THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

II. On the Mobilization of Acid Material within Cells, and the Reaction as Influenced by the Cell State.

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The general features of vital staining with litmus have been recorded in a preceding paper.[1] This present one is concerned with certain phenomena of special import.

Influence of Cell Injury and Death on the Reaction.

The litmus-containing cells of a peritoneal exudate withdrawn a day or so after injection of the indicator almost without exception show it in the red state coloring the granules of the cytoplasm. But there are exceptions. If the solution introduced contained litmus in particulate form a very occasional cell may be observed containing an opaque, deep blue particle, unaffected apparently by the cell activities. More significant is the occurrence of elements with blue granules or a diffusely blue cytoplasm. Counts show that there may be one or two such cells to every hundred with pink granules in the 24 hour exudate; or they may appear only later. Usually the blue is diffuse, both nucleus and cytoplasm having much the same hue, and no granular differentiation is to be seen. The nucleus of such cells stains at once and deeply with 1 per cent trypan blue in isotonic salt solution, whereas that of the cells with pink granules remains unaffected. The inference that the blue cells are dead is warranted not only by this fact[2] but by what is known of the altered intracellular distribution of vital

[1] Rous, P., *J. Exp. Med.*, 1924, xli, 379.

[2] Evans, H. M., and Winternitz, M. C., unpublished work, cited by Evans, H. M., and Schulemann, W., *Science*, 1914, xxxix, 443.

stains in general when cell death has taken place. Metchnikoff[3] long ago noted, in the case of some of the lower organisms, that intracellular granules rendered pink by litmus became blue on slight injury to the cell, as when pressure was exerted upon the cover-glass. A like change takes place, I have found, when rat leucocytes containing litmus are injured in this way or are kept in the exudate fluid at ice box temperature for a day or two. The first alteration to be observed under either of these circumstances is a bluing of the granules, after which the stain gradually escapes, coloring the cytoplasm but soon fading as the indicator diffuses out. The nucleus becomes blue slightly later than the cytoplasm, appearing then as in a line drawing.

It has been enlightening to discover these evidences of cell death here and there within rats injected some time previously with litmus. Blue cells are rare in the early peritoneal exudate, as just stated, but with the passage of days the proportion of them rises so that they soon come to preponderate. Thus, in the case of an animal yielding only cells with pink granules 24 hours after a litmus injection, there were found in the trace of exudate recovered on the 10th day seventeen cells diffusely blue or with blue granules to two with pink granulation. Numerous other instances of the sort could be cited. There is a tendency for the exudate cells to lodge on the peritoneal surfaces and especially to gravitate toward the pelvic region. In the female they accumulate in the cul-de-sacs next the bladder whereas in the male they pass on into the scrotum. The cell masses found in either of these situations a week or more after the injections may be several millimeters in diameter; and they are a diffuse deep blue everywhere save at the periphery, owing to a general cell death. At the surface of the mass, though, where conditions are compatible with the survival of some of the elements, a part of the litmus is still confined in pink intracellular granules. The precise state of affairs becomes clearly evident on inspection of small cell aggregates upon the omentum, which have undergone a central death for lack of a circulation. In consequence of diffusion of the indicator from the dead elements there has occurred a secondary granular staining of the macrophages round about. By such a process the scrotal connective tissue fre-

[3] Metchnikoff, E., *Ann. Inst. Pasteur*, 1889, iii, 28.

quently comes to be the most deeply stained portion of the body and that holding its color longest.

The 24 hour exudate of a few mice which survived the intraperitoneal injection of a litmus solution for some reason highly injurious, as shown by the death of rats injected at the same time, yielded about as many blue cells as cells with pink granules. A number of tests in mice have demonstrated that when harmless material is used the latter alone are to be found so soon after the injection. When the animals receiving the toxic material were sacrificed,—about 3 weeks after the injection,—further evidence of injury was forthcoming in the shape of focal aggregates of macrophages red with litmus scattered amid the liver tissue.

Cells dying after they have come to contain litmus are not restricted to the exudate elicited by its presence but may be encountered throughout the body. When the indicator has been given by intraperitoneal injection they are relatively frequent in the liver capillaries, in the sinuses of the spleen, and in the draining lymph nodes, more especially throughout the medullary substance of these last where they stand forth in blue against a red background of macrophages.

The Intracellular Changes in Reaction Preliminary to Death.

There is a period preceding death of the exudate cells when many of them contain granules of several colors, as a result of changes from the initial red or pink hue. Thus in the case of the animal above referred to that yielded seventeen blue cells to two with pink granules in a 10th day exudate there were twelve elements besides which held both pink granules and blue or violet ones of similar morphology. In another instance the trace of exudate procured on the 9th day was observed to contain six cells with pink granules, to one that was diffusely blue and eight that had both pink and blue or violet granules. All of the cells were macrophages. The cytoplasm and nucleus of those with a mixed granulation were not colored, and nuclear staining did not take place when they were submitted to a test solution of trypan blue, indications both that the cells still lived. The question is worth asking whether the decline and death of litmus-containing elements of the fixed tissues may not be signalized in some such way as that of exudate cells; namely,

by changes in the color and distribution of the indicator. By such changes if they occurred one might conceivably obtain information on rates of cell mortality and replacement. And they do occur. Kupffer cells containing blue granules amid the red, often large ones but of relatively pale hue, are not infrequent a week or more after an intraperitoneal litmus injection, whereas earlier they are rare. In an animal killed 29 days after the injection they were fairly numerous. But it is sometimes difficult to say whether the material distinguished by the blue hue may not be recently ingested débris from dead phagocytes previously stained. No such uncertainty exists with regard to the findings in rats killed between 4 and 7 months after injection. In them macrophages containing both pink and blue granules were frequent through all of those tissues in which a storage of litmus had occurred, except in the kidney where the granules of the individual cell were all of one color, here blue, there pink, in accordance, presumably, with the local physiological conditions. The blue coloration of the granules of the tissue macrophages cannot have been the result of the trauma involved by the microscopic observations. For none of this color but only pink and red ones were to be observed in preparations from recently injected animals treated in the same way and with the same litmus specimen. Furthermore, as in the Kupffer cells studied at an earlier period, the blue coloration was far paler than that observed when the red or pink granules of the same cell were designedly turned blue by trauma. One may suppose that part of the original litmus content of the blue granules had diffused out or been disposed of previous to the observations.

In animals killed early a diffuse blue coloration of individual Kupffer cells has now and then been noted, as also of connective tissue elements, but only under circumstances admitting of injury to the tissue at the time of inspection. When rats have been intensely stained with litmus for a week or more curious bodies may sometimes be observed within the lymph nodes and liver, "blanks" one might call them in the sense in which the term is used of round pieces of metal lacking the die stamp that makes a coin. They are rounded or ovoid, apparently homogeneous like hyaline, stained intensely pink or red, non-refractile, and often as large as neutrophil leucocytes. Some are undoubtedly extracellular.

The Intracellular as versus the Extracellular Reaction.

Plasma, lymph, peritoneal fluid, and edema fluid are, as one would expect, always rendered blue by litmus when tinted at all. The mass reaction of connective tissue which has become deep red with stored indicator still lies on the alkaline side of the range of the latter, as may be convincingly proven by trauma. Wherever a hypodermic needle is thrust in the colored tissue, it leaves a sharply demarcated blue track in the midst of the red. When such a test is made during life the influence of the circulating fluids upon litmus liberated from the torn cells cannot be ruled out. It is better to exsanguinate the animal under ether, by cutting across the vena cava just above the diaphragm, and immediately to excise a large piece of skin with connective tissue attached, which is folded with this latter inside and forcibly compressed here and there with forceps. Wherever the pressure is exerted a blue mark is left in the subcutaneous tissue amid the red, accurately duplicating the forceps imprint upon the protecting epidermis. There is present, of course, in preparations made as described some interstitial lymph, but the amount cannot be great in the compressed regions. Additional evidence that the connective tissue as a whole has a reaction on the alkaline side of litmus is to be found in the diffuse blue tinting of the intercellular portion of it.

The Predominant Reaction Sometimes Acid.

The formed elements in the peritoneal exudate are frequently so distended with pink granules, and the cytoplasm enclosing these latter is relatively so inconspicuous in bulk that it is difficult for one to suppose that the prevailing reaction in a cell mash could be other than acid. Oftentimes the merest thin rim of cytoplasm exists about an immense red globule. Some simple tests were made to determine the reaction predominating when material of this sort is forcibly broken up.

A rat of 103 gm. received on successive days 1.25 and 2 cc. respectively of litmus in salt solution, and, after an interval of 2 days, another injection of 2.5 cc. 3 days later several drops of the peritoneal exudate were aspirated into a well cleansed glass pipette. The subsequent steps were carried out rapidly. The pipette was sealed in the flame and briefly centrifugated, separating the turbid, deep red exudate into a heavy sediment of the same color and a clear, pale blue, supernatant fluid which was drawn off as completely as possible and discarded.

A little of the sediment,—which consisted almost entirely of macrophages distended with red granules and globules,—was placed between a glass slide and cover which on prior test had proved almost inert to litmus, causing it to become blue only very slowly; and crushing pressure was applied. The liberated red granules now observed to float away in the alkaline exudate fluid became blue practically at once, but those still held amid the flattened and broken tissue remained pink. For the purposes of a second test a slide and cover of mica were used that did not affect litmus at all, and the cells of the sediment were briefly rinsed in isotonic salt solution and again collected by centrifugation prior to the crushing. All of the granules liberated thereby, the free as well as those still intermixed with the crushed cytoplasm, remained pink for a half hour after, when the observations were terminated.

The possibility had to be thought of that carbon dioxide accumulated from the protoplasmic activities of cells massed by sedimentation might have had a hand in these results. To test the matter a drop of the turbid, red, peritoneal exudate from a rat injected like the last one was transferred, as soon as withdrawn, to a tested mica slide, the fluid taken off from the scattered cells with a capillary pipette, the merest trace of 0.9 per cent sodium chloride solution, which did not change litmus, was run on, and the cells were forthwith crushed by pressure upon the mica cover. Everywhere the granules, whether free or lying amid cytoplasm, retained the original pink hue. As time passed the nuclear material of the crushed cells gradually took on a bluish tint whereas the fragments of cytoplasm remained unstained. Since the surrounding fluid had held no litmus one must suppose that the indicator responsible for the secondary staining was derived from the cell granules by diffusion.

Another test of this sort, carried out with a glass slide and cover gave similar but less clear-cut results, not a few of the granules becoming blue.

Findings such as these yield no information as to the reaction of the protoplasm of the cells involved, owing to the fact that protoplasm is not water-soluble. One must suppose that the cell substance fragmented by crushing, and the nuclear material as well, remained discrete entities amid the tissue juice. But the tests do show that this tissue juice did not contain sufficient alkali to overcome the accumulated acid of the cell granules, turning the red litmus to blue. The bluing which is regularly noted on the death of such litmus-containing cells when surrounded by lymph or exudate may with good reason be laid to the penetration of such fluid in quantity.

Significance of the Litmus Coloration.

Authorities unite in the statement that litmus is a notably fallacious indicator, one subject to large salt and protein errors. Ac-

cording to Prideaux[4] and Kolthoff[5] its turning point when in watery solution is at about pH 6.5; but in the presence of salts more hydrogen ions are required.[5] The purified material I have employed is red at pH 6.24 in Sörensen's double phosphate buffer mixtures,[6] violet at pH 6.47, becoming a clear blue only as pH 8.04 is approximated, though the hues at pH down to 6.98 might be recorded as "blue" in the absence of the contrast afforded by a color series. The blue at pH 8.43 does not differ from that at pH 8.04 but both are so pronounced in color, as compared with the mixed though still "blue" tints of slightly less alkaline solutions, as to give the erroneous impression that the indicator must be present in larger quantity. The point is an important one for the interpretation of color differences within the tissues. When the litmus is in very dilute solution in the buffer fluids the blue element in the tinting manifests itself further toward the acid side. The indicator is then pink at pH 5.91, violet at pH 6.24, and "blue" before pH 6.98 is reached. Dr. D. R. Drury kindly made up the buffer solutions and controlled them electrometrically.

Needless to say, the figures just given do not enable one to determine the precise pH of tissues colored with litmus. But they have a by and large significance for the interpretation of the findings. Those tissues which appear blue may have a reaction approximating that of the blood or one somewhat more, or even slightly less, alkaline; and those wherein the litmus is red or pink are certainly possessed of a considerable acidity, segregated though it is into granules save perhaps in at least one dubious case, that of the liver.[1]

The Degree of Acidity Developed within the Cell Granules.

It has long been supposed that the granules of mammalian cells in which vital stains tend to be laid down are at the most feebly acid in character, insufficiently so to alter blue litmus.[7,8] The present find-

[4] Prideaux, E. B. R., The theory and use of indicators, London, 1917.

[5] Kolthoff, I. M., Der Gebrauch von Farbenindicatoren, Berlin, 1st edition, 1923.

[6] Clark, W. M., The determination of hydrogen ions, Baltimore, 1920.

[7] Metchnikoff, E., Immunity in infective diseases, Cambridge, 1905, translation from the French by F. G. Binnie. Zinsser, H., Infection and resistance, New York, 1st edition, 1917.

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

ings, while unexpected, demonstrate, not only that some cells are able to turn blue litmus to red and store it for long periods in this form, but that there are hosts of elements throughout the body which possess the ability. In order to determine whether the acid reaction is referable to some foreign ingredient in the litmus, as well as to procure further evidence on the strength of the intracellular acid, I have utilized certain indicators of the phthalein series, brom thymol blue, brom phenol blue, brom cresol green, and thymol blue. Mice were chosen for the work instead of rats, to conserve material.

Alkali salts of the indicators just mentioned were made by adding to 0.1 gm. of each the amount of N/20 sodium hydroxide required for the purpose according to Clark. Since the color regularly showed that the reaction still lay on the acid side of the range of the indicator a little more of the NaOH was stirred in, just sufficient to change the color to the alkaline side, and the material was brought to a bulk of 5 or 10 cc. with 0.9 per cent salt solution. The intraperitoneal injection of 1 to 2 cc. of such solutions was well borne by mice of 30 gm., save in the case of brom thymol blue which rendered them ill and gave rise to marked accumulations of fluid in the peritoneal cavity. The phenol indicators, being highly diffusible, do not lend themselves well to storage within cells, though they color the animal strongly; and injections on several successive days were required to effect it. Even then it was of such short duration that aspiration had to be performed at frequent intervals if one was to obtain an exudate free from the indicator in dissolved form yet containing it within cell granules. Some specimen protocols will be given.

Brom Thymol Blue.—Yellow at pH 6.0, blue at pH 7.6. A 32 gm. mouse received on successive days 0.5, 0.5, 1, and 2 cc. respectively of the blue indicator solution. Following the last injection the animal as a whole appeared yellow-green for a few hours but by next day it had again the normal hue. In the interval it had voided yellow-stained feces and urine, both of which turned deep blue when NaOH was added. The peritoneal fluid now withdrawn was very pale yellow, becoming faintly blue on the addition of alkali. Many of the macrophages in the exudate held brilliant yellow, non-refractile granules of the usual morphology, which turned through green to an intense blue when weak NaOH was cautiously run under the cover-slip. The cytoplasm of the cells containing the stored indicator then became blue secondarily whereas that of elements lacking it remained colorless.

Incidental observations had disclosed the fact that brom thymol blue, as already mentioned, causes injury. For this reason its use was discontinued before any peritoneal exudate had been procured wherein the indicator was strictly confined to the cell granules. Methyl red, which has a slightly more acid range, was found to be so rapidly changed by the living organism as to be unsuited

to the purpose in hand; so recourse was had to the recently described brom cresol green.[9] According to Cohen[9] this is yellow at pH 4.0 and blue-green at pH 5.6 to 6.0. In some of Clark's double phosphate buffer mixtures prepared by Dr. Drury and checked by him electrometrically I find the indicator to be yellow at pH 4.0, and greenish blue at pH 5.2. Mice tolerate well the intraperitoneal injection of considerable quantities of the sodium salt, made as above described, in a 2 per cent concentration.

Brom Cresol Green.—A mouse of 30 gm. received an intraperitoneal injection of 0.4 cc. of the 2 per cent indicator solution, on 2 successive days. After each injection the whole animal became blue within a few hours, it voided much green urine, and the feces were colored green as well. Within 24 hours it had regained the normal aspect. On the day after the second injection a little fluid, colorless itself but with the faintest yellow turbidity, was obtained by aspiration from the peritoneal cavity. Under the microscope many macrophages were to be seen in it, mostly colorless but frequently containing several typical, coarse to fine, non-refractile, translucent, granules stained lemon-yellow. There were also rare pale blue ones. When N/20 NaOH was run under the cover-slip the yellow granules turned, first pale green, then blue, so that the field of observation appeared brightly studded with this color. The surrounding fluid did not become tinted, nor did the cytoplasm of the macrophages during the brief period of observation. The peritoneal fluid obtained next day still held an abundance of the cells but none now showed color.

This experiment was often repeated. The change in hue of the granules could be brought about by a pressure upon the cover-slip which forced them out and into contact with the surrounding fluid.

In instances in which the indicator was present in the exudate fluid, rendering this latter bluish, hosts of macrophages containing granules of a clear yellow were encountered together with a few that had blue granules, and yet others, evidently dead, with a diffusely blue cytoplasm and nucleus. When such an exudate was subjected to alkali the yellow granules were converted into intensely blue ones.

Brom Phenol Blue.—Yellow at pH 3.0, blue at 4.0. A mouse of 27 gm. receiving 1 cc. of the indicator solution on each of 3 successive days was submitted to abdominal aspiration 24 hours after the last injection. It had become intensely blue for a few hours following each, and had put out dark blue feces and urine, but now it appeared normal. The slight amount of peritoneal fluid procured was colorless though faintly turbid with cells. A little was placed between a mica slide and cover and searched for colored granules. Not a few of the macrophages showed typical ones, lemon-yellow in hue, whereas only a single blue one could be found. N/20 NaOH was slowly run under the cover. Almost at once a considerable number of blue granules sprang into view, by conversion of those previously yellow. The surrounding fluid did not itself become blue.

[9] Cohen, B., *Pub. Health Rep., U. S. P. H.*, 1923, xxxviii, 199.

Efforts to obtain a granular staining with propyl red and with thymol blue were unavailing. These indicators are but sparingly soluble in salt solution.

The ranges of the phenol indicators employed were all further to the acid side than that of litmus. The outcome of the experiments supports the view that the acidity of granules storing litmus is independent of substances derived from the indicator itself or introduced with it. Furthermore the acidity within the cell granules would seem to be considerably greater than is demonstrable by alterations in the color of litmus.

DISCUSSION.

One of the earliest discoveries made with vital dyes was that of the changes in distribution of the staining which occurs on cell death. Cytoplasms and nuclei which before were untinted now become diffusely colored and granules often lose their stain. That the alterations in permeability of which this phenomenon is the expression may be of importance in disease states is evident. In a previous paper from this laboratory[10] the fact has been brought out that living phagocytes protect ingested microorganisms and red cells against homologous antisera but that when the cells are killed, the sera rapidly penetrate them and act upon the ingested objects. The healthy cell "protects" the acid reaction of intracellular granules, as the present work shows, and injury is promptly followed by a change to alkalinity within the granules. Metchnikoff[7] noted this phenomenon in mammalian cells stained with neutral red and attributed it to the influence of alkali contained in the protoplasm. Le Dantec[11] who followed it in infusoria that had taken up litmus was of the opinion that it may be due to a penetration of the cytoplasm by constituents of the alkaline body fluid round about. Both our present and past work point to this as the true explanation. Whether the color changes in the individual litmus-stained granules of sick cells are a consequence of local alterations in permeability, or of pathological activities within the cytoplasm, remains to be seen. In this connection the local exosmosis of chlorides which takes place after injury to the enormous cells of the plant, *Nitella*, has a suggestive interest.

[10] Rous, P., and Jones, F. S., *J. Exp. Med.*, 1916, xxiii, 601.

[11] Le Dantec, F., *Ann. Inst. Pasteur*, 1890, iv, 776.

By litmus staining one is enabled not merely to recognize when cells are dead,—for with many stains that can be done,—but readily to discern moribund elements of certain kinds. In this way one can trace out the fate of the wandering phagocytes. The polymorphonuclear ones which come into the peritoneal exudate that accumulates after an intraabdominal litmus injection are relatively short-lived. The vast majority of those ingesting litmus never leave the peritoneal cavity but tend to accumulate at its lowest point, in the pelvic cul-de-sacs of the female, and within the scrotum of the male,—which in the rat is essentially part of the peritoneal domain. After a week nearly all are dead, as the change in the staining shows. It seems unlikely that this is the result of toxicity of the litmus since tissue macrophages containing the indicator survive for months.[1] A few polymorphonuclear cells and free macrophages coming presumably from the peritoneal exudate are to be found after a week or more, dying or dead, in the sinuses of the liver, and in the spleen and bone marrow. 4 to 7 months after the injection many of the tissue macrophages which had originally taken up the indicator show a mixed coloration of the cell granules such as, in wandering cells, precedes death. And at this time macrophages recently dead, as shown by a tinting with litmus that has not yet diffused out, are far from rare. The observations suggest a method whereby rates of cell replacement in some elements of the fixed tissues might conceivably be determined.

In a preceding paper, on the general features of litmus staining, emphasis was laid upon the ability of cells throughout the organism to mobilize a large amount of acid material which is held within the cytoplasm in granular or globular form. The present work demonstrates that the quantity of it accumulating in the macrophages of a peritoneal exudate may on occasion be sufficient to ensure a dominance of the acid reaction when the cells are gathered together and crushed. This is scarcely to be wondered at when one considers that the material of the intracellular granules is acid to brom phenol blue, a finding that, under controlled circumstances, would indicate a pH of 3.0 or less.[9]

Observers are at one in believing that the granules in which dyes are laid down have many uses less foreign to the ordinary activities

than the storage of extraneous material.[12] In this connection one may recall the fact described in the preceding paper[1] that litmus tends to accumulate about the erythrocytic detritus normally present within certain large elements of the spleen and marrow, and also in the globules which form about lipoid matter within the cells of peritoneal exudates. As already stated the existence of an acid state in the granules storing dyes has not gone unrecognized by previous workers but the acidity has been deemed too slight even to affect litmus.[7,8] With the demonstration that it is considerable, questions arise as to its nature and significance for body processes. One thinks immediately of the fact that the proteolytic enzyme of macrophages acts only when the reaction is acid;[8] and also of the regularity with which some organisms, notably the pneumococcus, succumb in an acid medium. The conception that the cellular defense of the body may depend in some part on the development about phagocyted bacteria of a reaction inimical to them is not new. It led Metchnikoff to many experiments with litmus and mammalian cells, but for reasons already given[1] these failed to disclose the acidity for which he sought, one which actually develops as the present experiments show.

SUMMARY AND CONCLUSIONS.

The acidity of the macrophage granules in which litmus comes to be stored during life is considerable. It has proved possible to stain these granules *in vivo* with some of the phthalein indicators and the results, had they been obtained under controlled conditions, would indicate a pH of 3.0 or less. The amount of acid material which may accumulate within the cells of animals stained with litmus is great, sufficient in the case of the elements of a peritoneal exudate for the acid reaction to prevail when they are gathered together and crushed. The material is derived, not from the dye, but from living elements responding characteristically to a stimulus far from unique. Such responses may well play a rôle in normal physiological activities and in the cellular defense against microorganisms.

Vital staining with litmus demonstrates anew that the intracellular reaction during life is independent of that of the body fluids. By

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

means of color changes in the stored indicator one can distinguish sick as well as dead cells of certain sorts and follow their distribution and fate within the organism. There are data to suggest that with the aid of the indicator the normal period of survival of certain elements at least can be determined.

By the indicator method, of which the foregoing observations afford a crude illustration, much should be learnt in the future about body processes. The present paper is the second of a series upon the theme.