THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

III. Indicated Differences in the Reaction of the Blood and Tissues on Vital Staining with Phthaleins.

By PEYTON ROUS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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In the present paper and others to follow some color phenomena noted in animals stained during life with phthalein indicators will be described. It is a curious fact that these indicators have not been utilized heretofore for a general vital staining, in view of their multifarious applications to the study of body fluids. The findings in the stained animals will be presented objectively for the most part. Interpretation of them can be but tentative at best, in the present state of knowledge.

To come at the reaction within living cells and organs by means of indicators, one must have available substances of a sort which will penetrate readily, and not interfere with the normal activities; which will stain so intensely as to prevail on the color of the tissues themselves, yet not give false information. These are conditions difficult to satisfy. And with each indicator it will be necessary to determine exactly what is stained. No more need be said to show that the task is one of great complexity.

In previous communications the results of vital staining with litmus have been described.[1,2] The coloring matter diffuses poorly, and becomes largely segregated in red form within intracellular granules. The acidity of these granules, as indicated with litmus and some of the phthaleins of a more acid range, is far from negligible.[2] There are certain tissues which appear to stain diffusely with litmus, and these are mostly rendered blue by it. But in the case

[1] Rous, P., *J. Exp. Med.*, 1925, xli, 379.

[2] Rous, P., *J. Exp. Med.*, 1925, xli, 399.

of one at least, the liver, signs have been found that some portion of the indicator unsegregated within granules is pink.

The phthaleins are highly diffusible, as will be shown, and maintain their characteristics as indicators when introduced into living animals. Most of them are well tolerated by mice in amounts which color these creatures brightly. The staining is far more general than that with litmus and ordinarily is diffuse, though localization in the cell granules can, on occasion, be brought about.[2] Elimination, which occurs into the bile and urine, is, like the spread of the dyes through the body, extremely swift, and the coloration of the tissues usually is lost within a few hours.

Method.

Half-grown to adult mice were employed. The sodium salts of the indicators (Hynson, Westcott and Dunning) were prepared according to Clark's directions,[3] immediately prior to injection, and dissolved in 0.9 per cent saline solution. No sterilization was attempted. Ordinarily, each 10 cc. of the fluid contained 0.1 gm. of the original indicator, or 0.2 gm. in the case of such as were highly soluble. When thus made up, all of the solutions except those of brom phenol blue and brom cresol green had colors showing that the indicators were still in the acid form. A very little N/20 NaOH was added, just sufficient to change the hue to the alkaline side and, when the range permitted, to the color indicative of pH 7.4. It was found that subcutaneous injections of the material yielded but a weak staining because elimination nearly kept pace with absorption. When, however, the dyes were thrown into the peritoneal cavity, in amounts ranging from 0.5 to 3.0 cc., there resulted a pronounced coloration of the whole animal. As a rule the examination was carried out when this had reached a maximum, the mouse being put under ether and the tissues inspected. In order to avoid, so far as possible, the complication of acidosis incident to prolonged anesthesia, the dissections were made rapidly, and were in many instances confined to a few organs. The skin incision was carried around the site of injection to avoid a possible contamination with free indicator. The instruments were cleansed in boiling water and dried upon gauze that had been washed free of soluble material.

Preliminary observations brought out the fact that changes in the reaction of some of the tissues take place practically at once on exposure of them to air. For this reason, as a rule, the dissections have been carried out with the body immersed in washed paraffin oil in a tray with a layer of solid paraffin at the bottom. The oil had been tested and shown not to affect the indicators even in unbuffered solutions. The colors glow with a special brightness in it. The tissue bits for microscopic examination were clipped or sliced away and placed in oil between a slide and cover-glass of mica. Pieces of the larger organs were

[3] Clark, W. M., The determination of hydrogen ions, Baltimore, 2nd edition, 1922.

sliced in the tray with a Valentine knife. The inspection of color was carried out as swiftly as possible, first with the naked eye, then under low and high magnifications. Daylight was used throughout. In most instances there was a little of the indicator fluid still free on the peritoneal surfaces at the time of the examination, and, when necessary, this was taken off with gauze. It never interfered with the appraisal of the hue in the interior of the viscera.

The observations of the present paper and that to succeed it will be reported in the order in which the phthaleins stand in series, from thymol blue in its alkaline phase to brom phenol blue which has a range well to the acid side. The specimens of indicator employed have been checked up in Sörensen's double phosphate buffers, kindly provided and controlled electrometrically by Dr. D. R. Drury; and, with the exceptions of brom phenol red and chlor phenol red, they have been found to possess the ranges generally accredited to them. Checking has special importance in the case of brom cresol purple which may be so altered when the alkaline salt is improperly made that its range is narrowed and shifted toward the alkaline side.

The available range of all of the phthaleins is greatly narrowed when they are diffused throughout the body, for the reason that only outspoken color differences, not varying intensities of the same hue, can then be depended upon. For, needless to state, observed local differences in intensity may be the result of differing local concentrations of the dye, not of differences in reaction. The colors also are influenced by this factor, dilute solutions of an indicator at a reaction close to the middle of its range having often a different hue from more concentrated ones. The difficulty thus introduced was circumvented by comparing the hue of each stained tissue with those of the tubes of a series of buffer solutions containing the indicator in approximately the color intensity manifested by the tissue. For the purpose several graded sets of buffer mixtures containing different amounts of each indicator were requisite. Often a comparison was possible with the color chart given by Clark.[3] It may be recalled that for each hue depicted in this last the pH is given to which said hue corresponds under controlled conditions. The use I shall make of these pH numbers in recording the color of stained tissues must not be taken to imply a conclusion that the latter had the precise reactions for which these numbers ordinarily stand. They will be employed merely as index symbols, save where otherwise stated. To minimize the error introduced by the native hue of the tissues a staining sufficient to prevail over this was induced. Yet, needless to remark, it had always to be taken into account.

Control Observations.

In view of the great diffusibility of the phthaleins and the intense coloration they produced in the animals there could be no doubt that the dyes passed from the blood stream into the tissues generally.

Yet direct tests of the point seemed advisable. Accordingly, mice were injected with the amounts of cresol red, phenol red, and brom cresol purple employed in the experiments proper, and when the staining had reached its maximum they were rapidly etherized, the inferior vena cava severed just above the diaphragm, and warm Ringer's solution was slowly injected into the left ventricle of the exposed and beating heart. As result the animal exsanguinated itself in the course of a minute or two and washed itself out with fluid propelled by the ventricular contractions. The bloodless ears, lungs, connective tissue, epidermis, liver, and kidney were found in every case to be notably stained with indicator. There was less in the voluntary muscles, and still less in the heart muscle, though even here some was demonstrable. The parenchyma of the testicle was looked at in a few cases and found stained; but no general examination of the organs was undertaken. The presence of the indicators was of course to be expected in the liver and kidneys which excrete them.

According to Clark and Lubs[4] the phthaleins manifest much resistance to chemical influences such as they would be likely to encounter within the body. That the hues which they assume when distributed through the tissues are an expression of their behavior as indicators is plain from the characteristic alterations to be noted when the reaction is in any way changed. But it is essential to know whether the color distinctions remain the same in the body as when the indicators are added as such to buffer solutions. This is readily demonstrable. Animals given an indicator are exsanguinated under ether, and various of the stained organs are thinly sliced and the slices squeezed between dry, neutral gauze to remove still more of the blood. It would have been better to have employed organs washed out with salt solution. The slices are placed in water for a few moments in order to remove as much as possible of what is left of the tissue fluids, and are then distributed in a graded series of buffer solutions. I used Sörensen's double phosphate mixtures. Both in the case of phenol red and brom cresol purple, the most valuable phthaleins for tissue work, the hues assumed by the indicator diffusing out into the buffers corresponded absolutely with those obtained on

[4] Clark, W. M., and Lubs, H. A., *J. Bact.*, 1917, ii, 1, 109, 191. Clark, W. M., personal communication.

adding the phthaleins directly to a similar series of solutions, as furthermore with those given in Clark's chart. Brom cresol purple excreted into the bile retains the typical range.

The Staining with Thymol Blue.

Thymol blue in its alkaline phase is yellow in dilute solution at pH 8.2, but with increasing degrees of alkalinity changes until at about pH 9.4 it has become sufficiently blue for the color to dominate presumably over that proper to most tissues, should they contain it. The indicator is one of the few that is poorly tolerated. Mice receiving enough of the alkaline salt to color them markedly succumb as a rule within the next 24 hours. Somewhat smaller amounts appear to interfere little with health. The tinting develops rather slowly, reaching a maximum only after several hours and may not have wholly disappeared by the next day. A deep yellow urine and yellow feces are voided, both of which turn blue with alkali. Nowhere in the body is the blue color found. When the staining is at its height, the hairless surfaces are pronouncedly yellow like the tissues disclosed by dissection. The question of the precise distribution of thymol blue within the body need not be dwelt upon since tests with the other phthaleins show clearly that none of the tissues of the mouse is alkaline enough to come within its range.

Cresol Red.

Cresol red, when dilute enough to be comparable with the color scale of Clark, is yellow at about pH 7.2, becoming gradually red on alkalinization, until at approximately pH 8.4 this color is outspoken. The red component becomes more evident with increasing concentration of the dye, and, when this is ten times that just considered, the hue in a buffered solution (Sörensen) at pH 7.55 is damson-red, at 6.98 ruddy orange, and only at 6.24 is a clear orange attained. The amount of cresol red in the tissues, when animals are properly stained, permits of a direct comparison with Clark's table, whereas in the blood so much more is present that to gauge its hue properly recourse must be had to graded buffer solutions containing the phthalein in considerable amount.

Mice receiving 1 cc. of 1 per cent cresol red become a ruddy yellow

within an hour while within another the staining has largely faded and by next day has wholly disappeared. The urine is sometimes deep yellow and again intensely red. The animals at no time appear disturbed. On close scrutiny of the ears it can be seen that the red element in the coloration is confined to the blood (and lymph ?). For when the ear is rendered bloodless by gentle pressure between glass slides, it appears clear yellow. The same holds true for the subcutaneous tissue adherent to a skin flap everted under light ether anesthesia with the animal submerged in oil. A like yellow coloration is to be found almost everywhere, save in the brain and nerves which are not stained. In special the connective tissue, adipose tissue, tendons, cartilages, epidermis—except for the horny layer—the lymph nodes, kidney, spleen (Malpighian bodies), liver, gall bladder wall, pancreas, heart muscle, and voluntary muscle are all yellow with the dye, as is the medulla of the suprarenal and the more vascular regions of the bones. Suprarenal cortex and laminated bone appear unstained. The yellow color, if encountered under controlled conditions in a buffered solution, would mean a reaction at or below pH 7.2. The finding does not stand alone. Tests with indicators of less alkaline range have given results in close agreement with it, as will be shown.

Phenol Red.

Mice behave normally when deeply colored with phenol red,—as after the injection of 1 cc. of a 2 per cent solution into a 30 gm. animal; and no impairment of health occurs subsequently. The staining is at its height within an hour and has almost disappeared within another. Ruddy to crimson urine is voided. The indicator is clear yellow in dilute solution at pH 6.6, changing toward old rose with gradual alkalinization until at pH 7.8 the hue is outspoken. In concentrated solutions the rosy element extends much further down the pH scale, as in the case of cresol red. It follows that the color differentiation of the tissues is not well seen when vital staining is intense.

In stained animals the hairless surfaces appear pink; yet on scrutiny the color is seen to be mixed with yellow, a state of affairs most evident

in the ears by transmitted light. Wherever vessels are few, as near the margin of the auricle, the color is yellow, and, where many, a deep pink. Pressure sufficient to drive out the blood leaves the tissue yellow. Clippings of the epidermis of the tail, feet, or abdomen are all brilliant yellow, save in the horny layer which is unstained. The adipose tissue attached to some specimens is also bright yellow. In lightly etherized animals with the body submerged in oil the only stained fabrics which do not appear pure yellow are those of the tendons and connective tissue. Everywhere throughout the body the latter is yellow-pink to ruddy orange, according to the amount of phthalein present, the hue corresponding ordinarily to that of pH 7.2 in Clark's scale, though often it is more yellow, a slightly pinkish yellow, as the tendons regularly are. The brain and nerves are not colored; and the distribution of the dye in the other organs closely accords with that of cresol red, so there is no need to designate those rendered yellow by it. Occasionally the cortex of the kidney is red, but ordinarily it is yellow, the individual differences being dependent, doubtless, on functional conditions.[5] The prevalence of yellow throughout the body is the more noteworthy because the blood, as viewed within the vessels, appears either old rose or damson-red, depending on how much indicator is present. The inguinal lymph glands, inspected *in situ*, have a rosy interior, but when they are rapidly excised and broken open between mica slides, by pressure the color is found to be confined to the lymph and blood, the tissue proper to the gland being a clear yellow.

The coloration with phenol red, if accepted at its face value, would mean that none of the stained organs except the connective tissue and tendons can have a pH greater than 6.6. The hue assumed by the blood on the other hand would indicate that it is relatively alkaline, a finding in agreement with what has been noted of cresol red animals. But the differing concentrations of the extra- and intravascular indicator may conceivably be responsible for much of the color difference. In some further, more precise, observations account was taken of this factor.

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

Effects of Ether Anesthesia on the Vital Coloration.

The animals from which stained blood was to be taken were placed under ether. When light anesthesia had been rapidly induced, no change was to be noted in the color of the ears, tail, or other hairless surfaces; but when it was heavy, or had been maintained for some time during a detailed inspection of the organs, the body surfaces showed noteworthy alterations in hue indicative of a changed reaction of the blood. In cresol red animals the vascular portions of the ears no longer appeared ruddy yellow but were yellow, the vessels now showing merely as fine orange lines, not red ones as previously. An identical alteration was to be noted throughout the blood of the organs. This phenomenon, indicative of an acidosis, was not quite so plainly to be remarked in phenol red mice, presumably because of the circumstance that the range of this indicator lies further to the acid side, the alteration toward acidity during life being never sufficient to convert the color completely from damson or rose to yellow. The pink of the hairless surfaces was altered by the anesthesia to, at most, a pinky yellow. With the brom phenol red recently described by Cohen, my specimen of which had a range only slightly different from that of phenol red, being yellow at pH 6.47 when in dilute solution, and old rose at 7.38, the changes were much the same. The ears of the stained animal were ordinarily pink, with an orange admixture where there was little blood, but during prolonged ether anesthesia they became yellow save for a few of the larger vessels, which retained the red hue. With the phthaleins of a more acid range color changes fail to occur during anesthesia.

The development of acidosis during ether anesthesia is, of course, a well known phenomenon. However, its manifestations in the stained mice have an interest both as proving that the circulating phthaleins maintain their indicator characteristics, and as pointing to a source of error in animals examined while anesthetized. The acidosis is evident even when the breathing is so hastened and exaggerated as to give the impression that an overventilation must be taking place. The question as to the extravascular implications of the change in blood reaction cannot be answered with the red phthaleins now under consideration, owing to the fact that nearly all of the tissues have a reaction beyond their range on the acid side.

Connective tissue is an exception in that it is colored yellow-pink with phenol red. I have not been able to observe any certain alteration in its hue during prolonged anesthesia unaccompanied by asphyxia. In the experiments now to be detailed, to compare the blood and tissue reactions, the etherization has been light and brief, yet some slight change in the color of the hairless surfaces has been not infrequent.

Differing Coloration of the Blood and Tissues.

General Experimental Procedure.—Mice stained with cresol red or phenol red were lightly etherized, placed at once in the oil bath, and the skin rapidly stripped back from over abdomen and chest. The color of the subcutaneous tissues was noted *in situ;* the thorax was laid open with scissors; and blood was aspirated from the right ventricle directly into one, or, when possible, two tubes containing a column $1\frac{1}{2}$ to 2 cm. long of paraffin oil. The tubes had a bore of about 0.35 cm. and were of "Perfection" glass, which contains practically no free alkali. An end had been drawn out into a wide-angled but sharp point to thrust into the heart. Of each pair of tubes one had been rinsed in twice distilled water, just before oil was drawn into it, and the other had been coated with paraffin and then rinsed prior thereto. The blood never came in contact with air. Immediately that it had been withdrawn the unsealed sharp tips of the pipettes were thrust deep into rubber stoppers and they were centrifuged in chilled containers for 2 or 3 minutes at high speed. In this way a clear plasma was obtained which was compared, at room temperature, in buffered solutions of indicator. In the case of cresol red several series of such solutions were used containing different concentrations of the indicator; and a match in color and intensity for the blood plasma was found by taking up the appropriate buffer solution into a tube like that containing this latter. Duplicate plasma specimens were found to have always the same hue. The presence of dissolved hemoglobin as a complicating factor was ruled out with the spectroscope, and the influence of oil, paraffin, and glass by preliminary tests with dilute watery indicator solutions introduced into the tubes. Even after 24 hours at room temperature the stained plasma, now clotted of course, had changed but faintly in hue, toward the acid side. The blood of some mice was procured when the staining was at a maximum and of others when it was fading. The hue of the plasma colored with cresol red corresponded in general with that evident at pH 7.38 in a buffer solution, but in some individuals approached that at pH 7.55. So, too, with phenol red. It has been interesting to learn from Dr. James A. Hawkins, since the observations were completed, that he has independently obtained the same values for mouse blood by means of his accurate colorimetric procedure.[6]

[6] Hawkins, J. A., *J. Biol. Chem.*, 1924, lxi, 147.

Only the skin flaps of the mice from which blood was procured were looked at during life, but the colors there visible sufficiently indicated that the staining in these instances was typical.

The findings with cresol red and phenol red accord despite the differing ranges found for the phthaleins under controlled conditions. With both the color of the indicator in plasma from the right ventricle is such as would indicate a reaction varying from pH 7.38 to 7.55. It may be recalled that the hue of the tissues stained with cresol red, if taken at its face value, would mean that these had a pH at least as low as 7.2 and possibly less. Phenol red will indicate pH 7.2 with exactitude, at least under controlled conditions, and the connective tissue had a hue approximately corresponding thereto and the tendons one not very different; whereas the hue of all the other structures fell outside of the range of this phthalein, on the acid side, being a yellow such as is associated with pH 6.6 or less. There is good reason to believe that the findings in the blood are indicative of the actual state of affairs as concerns it. But what is one to suppose of the color manifestations within the tissues? As a first step toward answering the question tests have been made with other phthaleins.

Brom Phenol Red.

Recently Cohen has reported upon dibromo-phenol-sulfonphthalein (brom phenol red) which has, according to his brief statement, a range from pH 5.4 to 7.0. My specimen of the indicator, supplied by Hynson, Westcott and Dunning, is yellow at pH 6.47 and old rose at 7.38 when in dilute solution (0.1 per cent in a double phosphate buffer), whereas when ten times as much is present it is yellow at pH 6.24, orange at 6.64, and orange-red at 7.38. The range differs but slightly, then, from that of phenol red. 1 cc. of a 2 per cent solution is well tolerated by a 30 gm. mouse, and the staining is such that the tissue hues can be checked against the more dilute of the buffer series just mentioned. The coloration is at a maximum within 1 hour, has largely faded within another, and vanishes completely overnight without appearing to have affected the health. The animals void dark red urine.

As already remarked, the yellowish pink of the hairless surfaces of

stained mice nearly resembles that after phenol red, and a like difference between the color of the blood and tissues is demonstrable in the ears. During prolonged or heavy etherization the hue of the body surfaces changes from pink toward yellow. Clipped fragments of epidermis are always yellow, like the adipose tissue attached thereto. As with phenol red most of the tissues are yellow but the brain and nerves fail to stain. As in its case, too, the connective tissue and tendons form notable exceptions, the former being yellow-pink, as is the indicator in a buffer solution of pH 7.2 and the latter pinkish yellow. In their apparent significance the findings correspond absolutely with those obtained with the other indicators.

The results with brom cresol purple, chlor phenol red (Cohen), and indicators of more pronouncedly acid range will be detailed in a paper to follow. Suffice it to say that, in so far as they bear upon the findings here described, they are in consonance with them. Brom thymol blue cannot be employed because it is even more toxic than thymol blue. An amount sufficient to produce staining brings about an extreme general acidosis, as evidenced by the greenish yellow color of the prostrated animal, a hue which, under controlled conditions, would indicate that the blood had a reaction as acid as pH 6.2. Death ordinarily takes place within a few hours.

The Factor of Asphyxia.

Asphyxia notably influences the hue of tissues stained with the phthaleins. In mice killed after receiving cresol red there occurs within a few minutes an alteration in the color of the blood within the smaller vessels from red to yellow. The tissues have, during life, the latter hue, that is to say are apparently so acid as to be beyond the range of the indicator and, as would be expected, no change is visible in them post mortem. The same holds true for most of the tissues stained with phenol red. With this dye, however, as will be recalled, the connective tissue is a yellowy pink during life, and the body surfaces appear pink both because it underlies them, and because of the ruddy blood. Soon after death, though, the surfaces change from pink toward yellow; and the connective tissue then exposed is found to have this hue, while the blood in the smaller vessels is far less pink than during life. Analogous changes are to be noted when brom phenol red is the

stain. All may be attributed with good reason to asphyxia, which is known to make for acidity within the organs. The hue in many of the tissues of the dead animal veers toward that indicative of alkalinity when they are exposed to air. Even during life the ventilation of raw surfaces results in local changes in the reaction, as will now be shown.

Ventilation from Exposed Tissue Affects Its Reaction.

When etherized animals stained with cresol red are laid open in the absence of the protection afforded by an oil bath, the color of one of the tissues exposed *in situ* to air, but only one, namely the connective tissue, is observed gradually to change, turning from yellow to red. When phenol red has been employed, the change is rapid, indeed very rapid in subcutaneous layers stripped away from the adjacent structures; and not only is the connective tissue affected, if this most obviously, but tendons, cartilage, and bony surfaces as well. Within a few minutes all are purply red. With brom phenol red similar alterations and of the same tissues are observed. The limitation of the phenomenon in the case of cresol red falls in with the fact that its range is relatively alkaline. One may suppose that only in the case of the connective tissue, the most alkaline apparently, of the fixed constituents of the body is the further increase in alkalinity great enough to affect the indicator color.

There are many conceivable explanations for this change in reaction occurring upon exposure to air, but the most reasonable is a ventilation from the raw surfaces, carbon dioxide escaping therefrom. Since the change does not occur when the dissection is made under oil, one can be certain that altered circulatory conditions are not responsible for it. That it is not irreversible follows from the observation that everted skin flaps, containing phenol red, which have become rose-red following exposure to air, return to the original orange hue when they have been replaced against the body for a few minutes. The cause of the alterations in color was demonstrated as follows:

A mouse stained with phenol red by intraperitoneal injection was anesthetized and two symmetrical, slightly curved incisions were made through the skin of abdomen and chest, from the pelvis to the neck. Between them was left a small isthmus of skin containing the needle puncture—this in order to avoid a possible

contamination of the fields of observation with indicator solution leaking from the peritoneal cavity. Threads were tied into the upper and lower ends of the skin flaps outlined by the incisions, and the flaps themselves were rapidly separated from the underlying structures by blunt dissection with forceps and at once replaced in the original position. There was no bleeding. The animal was now placed on its back upon wet blotting paper covering a platform warmed by a water circulation; and a large glass funnel was inverted over it (Fig. 1). At one edge of the funnel rim was a notch patched with a piece of rubber dam moored

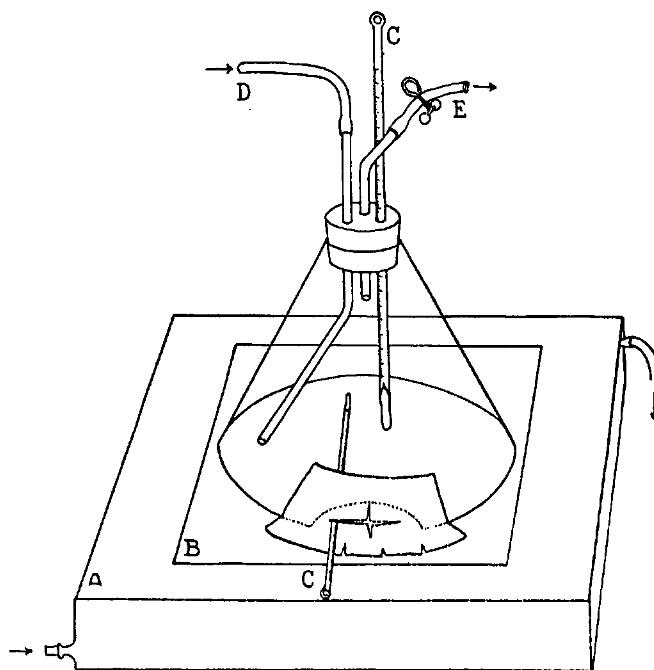

FIG. 1. *A*, metal box with circulation of warm water; *B*, wet blotting paper; *C*, thermometers; *D*, inlet for gases; *E*, outlet.

to the glass with adhesive plaster. The head of the mouse was brought to the outside through a slit in the rubber which fitted the neck snugly. Thus the animal continued to breath the room air, while the body inside the funnel could be submitted to any gas one chose to introduce. The neck of the funnel had been cut off and a three-hole rubber stopper inserted in its place. One of the holes contained the shaft of a thermometer, the bulb of which was fixed just above and to one side of the mouse. Another thermometer, passing horizontally through the rubber dam, rested on the blotting paper. A bent tube ex-

tending down almost to this latter, through the stopper, but ending well to one side of the animal, was employed to introduce the gas for test. Another tube, barely projecting into the funnel, which could be closed at will with a pinchcock, furnished an outlet. The funnel rim fitted the blotting paper closely. The gas to be introduced was moistened and heated to body temperature by bubbling it through warm water. Little difficulty was experienced in keeping the temperature constant within the funnel, at 36–40°C., according to desire. When the animal was first put in, it was stretched on the back by means of cords attached to the legs and extending under the funnel rim. The threads from the skin flaps also passed out under the rim, but the flaps as yet lay in place.

A specimen protocol follows (see also Table I):

A female mouse of 30 gm. was given 0.4 cc. of a watery 20 per cent urethane solution, into the subcutaneous tissue of the back of the neck at 10.19 a.m., followed at 10.32 by 2 cc. of a 1 per cent solution of phenol red into the abdominal cavity. At 11.42 the anesthesia was sufficient for the operative work which was finished by 11.50. In freeing the flaps care was taken not to interfere with the supplying vessels from the groins and axillæ. There was no hemorrhage. At 11.55 the mouse was placed under the funnel, and observed until 12.08. During the 13 minutes the rate of respiration varied between 186 and 190 per minute. The temperature within the funnel was kept between 35° and 36°C. During later periods it was held close to 35°.

12.08½ to 12.13. *Respired air of an observer breathed directly into the funnel,* the lungs often being emptied more fully than usual.

12.13. *Left skin flap exposed* by traction on the threads. It appeared ruddy orange as usual, owing to the blended yellow of fat and epidermis with the pinker color of the connective tissue and blood. At 12.16 there were 190 respirations per minute, at 12.19, 182, and at 12.22 and 12.23½, 178 per minute. Respired air still breathed in until 12.24.

12.24. *Right flap pulled open and compared with left.* The latter as viewed by itself, appeared scarcely to have changed color, but it was now seen to be slightly the more ruddy of the two, indicating a change in it toward greater alkalinity, despite the exposure to expired air. The respirations had now fallen to 182.

12.24 to 12.32. Funnel repeatedly ventilated with room air and adjustment made of flaps to expose them more fully.

12.35. The flaps had become purply red. There were 182 respirations per minute.

12.39. Respirations 176 per minute. *Pure oxygen was run into the funnel* and the flow kept up until 12.51½. During this period the exposed flaps became more purple. The respirations, taken every other minute, varied between 172 and 180.

12.52. Respirations 178 per minute. *The oxygen was shut off and a flow of*

Time.	Period.	Procedure.	Color of exposed tissue.	Respirations per min.	Remarks.
	min.				
11.55 to 12.08	13	Mouse under funnel.	Tissue unexposed.	186 to 190	Control period. Funnel temperature 35–36°.
12.08½ " 12.13	4½	Funnel filled with respired air.	" "		
12.13		Left skin flap exposed.	Ruddy orange.		
12.13 to 12.24	11	Funnel kept full of expired air.		190 " 178	
12.24		Right skin flap exposed.	The one already bared found slightly the more ruddy.	182	
12.24 to 12.39	15	Funnel repeatedly flushed with room air.	Flaps gradually became purple.	182 to 176	
12.39 " 12.52	13	Oxygen run in.	Flaps became more purple.	172 " 180	
12.52 " 12.54	2	Oxygen shut off; CO_2 run in.	Rapid change to yellowish orange.	180	
12.54 " 1.12	18	CO_2 still run in.	Brilliant orange-yellow.	Progressive increase to 204.	Exaggerated respiration.
1.12 " 1.25	13	CO_2 shut off; oxygen run in.	Very gradual change to purple-red.	Decrease to 180.	Rapid return to normal.
		Experiment discontinued.			Funnel temperature 35°.

pure carbon dioxide started. Practically at once the color of the flaps altered toward orange. By 12.54 they had become an outspoken yellowish orange with no hint of red. The respirations were still 180 at 12.55, but as the gas continued to flow into the funnel they gradually quickened until at 1.02 there were 204 per minute. The breathing had become very exaggerated, causing the whole body to move. No signs of returning consciousness were visible. Until 1.12, when oxygen was substituted for the carbon dioxide, the breathing continued rapid and exaggerated. At 1.12 there were 196 respirations to the minute. The flaps were a brilliant orange-yellow.

1.12 to 1.25. *Oxygen run in.* The flaps were noted to have become somewhat ruddy 3½ minutes later, and the breathing to be less agitated. By 1.17 the respiratory rate had fallen to 180, and it did not alter thereafter, being 180 at 1.24. The flaps had gradually become purple-red.

The animal, in excellent condition, was sacrificed. There was no free fluid upon the raw surfaces. The phthalein which had so frequently changed color lay within the tissue.

Analysis of the phenomena just described leaves no doubt that carbon dioxide is rapidly given off from raw subcutaneous surfaces and taken up by them, with concomitant changes in the local reaction of the tissues. The observation that a raw surface submitted to expired air fails to undergo the change to ruddy purple, which takes place in room air, does not of itself suffice to show that a loss of carbon dioxide is responsible for the alteration in hue. The final proof has been supplied by exposing the surface to expired air which had been passed through soda lime to remove the dioxide. Under these circumstances the change to purple was a rapid one.

It will have been noted in the protocol that when the large skin flaps had been for some time exposed to an atmosphere of carbon dioxide—in which they soon became orange-yellow—a gradual, but eventually marked, increase in rate and amplitude of the breathing took place, and that the changes lasted so long as did exposure to the gas, disappearing after oxygen had been substituted. The quickened breathing did not occur in animals unprotected against loss of body heat, the rate then falling progressively, irrespective of the gas to which the surfaces were exposed. The question whether the hurried and gasping respiration was the manifestation of a general acidosis consequent on absorption from the exposed tissues was answered in not a few instances by a concomitant change in the color of the ears from pink toward yellow, indicating that the blood had become more acid.

When mice thus affected were removed from the funnel to an incubator, and kept at 38°C., the skin flaps having first been put back in place, many minutes elapsed before the hairless surfaces recovered the pink hue, so profound had been the disturbance of the acid-base equilibrium. The most marked instances of the sort occurred in animals given ether. In them, of course, intercurrent respiratory changes took place that were attributable to the anesthetic itself, but over and above these one could note marked and characteristic changes in the breathing as the acidosis with carbon dioxide developed. Incidentally it may be remarked that, irrespective of other possibilities and probabilities, the acidosis consequent on the anesthesia itself will suffice to explain why a protected raw surface usually appears slightly less alkaline than one exposed for some time to expired air.

The only tissue which was observed to change color during the experiments on ventilation was the connective tissue forming the raw surface of the skin flap and of the body whence the latter had been stripped. The underlying adipose tissue, muscle, and skin retained the initial yellow. In the case of the connective tissue, the intense rose-purple hue assumed on exposure to air is such as might be taken to indicate that the alkalinity had increased to pH 8.0 or more. The well known fact may be recalled in this relation that blood serum set in the air undergoes a considerable increase in alkalinity.

DISCUSSION.

However great the demerits of indicators may prove to be as gauges of local reactions within the body, the utilization of them here described has demonstrated, if only anew, facts which render their employment justifiable. Principal among these is the complexity of the conditions to be coped with if the precise pH prevailing in the interior regions of the organism during life is to be determined by other than intravital methods objective in manifestation. The least interference with blood supply, the giving of an anesthetic, the exposure of the tissue to air, postmortem changes, all alter the reaction swiftly. So, too, must many another influence, for example, trauma. A gradual recognition of these possibilities of error in the application to the tissues of the electrometric method may be traced in the litera-

ture of the subject, beginning with Michaelis' attempts[7] to determine the reaction by a procedure which involved hashing the organs in the air and extracting them with water, after cooking in some instances, and progressing to the observations of Schade and his collaborators[8] who introduced a hollow needle electrode directly into the tissues, and foresaw and made provision to meet errors incident to differences in carbon dioxide tension. Precise determinations have been reported with Schade's apparatus, but only on tissue fluids and in superficial regions.

The question of the meaning, in terms of pH, of the hues assumed by tissues stained with a given indicator must be left unanswered for the time, like that of the situation of the stain, whether only in the lymph or upon or inside of the cell, or in the intercellular substances. It will suffice at the moment to speak merely of indicator within the tissues as distinct from that within the blood. The findings with undiluted plasma more or less heavily tinted with indicator, as procured by aspiration from the heart, are readily subject to control by the micro method of Hawkins.[6] *A priori* much the same objections might be lodged against the interpretation of the tinting of the plasma in terms of pH as against a similar step in connection with the color of the tissues. One thinks immediately of protein errors and of a possible loss of carbon dioxide through the oil. But, as already remarked, Dr. Hawkins' unpublished data prove that the pH of mouse blood has essentially the range which it would appear to have from the hue on vital staining with the phthaleins as I have described it. One is justified in saying with regard to the tissue coloration merely that the evidence with different phthaleins accords remarkably in that it indicates the reaction within them all to be less alkaline than that of the blood and in most cases to be frankly acid. In a succeeding paper results with indicators suited to the determination of this presumptive acidity will be described.

It may be objected, in connection with the examination made of bits of the viscera, that the conditions of study were supravital rather than vital. The objection is valid, no matter how swiftly the bits are re-

[7] Michaelis, L., and Kramsztyk, A., *Biochem. Z.*, 1914, lxii, 180.

[8] Schade, H., Neukirch, P., and Halpert, A., *Z. ges. exp. Med.*, 1921, xxiv, 11.

moved and inspected. It will lose force only when a method has been devised whereby the reaction within highly vascular or well encapsulated organs can be discriminated while they are still *in situ*. Fortunately such a discrimination is possible in the case of many tissues; and some of them, cartilage from the tip of the tail, epidermis, connective tissue, fat, can be viewed directly in the absence of any complicating acidosis such as anesthesia might produce.

The demonstration of a relative acidity within tissues is foreshadowed by not a little previous investigation. Theoretically, as Schade remarks, every cell constitutes a focus from which acid streams out, with result that one would expect a more or less gradual decline in the hydrogen ion concentration as the products of activity of the cell pass from this latter to reach eventually the blood of the right heart. He states that the pH of the intercellular fluids of the subcutaneous tissue is definitely, if very slightly, less than that of the blood. The reaction of the perimuscular fluid of the exercised rabbit falls as low as pH 6.6. Inflammation is accompanied by a marked local acidosis of the tissue fluids; and the pus of furuncles may be acid as pH 5.96. Crozier[9] has noted that certain ciliates and some of the cells of insect tissues stain with brom thymol blue in such wise as to indicate a condition of acidity. The dye is toxic for the mouse, but seems not to be for insects. Crozier suggests that protoplasm in general may be acid.

Krogh has shown the case with which carbon dioxide passes through animal membranes;[10] and the change in reaction of raw surfaces exposed to air cannot, in view of this fact, be considered as a surprising one. But the demonstration that a general acidosis may be produced by the exposure to the gas of large raw surfaces of notably poor vascularization speaks much for its diffusibility. Were carbon dioxide the only substance involved in respiration, it is conceivable that large subcutaneous cavities with highly vascular walls, such as might readily be prepared by operation, would serve the part of lungs, if due care were taken to ventilate them. But, unfortunately for such an experiment, oxygen diffuses with relative difficulty, about thirty-two times as slowly as the dioxide.[9]

[9] Crozier, W. J., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 58.

[10] Krogh, A., *J. Physiol.*, 1918–19, lii, 391.

SUMMARY.

Mice can be vitally stained with many of the phthalein indicators. The staining is diffuse, appearing to interfere not at all with health in the case of the majority of the dyes. The color phenomena show that these retain the character of indicators. A special technique has been evolved for the determination of the hues of the various organs, which are readily modified by extraneous influences. The ability to recognize that the pH has thus been altered is a signal advantage of the indicator method.

Phthaleins of slightly alkaline range or one that trenches slightly on acidity have been employed for the work here reported. Cresol red, phenol red, and brom phenol red have proved especially useful. The observations with the three agree closely in pointing to the existence of notable differences between the reaction of the blood and that within the tissues generally.

The hue of blood plasma from the right heart is such as to suggest that its reaction lies at about pH 7.38 ordinarily, whereas that of the most alkaline of the tissues, judging from its color, the connective tissue, would appear to have a pH of 7.2 or slightly less. The tendons seem to be nearly but not quite so alkaline. The other stained tissues without exception, are of a hue which would indicate that the reaction lies beyond the range of phenol red on the acid side, that is to say is at least as acid as pH 6.6. In a subsequent paper observations which accord with these findings, carried out with indicators of frankly acid range, will be described.

On the exposure of tissues to air, without disturbance of the circulation, some of them become alkaline. In the case of connective tissue, at least, the change is a consequence of the escape of carbon dioxide. The gas passes readily in and out, exerting a practically immediate influence on the color of the tissue bared by eversion of a skin flap; and so much may be absorbed on exposure to pure carbon dioxide, when the surface is large, that a general acidosis results.

The precise interpretation of the color changes in terms of pH waits necessarily upon further work.