus, to ascertain the reaction of living kidney tissue under various conditions. He injected the indicators intravenously, sacrificing the animal a few minutes later. The amounts he employed seem not to have been sufficient for a general tinting of the body,—at least no mention is made of any such occurrence,—but the urine was well colored and the renal tissue also. Stieglitz was thus enabled to confirm the classic observation that the kidney cortex is acid while elaborating an alkaline urine, and he concluded further that when an acid urine is being secreted the normal cortex is alkaline, whereas an injured one is acid. He does not state whether the vital staining was granular or diffuse.

The experiments here to be detailed, demonstrate that there exist throughout the mammalian body, cells in great number which are capable of developing a segregated acidity more than sufficient to turn blue litmus to red, in some instances far more. The fact has escaped recognition in the past principally because litmus has not

[6] le Dantec, F., *Ann Inst. Pasteur*, 1890, iv, 776.

[7] Greenwood, M., *J. Physiol.*, 1887, viii, 269.

[8] Metchnikoff, E., Immunity in infective diseases, Cambridge, 1905; translation from the French by F. G. Binnie.

[9] Opie, E. L., *Physiol. Rev.*, 1922, ii, 552.

[10] Stieglitz, E. J., *Arch. Int. Med.*, 1924, xxxiii, 483.

heretofore been utilized to stain the living mammal. The periods of observation during *in vitro* work with the indicator have not been prolonged enough to enable the cells to cope with the alkaline material ingested with it. Among the older observers, le Dantec and Loisel[11] alone seem to have realized the importance of purifying the indicator; and their work was carried out with invertebrate creatures. Metchnikoff employed crude litmus in particulate form, as already mentioned, and he looked for changes in the reaction about phagocyted objects, such as bacteria and red cells, at a time when any acid elaborated by the cell might well have gone into combination with the substance of these objects, practically as soon as formed.

Method.

Kahlbaum's cube litmus was purified by extraction with hot water, treatment with acetic acid, and precipitation with alcohol, according to a method given by Sutton.[12] It was then dried, powdered, and placed in ether for some days to sterilize it. The indicator, which is dark blue as thus prepared, contains very little alkali, and keeps well. For the purpose of localized tissue staining the litmus particles as such were added in a little of the ether to a 1½ per cent solution of purified agar in 0.9 per cent salt solution which had been cooled to 42°C. after boiling; and immediately that the ether had come away the agar was drawn up into a syringe, and, while it cooled, the particles of litmus were kept from settling by revolving the syringe slowly upon its long axis. The material was next chilled in the ice box to render it solid and injected through a large bore needle, either beneath the skin, where it lodged as a discrete "button," or into the peritoneal cavity. Passage through the needle fragmented the stiff blue jelly, of course. For vital staining of the organism as a whole the ether was poured off from the litmus as far as possible, the remainder evaporated by warming, and the powder was taken up in warmed 0.9 per cent salt solution. About 1 gm. of the indicator material was present in every 15 cc. of solution. Rats weighing from 70 to 110 gm. and a few adult mice were the animals injected. 0.6 to 0.8 cc. of the agar mass was sufficient for a localized subcutaneous staining in the case of rats; while for intraperitoneal staining 2.0 cc. was used. General coloration of the body was accomplished by the introduction into the peritoneal cavity of 1½ to 2½ cc. of the litmus solution on each of 3 or 4 successive days. When the animals were finally sacrificed cultures were taken on agar and in bouillon and smears were made as well. Infection was rare. Some specimens of litmus proved toxic even after careful purification, killing the animals within 24 hours. Others were well tolerated in the largest amount just mentioned.

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[11] Loisel, G., *J. anat. et physiol.*, 1898, xxxiv, 187.

[12] Sutton, F., Volumetric analysis, Philadelphia, 10th edition, 1911, 35.

In order to avoid so far as possible all supravital changes in tissue reaction the organs of many of the animals were examined while they were under ether. No differences were discernible from the findings immediately after death. Mica slides and covers were largely employed since the alkali from ordinary glass ones often quickly turns intracellular litmus from red to blue despite the protection afforded by the protoplasm. The change, which can be delayed by flooding the preparation with salt solution, is on occasion not without usefulness both to show that the intracellular indicator is still capable of reacting and to bring out its presence in slight amount, since the blue form of litmus is far more easily discernible in the tissues than is the red. The liver and kidneys were sectioned immediately on removal, with a Valentine knife. Thin fragments of the other tissues were clipped off with fine scissors, and flattened under the object-glass or teased out with needles.

Localized Subcutaneous Staining.

Within 24 hours after a litmus "button" has been introduced into the subcutaneous tissue of the shaved side a wide blue areola fading gradually at the edges and especially pronounced in the direction of the draining lymphatics can be noted through the translucent skin. Evidently the particulate litmus embedded in the agar is dissolving out. There may be a slight local edema at this time but by next day it has disappeared. The areola is now less extensive and has a violet cast. By the 4th day it has a spread of only 2 to 4 cm. about the agar but has acquired a brilliant rosy hue which it retains while fading during the weeks that succeed. The button in its midst is gradually encapsulated by a vividly pink tissue. The litmus particles distributed through the agar become gradually paler, as is disclosed at autopsy, and the tinted agar also, but even after 24 days sojourn in the animal—the longest time yet allowed to elapse—both are still blue, and the zone round about is still brilliant pink and 2 cm. or more wide.

The pink coloration of the subcutaneous tissues appears diffuse to the unaided eye but microscopically it proves due to an immense number of granules contained in tissue macrophages, and, as time passes, in what appear to be fibroblasts. The morphological picture closely resembles that encountered by Evans and Scott[13] in the vital

[13] Evans, H. McL., and Scott, K. J., *Carnegie Institution of Washington, Pub. No. 273, Contributions to Embryology*, 1921, x, 1.

staining of connective tissue with the acid azo dyes. Occasionally a variant from it is seen a week or more after the injection in the presence of scattered ovoid or pear-shaped cells with a small, eccentrically placed nucleus, which are so crowded with fine, lightly stained granules that the cytoplasm as a whole has the appearance of pink ground glass. No attempt has been made to ascertain the nature of these cells. They can scarcely be wandering macrophages for the stained granules of the latter are at this period deep pink or red, and coarse, some of them being as large as an erythrocyte. The ruddy hue of the capsule of reactive tissue enclosing the agar button is due to the presence in it of immense numbers of such macrophages swollen with their content of red granules. Within the agar, by contrast, no pink granules are met despite the presence of a host of invading and organizing cells.

Intraperitoneal Staining.

The changes occurring about litmus agar introduced into the peritoneal cavity differ from the foregoing in some respects.

After 24 hours the peritoneal lining is everywhere markedly blue as are the agar fragments which are now for the most part closely involved in omentum. But here and there in the injected material pink foci with a maximum diameter of about 1 mm. may be discerned. These are found to consist of crowded cell aggregates, the greater number of the cells containing coarse, highly refractile, pink granules, three to eight in each as a rule. Most such elements are polymorphonuclear in type though a few macrophages are present.

Within 5 days after the injection the peritoneal lining of uninfected animals has become an intense ruddy pink owing to the many colored granules in the macrophages of the subperitoneal tissues. There is practically no free fluid. The fragments of litmus agar are widely distributed throughout the abdomen, some of them fixed on the surface of the larger viscera but most enveloped in omentum. They now appear as small and scattered, pink, translucent masses, each containing one to several red-violet points. These latter are particles of litmus as yet undissolved and retaining the original blue hue, but so overlain and enclosed in a layer of cells with pink and red granules that the combined hue is violet. Further away in the agar the thronged cells lie separate. The grouping about each particle of the indicator much resembles the gathering of a swarm about a newly alighted queen bee. Most of the elements are still of polymorphonuclear type but there are numerous macrophages as well, and the relative proportion of them rapidly increases as time goes on.

The liver may show a granular pink staining of the Kupffer cells, some days after the injection, and the macrophages of the mesenteric lymph nodes may be pink with granules too. These evidences of a wide distribution of the indicator will be considered in the section on staining of the body as a whole.

From what has been said it will be noted that whereas in the subcutaneous tissue the cells ingesting litmus are of the ordinary kinds taking up the vital dyes in granular form, that is to say macrophages and fibroblasts, within the peritoneal cavity the elements concerned during the first days are predominantly polymorphonuclear in type; and whereas the subcutaneous agar is practically free from penetration by stained cells, that within the peritoneal cavity everywhere contains them. This latter difference is referable in part to the difference in the character of the cells invoked by the foreign body in the two situations. For when the subcutaneous agar happened to become infected, with result in an accumulation of polymorphonuclear elements followed by macrophages, the presence within it of pink-granulated cells was noted, just as under sterile conditions within the abdominal cavity.

Litmus Staining of the Body as a Whole.

That the agar or agar derivatives could not have been primarily responsible for the findings described became evident from observations on the animals given repeated intraperitoneal injections of a saturated solution of litmus in normal saline. This solution was deeply and intensely blue. A little of it frequently escaped along the needle track into the subcutaneous tissue of the abdominal wall; and here from day to day the same cycle of changes in color, from blue through violet to pinky red, was witnessed, with the same granular engorgement of the cells, as around litmus agar buttons. The rat turned blue within a few hours after each injection, the intensity of the hue and its duration depending on the amount of the indicator administered. The urine was for a day or more pinky red, promptly changing to blue on the addition of alkali.

Repeated injections of litmus on successive days resulted in a persisting coloration of the animals. The blue hue of the body surfaces was succeeded within 24 hours by a change to violet, and,

after 48 hours or more, to pinky violet, but never to clear pink. The pink-violet faded gradually, being discernible in some instances as long as 10 days after the injections. During the earlier portion of this period the urine contained a little pink litmus, as was apparent when it was caught on neutral white absorbent cotton and tested with alkali.

One would have supposed from the local findings that animals stained as a whole would be rendered an outspoken pink. Instead they had, as just mentioned, a definite violet cast. Yet when the skin was stripped back the staining of the subcutaneous tissue proved to be reddish pink just as around a litmus agar button, while the larger viscera were all likewise stained an undeniable pink. The violet cast of the intact body surfaces was found to result from the presence of a blue coloration in the epidermis overlying the pink-granulated connective tissue. Clippings of this epidermis proved startlingly blue throughout, changing to pink with acid. This specialized diffuse staining was encountered in the case of a number of the other tissues. In some of them cells containing granules of pink litmus stood out against an azure background. The blue could not be attributed to the presence of indicator in plasma and lymph, for blood serum taken at the time when the diffuse coloration of the tissues was at its height, appeared at most only negligibly tinted and often not so at all; while furthermore the diffuse coloration lasted for weeks and in certain situations for months. Needless to say, the lymph, in the case of so colloidal a dyestuff, can have contained only relatively little of it at any time.

The findings in the individual organs of animals examined when the vital coloration was most pronounced, that is to say about a week after the last injection, will now be set forth, in the order in which the organs were inspected during ether anesthesia.

Just prior to removal of the larger viscera the blood vessels were swiftly clamped off. Organs which could not well be inspected until after death were observed immediately upon it. Reflected light was found to serve far better than transmitted for the detection of diffuse staining, and the hue evident in the gross was often a great help in this connection. Tissues occurring at many points of the body will be dealt with under the head of first mention.

The peritoneal exudate: Peritoneal aspiration was performed from time to

time in many instances. Even as early as an hour and a quarter after the injection some cells showed a pink, granular staining. For the first day or two the peritoneal fluid was deep blue, containing sometimes few, sometimes many cells with pink granules. Later it was a paler blue with a definite pinkish shimmer, owing to the many suspended elements heavily granulated in red. After 6 or 7 days it often appeared a cloudy, deep red because of its content of such cells, and only when these had been separated out by centrifugation was it perceived to be clear and pale blue. Its amount, which was never more than a few drops, now diminished and after 9 to 10 days a trace only was obtainable, and most of the cells therein were free from the indicator. As a rule, no fluid was to be had later, although occasionally cells with colored granules could be recovered even after 15 days. Most of the formed elements present during the initial 24 to 48 hours were polymorphonuclears, as has already been stated, but soon their place was taken by macrophages. It was noteworthy that, irrespective of the kind of cell, the colored granules were practically all pink or red during the first few days, whereas blue granules were to be found later, together with not a few diffusely blue cells. The observation is a significant one, to be discussed in a succeeding paper.

The blood: None of the cells was stained. The serum varied from deep blue a few hours after the injection through pale blue to colorless a week or more after it.

Cartilage: Both to the unaided eye and microscopically the cartilage of the tail was diffusely and brilliantly sky-blue and so too with that of the knee joint, ensiform, ribs, and skull of the growing animal. The tracheal cartilages and those of the larynx were in contrast wholly uncolored. The cells themselves did not appear to be stained, but only the matrix, though pronouncement on the point is difficult.

Bones: Those of the tail, legs, ribs, sternum, and skull, the only ones examined, were even bluer than the cartilage associated with them. The regions where ossification was going on in growing animals were especially colored and those extending across the shafts of the long bones at either end were marked out as sharply blue lines. When the femur was split open longitudinally and its inner surface looked at, cells with the general appearance of osteoclasts, containing rosy granules, could be made out seated here and there on the blue, irregular lining. The dye appeared to be laid down in fibrillar strands in the case of calcifying cartilage and to be diffusely distributed in osseous tissue. The cells of this latter had in some instances a dubious pink shimmer but a close scrutiny of them was not made. The general staining was so intense that during life the bones of the leg and foot appeared deep purple through the tinted overlying tissues.

Marrow: The fatty tissue never showed staining but the red marrow contained few to many cells of macrophage type more or less crowded with red granules.

Owing to the differing hues and color intensities of bone, calcifying tissue, marrow, cartilage, and the periosteal connective tissue, which last held many

macrophages red with granules, there were exquisite color nuances to be observed wherever bony growth was in active progress, as where the cranial plates came together over the occiput of young animals, and at the costochondral junctions, and in the small joints of the tail.

The tendons of the tail, like those elsewhere, were uncolored but the tendon sheaths were diffusely and palely blue.

The lymph glands of the groins were in the gross intensely red on their flatter side, and so too with those of the neck, peritoneal cavity, and thorax. The hue was occasioned by litmus concentrated within macrophage granules. The lymphoid tissue appeared uncolored save for an occasional wandering macrophage or polymorphonuclear cell granulated in pink.

The skin epithelium: This was everywhere diffusely and brilliantly blue, even to its dippings-down about the hair follicles. Since the corium was pink, owing to its content of colored macrophages, the contrast was an arresting one.

The connective tissue appeared pink to pinky red wherever it was markedly cellular owing to the amount of indicator stored within granules. But where cells were scattered sparsely one could see by reflected light that all the tissue lying between was sky-blue. The tinting was far less intense than that of epidermis, cartilage, and bone.

The nerves, the fat, and the voluntary muscles were everywhere unstained, but many cells of the connective tissue between and round about were brilliantly granulated in pink. The same facts held true for the *thyroid* and the *salivary glands*.

The spleen contained few to many macrophages more or less swollen with red granules, and there were also present, in the days immediately following the injection, some polymorphonuclear cells as well, with colored granules. For the rest, the organ appeared unstained.

The peritoneal lining, both visceral and parietal, appeared deep red in the gross, owing to the great number of cells with pink granules in the connective tissue immediately beneath its surface. These cells were of the kinds encountered elsewhere.

Pancreas: The organ appeared pink to the unaided eye, but beneath the microscope the gland cells proper were seen to be unstained, though everywhere in the connective tissue scaffolding were immense macrophages packed with red granules. The intracellular accumulation of litmus was more pronounced here than almost anywhere else among the fixed elements.

Kidneys: The cortex was deep pink in the gross but the microscope disclosed throughout it a regular patterning of blue upon pink. The colors were due to a granular staining within the cells of certain tubules. This finding, of local differences in reaction within the elements of the renal secretory system, has an obvious importance for the understanding of kidney function, and will be the subject of a later communication. The medullary tissue appeared unstained.

As a rule the animals were killed when the examination had progressed thus far, by laying open the thorax and heart with large scissors. The exsanguination thus accomplished made inspection of the remaining organs easier.

The liver, now practically bloodless, was at once sectioned. It appeared pink owing to a close network of Kupffer cells with red granules, present everywhere between the unstained cords of parenchyma. The granules were always diffusely colored, even in the first few days when small; and they were non-refractile and clustered about the nucleus. Later, in cases in which much litmus had been given, some very large and deeply stained granules or globules were to be seen here and there amid smaller ones in the distended cells. Still later the number of colored granules in each cell had much lessened but those that remained were coarse and deeply stained. The same change was observed in the colored granules of the renal tissue as well.

The lungs showed no staining except of the peribronchial connective tissue which was diffusely blue, as the cartilage of the smaller bronchi also appeared to be. There were some scattered pink-granulated macrophages. None was to be found in blood expressed from the vessels.

The suprarenal glands showed in the medulla frequent macrophages containing litmus and occasional ones in the cortex. In addition the medullary tissue was frequently a diffuse blue.

The heart: The muscle was not stained, but here and there amid it were macrophages red with stored litmus, and these cells were so numerous in the endocardium as to give to the inner surface of the heart a notable red hue. The heart valves, though, were brilliantly and diffusely blue.

The aorta was a marked diffuse azure save for the adventitia which was rendered pink by its content of heavily granulated macrophages.

The thymus was unstained except for certain small angular elements having yellow-brown granules that were definitely pink with litmus on the surface.

The stomach and intestines: The peritoneal surface, both visceral and parietal, appeared pink everywhere owing to the many underlying macrophages which had stored litmus. The presence of similar cells was responsible for a slight pink cast here and there throughout the gut. The epithelium of the mucosa was unstained.

The sex glands were not carefully studied. They were usually pink in the gross because of litmus stored in the cells of the connective tissue. The elements lining the seminal tubules were not stained nor were the intact ova. Ruptured and organizing ovarian follicles often held aggregates of cells intensely colored with red granules.

The brain tissue proper was never stained; and of the *eye* only the connective tissue about the ball contained litmus, in granulated macrophages like those elsewhere.

There was present in some animals a thin layer of newly formed *granulation tissue* here and there on the peritoneal surface. This was practically free from the indicator, as would perhaps follow from the circumstance that many of the component cells had only just come into being.

Among the points brought out by the foregoing description there

are two especially deserving emphasis. One is the tendency of certain tissues to stain diffusely with litmus, the other the existence throughout the body of elements in immense number which contain notably acid material under the circumstances of the vital staining. The quantity of this material is so great that many of the tissues are rendered pink through alteration it in the litmus segregated in them. The cells effecting the segregation and color change are those ordinarily concerned in vital staining with the acid dyes by the process of storage (*Speicherung*).

Duration of the Staining.

How long the staining can be made to last is not yet certain. In rats receiving four intraperitoneal injections of the indicator in the amounts already mentioned the color of the hairless body surface returns to normal within about 10 days. But even after $4\frac{1}{2}$ months,—the longest period yet allowed to elapse,—the bones are still so blue with litmus as to attract attention at autopsy; and elsewhere within the organism there is not a little of it to be found. In an animal killed 136 days after the last injection and weighing 180 gm. instead of 103 gm. as at first, the leg bones appeared sky-blue in the gross owing to the presence of a thin, deep blue layer on the inner surface. The walls of the large blood vessels also were markedly blue. Nowhere else was diffuse staining now to be seen. But there were a few macrophages, pale pink with litmus-containing granules, in the lymph glands, the red marrow, and the thyroid, more of such elements in the spleen and the interstitial tissue of the pancreas, an irregular network of them with markedly red granules throughout the medulla of the suprarenal glands, and a great many that were similar in the subperitoneal tissue layer everywhere. Curiously enough, the Kupffer cells of the liver, always heavily granulated in red during the early stages of the staining, now showed no trace of color, although in the interlobular tissue, a few macrophages with pink granules could be discerned. The cortical tubules of the kidney appeared curiously dotted with scattered cells which stood forth amid the unstained majority by reason of the aggregates of coarse, heavily tinted granules within their cytoplasm. The granules of each cell

were all of one color, blue or red, but neighboring elements frequently differed in color. The oxalated blood showed not a trace of litmus.[14]

Almost identical findings were encountered in another rat killed after 112 days. The blood serum was free from the dye at autopsy and the animal was half again as large as at first, weighing now 163 gm. instead of 109 gm. The subcutaneous tissue over the abdomen, into which a portion of the injection material had originally escaped, was stained pink and the microscope disclosed many red-granulated macrophages lying in a diffusely blue tissue. The epithelium overlying the colored patch was also pale blue, as if by diffusion of the indicator, but everywhere else was unstained. In this case there was no indicator to be seen anywhere within the liver. Many of the splenic cells that held litmus were of the sort which engulf and break down erythrocytes; and in them the indicator was often localized to the surface of the yellow and brown débris from such elements.

In a rat examined 29 days after last injection, the Kupffer cells still showed many colored granules, but the connective tissue macrophages throughout the body were free from litmus save in situations where at first there had been a great deal. The scrotal connective tissue was a deep ruddy pink, owing, as investigation showed, to a secondary staining of the macrophages as result of the accumulation and death within the scrotal sac of litmus-containing cells from the original peritoneal exudate. The ensiform cartilage was now dubiously blue. The kidneys were unstained with the exception of a few macrophages in the capsule.

The staining was intense in most of the organs after the lapse of 15 days, but the cartilage proved already well-nigh colorless and so too with the heart valves. In the marrow of an animal examined 6 days after the last of four litmus injections there were crowded aggre-

[14] In a rat killed recently, 7 months after injection, the bones and aorta were still notably blue with litmus, and there were many macrophages containing it in the pancreas and medulla of the suprarenal with a scattering in some other situations. A few epithelial cells in the kidney cortex held coarse, dark blue granules, and in the interstitial tissue near them an occasional small macrophage could be made out which contained pink ones. The liver appeared to be litmus-free. The animal weighed 200 gm. when sacrificed as against 89 gm. at the time of injection.

gates of macrophages intensely red with stored indicator and immediately about them a diffuse blue zone, fading at the edges. The cell nuclei were not stained and elsewhere the marrow was uncolored. It seems likely that here, as certainly in the abdominal patch of the animal killed after 112 days and in the scrotal sac of the 29 day rat, intracellular litmus was being liberated into the surrounding tissue fluid. Not improbably it is by this process, followed by elimination through the kidneys, that animals eventually become decolorized. For the indicator seems markedly resistant to destruction within the body.

Does a Diffuse Pink Staining with Litmus Occur?

The cytoplasm of the individual cells storing litmus in red granules usually appears colorless. But as von Möllendorff has remarked, dyes which are stored have after all to enter the cell. And it has been interesting to note, during the days when the indicator is being laid down in tissue macrophages, occasional, extremely minute, translucent, pale blue points or flecks in the cell substance between the relatively immense ruddy globules. They may, of course, be partially dissolved litmus particles so small as themselves to have escaped attention. The nucleus of an occasional macrophage has a bluish shimmer; but this has never been observed except under circumstances when the cells might have been injured.

The question arises whether there may not be a diffuse pink staining within certain tissues comparable to the blue of others but so masked by the pinkish sheen of intracellular granules as readily to be overlooked. *A priori*, the possibility might seem unlikely in view of the existence of a remarkable mechanism whereby alkalinity is assured to the blood; yet one cannot be certain of the state of affairs in the intercellular fluid of actively functioning organs. The liver of animals recently injected with litmus has, on fresh section, a notable pinkish hue. An attempt was made to determine whether this is referable merely to granules within the Kupffer cells or, in part at least, to unsegregated pink litmus.

Several rats given a single intraperitoneal injection of litmus solution were etherized 2 to 6 hours later and placed in a bath of washed paraffin oil which

did not affect the indicator. The blood serum was notably blue. Portions of several of the organs were one by one snared off, and immediately sectioned under oil and examined. The extraneous color of the renal cortex, which like the urine had an old rose tinting, proved due in great part if not entirely to litmus present in association with the intracellular granules of the tubular epithelium. The gland cells of the pancreas had not stained and the supporting tissue was diffusely blue. The spleen section appeared greenish yellow in the gross owing to the mingled hues of the erythrocytes and the blue serum. The section of the liver parenchyma on the other hand appeared rose-pink to the unaided eye and so too under the microscope, while the interlobular connective tissue was frankly blue as was the blood serum. No red granules could anywhere be discerned in the Kupffer cells. The few of these cells having a definite litmus content were rendered pale blue through the presence within the cytoplasm of minute solid particles of the indicator. When weak alkali (N/20 NaOH) was run under the mica cover-slip the pink of the liver section changed to a sharp blue, prior to any evident dissolving effect of the reagent on the particulate litmus just mentioned.

Under the circumstances of these experiments it was impossible to rule out a beginning granular segregation of the litmus as responsible for the findings. Within so short a period as $1\frac{1}{4}$ hours after an injection of the indicator some of the cells of the blue peritoneal fluid show, as already mentioned, a faintly pink granular tinting. No such segregation could be perceived in the liver, though, and the general impression derived from the observations was that there existed a non-granular distribution of pink litmus through the hepatic tissue, if only in the intercellular fluid. Tests with the phthalein indicators, to be described in a succeeding paper, have shown that this impression was probably correct.

The Litmus Storage within Granules.

Litmus is stored in the acid form within granules of several distinct sorts.

The cells showing the dye first after an intraperitoneal injection are, as already stated, the polymorphonuclear elements of the exudate fluid. After 24 hours these are brilliantly pink with indicator localized at the surface of several coarse, highly refractile, yellowish, irregularly rounded granules, such as, in the unstained state, may be seen in many other cells of like kind. The granules are in general composed of lipoid material, turning deep blue with Nile blue sulfate as a rule, or, rarely, red. The litmus never penetrates them though often highly concentrated round about; for they may be forced from the cell by pressure

and shattered into angular fragments, which latter are then seen to contain no stain. Their importance in connection with the storage of the indicator appears to lie in the circumstance that they serve as centers about which it accumulates together with acid material elaborated by the cell. After the passage of several days so much litmus and its acid menstruum have accumulated that each refractile granule appears to lie within a rosy globule; and a little later these globules come together as a single, immense, red one having within it the several refractile bodies that served originally as separate centers, now clustered together in an irregular rosette. This is the usual finding 5 or 6 days after the last of four litmus injections. Many of the cells then containing the indicator present a seal-ring appearance, so large is the red globule within. The further changes have not been followed.

Macrophages which come early into the exudate may likewise carry highly refractile granules, and develop a similar large globule containing the indicator; but there are always also present, scattered through the cytoplasm, the oft pictured granules[13] in which the acid vital stains as a group come to be stored. These granules are fairly numerous, relatively small, though sometimes reaching the diameter of an erythrocyte, and non-refractile. The tissue macrophages never show the highly refractile granules with litmus round about which are so prominent at first within the wandering elements of exudate. As the ordinary storage granules become large and deep colored they may become massed close together but without fusion, so far as I have been able to note.

There has been much discussion among students of vital staining as to the precise situation of stored dyes, whether upon the surface of cell granules or within. Litmus is always limited to the surface of the highly refractile granules above discussed, and in the early stages of staining it is seen to be concentrated near the surface of the ordinary storage granules of macrophages. Yet quite as certainly it becomes evenly distributed throughout these latter later on. For when such granules are pressed out of the cells they float about as discrete, non-refractile bodies of an even hue, pink at first, then blue as the surrounding body fluid acts upon them. The contents of the large globules that develop about lipoid granules can also be forced out and the material of which they are composed is likewise found to be evenly colored with litmus. When thus freed this material does not dissolve, at least at room temperature, but spreads so readily on pressure as to suggest that it is semifluid, an impression strengthened by a knowledge of the fusion whereby the globule came to be formed. The ordinary storage granules of the macrophages appear by contrast like a stiff gel; and they are, moreover, relatively insensitive to changes in the reaction of the surrounding medium. When a drop of exudate containing intact macrophages in which there is litmus is placed between a glass slide and cover yielding alkali the red "fusion" globules very soon turn blue, long before any of the ordinary storage granules of the same cell are affected; and when mica is used and bodies of both these sorts are forced out and into contact with the exudate fluid, the same difference in the rate of the color change may be observed.

The morphology of the colored granules within the Kupffer cells of the liver and the elements of other organs will not be taken up at this time. The phenomena occurring within the cells of the peritoneal exudate have been dwelt upon at length because they yield evidence of two sorts of acid segregation, frequently to be seen side by side within a single cell, one involving the presence of acid in the so called storage granules, the other an accumulation of acid material together with litmus about lipoid cell inclusions that are obviously pathological in derivation.

DISCUSSION.

Litmus is not lipoid-soluble and it is highly colloidal,[3] for which reasons it might be expected not to enter cells readily. On the basis of the general experience with dyes of the sort, one would predict that vital staining with it would come about through storage within cell granules. Such a process is responsible for the color of many of the tissues, as has been shown. Yet there can be no doubt that in some of them, notably bone, cartilage, epidermis, and the connective tissue, including that of heart valves and tendon sheaths and blood vessels, there occurs a diffuse blue coloration in contrast with the ruddy pink of the stored indicator, and one that persists long after litmus has disappeared from the blood. True, only the matrix or intracellular substance of most of the tissues mentioned appears to be colored, the cells themselves remaining unaffected, or at most containing the indicator in red granules. But there is a notable exception, the epithelium of the skin, which appears evenly and markedly stained throughout. It is possible, of course, that in its case the dye collects on the cell faces. The staining of the bones persists for months, reminding one by its intensity and narrow localization of staining with madder. Whether litmus can be utilized like this latter for the study of bone physiology and pathology remains to be seen. It is far more slowly eliminated than the essential component of madder, and the initial staining is more widespread through the organs. However, findings in animals sacrificed long after injection would appear to indicate that the secondary shifting of the indicator from one tissue to another is of insufficient magnitude to result in staining save under exceptional

circumstances, such as, for example, obtain about gross accumulations of dead, litmus-containing cells. The granules in which litmus is laid down are for the most part of the kind in which acid dyes as a class are stored; that is to say, especially those of the cells of the reticulo-endothelial system. But the polymorphonuclear leucocytes assembling in response to the injection of the indicator also take it up in quantity.

Much uncertainty exists as to whether the granules in which dyes become stored are preformed structures. In the connective tissue macrophages of mice rapidly stained *in vivo* with brom cresol purple I have been able to see intracellular granules, corresponding in morphology to those in which litmus is laid down, as colorless vacuoles against a purple background. Within little more than an hour after an intraperitoneal litmus injection one can recover from the peritoneal fluid polymorphonuclear cells containing good-sized nonrefractile granules or vacuoles that are colored a faint pink. It is difficult to suppose of these bodies that they have suddenly started into being in response to the presence of the stain, which they as yet contain only in traces. There can be no doubt on the other hand that the large, intracellular globules and granules holding litmus develop slowly as the stain is stored. The substance of the macrophage granules wherein it is laid down differs in notable respects from that of the litmus-containing globules developing in the same cells about particulate matter as, for example, about lipoid inclusions. Not impossibly these globules correspond in primary significance to the digestive vacuoles of the protozoa.

Vital staining with litmus discloses the fact that myriads of the body cells, both those fixed in tissues and others appearing in exudates, are able to develop on occasion a not inconsiderable granular acidity. In a paper immediately following this experiments will be detailed which prove that the degree of acidity is notable and that the accumulation of it is not to be thought of as constituting a unique response to the injection of litmus.

SUMMARY.

The present paper is the first of a series of reports on the relative reaction of living tissues as determined by vital staining with in-

dicators. It is possible to bring about a localized and a general coloration of living rats and mice with litmus. The animals remain in good health and the coloration of some of the tissues persists for months. Much of the dye is stored in cell granules, especially in those of the reticulo-endothelial elements, but a diffuse staining of certain tissues occurs, notably of bone, epidermis, cartilage, and connective tissue everywhere. In the intensity and localization of the bony coloration litmus has resemblances to madder. Diffuse staining with it renders blue most, if not all, of the tissues affected, while a granular staining causes others to become notably pink, owing to the fact that the indicator, though introduced into the organism in the blue form and circulating as such in the body fluids, is ordinarily red when stored in cells. The polymorphonuclear elements and macrophages of a peritoneal exudate, may become so laden with material colored red by litmus that the blue color of the fluid constituent is masked and the exudate appears a deep, turbid red. The phenomenon is but one manifestation of a notable acidity within cell granules throughout the organism. Like many another in the stained animals it would appear to be of physiological import. Some of the questions suggested by the work will be dealt with in the paper immediately following.