THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

VI. FACTORS DETERMINING THE REACTION OF SKIN GRAFTS; A STUDY BY THE INDICATOR METHOD OF CONDITIONS WITHIN AN ISCHEMIC TISSUE.

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In previous papers from this laboratory a technic has been described for the examination of tissues vitally stained with litmus or with indicators of the phthalein series (1), and some observations on the apparent reaction within various organs have been recorded (2). Recent control tests on the influence of tissue materials upon the colors manifested by phthaleins staining them have shown that these colors are not conditioned to any important degree by "salt and protein errors," but that they are really indicative of the prevailing pH (3). By means of vital staining with selected indicators one should be able to apprehend, and may even come to understand, certain physiological states inaccessible to study by approved quantitative procedures. One such state, that of "outlying acidosis," has already been briefly reported upon (4). The present paper is concerned with the conditions in a tissue, the skin, which survives when its blood supply has been cut off. The problem has both practical and theoretical ramifications. In corollary to it the changes which result from superimposed injuries to the tissue elements will be considered.

The Vital Staining of Skin Grafts with Phenol Red.

Male white mice of 25 to 30 gm. were shaved over the sides and back, from fore legs to hind, and under ether anesthesia pieces of the skin were excised, midway between axilla and groin, and at once sown back in place again. These pieces were roughly circular and from 1.0 to 1.6 cm. in diameter. They had the entire
thickness of the integument and contained, in addition to epidermis and corium, a varying quantity of fat and the thin layer of striped muscle which in the mouse and rat extends superficially over almost the whole body. It was frequently impossible to remove such large pieces without cutting cutaneous vessels that supplied neighboring regions, and in consequence of this occurrence the grafts often failed to become vascularized promptly and perished after some days. In females the mammary gland proved a serious complicating factor, and after a few trials males alone were used. The best results were had when the graft was not separated all at once from its surroundings but left attached by an isthmus that was severed only after the major portion had been sewn in place. By this procedure the swabbing of the raw surfaces with saline solution to prevent drying was reduced to a minimum; but great care had to be taken in the dissection else some of the loose web of tissue connecting the skin with the underlying parts escaped being cut, with result that total ischemia of the graft did not develop. For the sewing a fine curved needle carrying a single strand of the three which are wound into No. 2 surgeon's silk was employed; and an over and over stitch. Perfect approximation of the wound edges proved essential, for when a part of the wound bed was left exposed necrosis usually spread from it (5). Aseptic conditions were maintained during the operation; and no dressing was put on afterwards. The mouse had to be prevented from removing stitches and graft however. This was accomplished by passing its head through a hole in the center of a flat disc of pasteboard. The projecting collar thus formed, which stood out like the ruff of a clown, did not interfere with the animal's movements but kept it from gnawing at the graft. The stitches were removed on the day following the operation, and thereafter for nearly a week the attachment of the implanted skin was but frail.

Each day the mouse was injected into the peritoneal cavity with 0.5 cc. of a watery 4 per cent solution of phenol red, made as follows: 2 gm. of the phthalein (Hynson, Westcott and Dunning) is ground to a paste with a little water, 9.7 cc. of N/1 NaOH added, and then water to 50 cc. Such a solution is at pH 7.4 (as determined with the potentiometer) and approximately isotonic with 0.9 NaCl. The quantity of alkali employed somewhat exceeds that required, on theory, to bring the indicator to the hydrogen ion concentration mentioned, presumably because of acid impurities in the material. In order to effect the injection into the mouse without a struggle that might have entailed separation of the graft it was briefly anesthetized by dropping it into a jar containing cotton soaked in ether.

During most of the 1st week after implantation the graft appeared pallid,—save when vitally stained,—and bloodless. As is well known vessels begin to penetrate into transplanted skin during the second 24 hours, but there is certainly no effective circulation of blood for a much more considerable period in grafts of the size studied in the
present work. This is evident not merely from the aspect of the graft under ordinary conditions but from the slowness with which it colors up after an intraperitoneal injection of phenol red. On the 3rd day after operation it stains no more rapidly than on the 1st. Thereafter, though, it lags in this respect less markedly as compared with the skin round about, and by the end of a week, in successful instances, it colors as fast as the latter.

The rate of coloration provides enlightening data on the fluid interchange taking place within engrafted tissue. Phenol red is very highly diffusible, coloring mice deeply within a few minutes after an intraperitoneal injection; and the brilliant, ruddy color of the stained animals is due for the most part to an extravascular penetration of the dye. This has been shown by perfusing the stained animal until free of blood, with warm Locke's solution introduced into the beating heart, after the inferior vena cava has been snipped across (6). But the fact emerges even more strikingly from the observations on the skin grafts of the present work. These color deeply, evenly, and surprisingly fast with the phthalein at a time when it is certain no blood can reach them, that is to say within a few minutes after they have been separated and sewn in place again. The hue they manifest is referable to staining of the subepidermal tissue fluids and tissues, especially the corium. Muscle stains but slightly with the dose of indicator I have employed; fat scarcely at all; while the epidermis is so thin and so lightly stained that it may be dismissed from account.

In recording the hues of animal and graft Ridgway's "Color standards and nomenclature" (7) has proved of great service. Wherever the hues provided by this book are mentioned in describing the findings italics will be employed. For the matching, one of the standards at a time was exposed through a hole cut in a sheet of white paper, and compared with the skin color. A normal mouse of 28 gm. given 0.5 cc. of phenol red ordinarily becomes deeply stained within 15 minutes, and reaches a maximum color, one varying between jasper red and eugenia red, about 30 minutes after the injection. The healthy avascular graft usually remains entirely unstained for from 10 to 15 minutes (Fig. 1) after the injection,—a staring, pallid patch in the midst of the red body surface,—and then it begins to turn yellow here and there. The staining reaches a maximum intensity, apricot
orange ordinarily, after a little less than an hour in all, and then appears even (Fig. 2). The hue of many mice will already have begun to fade, owing to elimination of the phthalein into bile and urine; but long after the general decoloration has taken place, a process usually completed by the end of 2½ hours, the graft remains brilliantly tinted (Fig. 3). It is still yellow more than 5 hours after the injection; for the dye leaves the avascular tissue far less rapidly than it enters it.

These are the happenings whenever the animal is stained during the first 3 or 4 days after the implantation,—and it can be stained again and again without evident injury to either it or the graft. Later, as the new vessels become effective and the penetration of the phenol red into and out of the implanted tissue does not lag so noticeably, the color of the graft comes day by day to have less of yellow and more of red in it. By the end of a week the “take” is usually perfect, and the phthalein coloration differs practically not at all from that of the host.

Abnormal Reaction of the Grafts.

The color of the stained graft seems to indicate that it is acid as compared with the normal skin round about; and there is every reason to believe that this is the actual case. Phenol red is a stable indicator, not liable to error through its association with tissue components (8) with the important exception of the proteins of plasma. Kendall (9) states that some destruction of it by reduction occurs within the organism; but the amount changed in this way is negligible when large quantities are given, as was the case in the work here reported. Dr. D. R. Drury, of the laboratory staff, has recovered from the urine of a rabbit more than 96 per cent of the phenol red required to stain it vitally, and a part of the missing 4 per cent was present in the feces, which were not extracted. For nearly 40 years (10) the fact has been generally recognized that acidity develops in tissues when their blood supply is interfered with. If a string is tied tightly around the shaven leg of a rat stained with phenol red the color of the leg turns from red to orange within a few minutes, only to become red again shortly after the cord is loosened and circulation resumed. If one evert a flap of living subcutaneous tissue vitally stained with the dye it turns from
red to purple in proportion as carbon dioxide escapes from the raw surface; and the purple can be converted to orange-yellow by brief exposure of the tissue to an atmosphere of CO$_2$ (11). These are simple instances illustrating the readiness with which the phthalein reacts under in vivo conditions and showing that it reacts characteristically.

One can appraise the color of the stained body surface of the living animal by oiling it, placing over it an Autenrieth wedge filled with water, and comparing the color as thus viewed with that obtained by superimposing a similar wedge, filled with an appropriate buffer solution colored with phthalein, over the oiled and shaved skin of a normal animal. By varying the buffer solution and moving the colored wedge until precisely the right depth of fluid is obtained, one can closely approximate the hue of the stained tissue. Apricot orange corresponds with a pH of about 6.8, jasper red with pH 7.4, and eugenia red with pH 7.6. The actual figures can be disregarded. It is their relation to each other which tells the story. Evidently vigorous skin grafts of the mouse are, relatively speaking, about pH 0.6 more acid than the normal skin, and they are able to survive this state of affairs for several days. The acidity is referable, at least in the beginning, to the elements proper to the tissue, not to the many cells that wander in (12), as is sufficiently shown by its development within an hour after implantation of the graft.

**Vigorous Grafts Are Acid, Weak Ones Alkaline.**

At an early period in the work, when the operative technic was uncertain, grafts coloring red with the phthalein were not infrequently encountered. It was natural to suppose that these, as manifesting a tissue reaction close to the normal, would be the ones to survive; and not until some thirty orange or red examples had been followed did the fact emerge that it was precisely those grafts which showed for days the orange color of an abnormal acidity that lived and healed into place. Whenever the implanted bit of skin, or a portion of it, repeatedly stained red during the early period after operation when it should have been reestablishing itself, that graft or portion was noted soon after to perish. Once this had been realized reasons for the color difference were not far to seek. Owing to poor fluid interchange the acid products of the cell metabolism of healthy grafts would tend
to heap up, just as they accumulate in subcutaneous areas temporarily deprived of circulation through the vascular contraction following on a local injection of epinephrine (13). The cells of injured tissue on the other hand not only work less actively, or dying, cease to work at all, but to a greater or less degree they lose that semipermeability which characterizes them during life. As result they are penetrated by the alkaline lymph which acts to sweep away such acid products as may arise through autolysis, and, coming gradually into equilibrium with this fluid, they tend to approach it in reaction. One may doubt whether the interstitial fabric which forms so large a part of the corium, though staining deeply with the phthalein (14), possesses life of its own in any proper sense. One must think of its reaction as determined preponderantly by the activities of the cells dispersed through it.

_Grafts Injured Experimentally Are Alkaline._

To test this explanation of the findings pieces of skin of the usual size were damaged prior to implantation. The intention was to inflict the minimum insult that would ensure an eventual failure of the graft. Heat was employed in some cases, but repeated freezing and thawing proved better for the purpose.

In order to heat the pieces of skin they were folded upon themselves with the raw surface inwards, and placed far down in test-tubes already in a water bath at 50-51°C. The grafts adhered to the sides of the tubes which had moist gauze at the bottom to prevent drying. They were heated for from 7 to 10 minutes.

When a graft was to be frozen and thawed it was spread upon a sterile mica slide with its raw surface against the latter. To prevent drying the edges were sometimes folded under; but more usually a drop of salt solution, or Ringer’s solution, was run around the edge of the tissue. The slide was then placed on the freezing disc of a microtome, the tissue frozen solid by the escape of CO₂, and the preparation removed and thawed at once with the warmth of the hand or that of a metal plate at about 38°C. The processes of freezing and thawing were carried out three or four times as rapidly as possible, and the graft was replaced in position. Meantime the skin defect on the body of the animal was covered with a sponge moist with salt solution or Ringer’s solution.

It proved easier to return the grafts to their original position when they were asymmetric or had been cut with a slight projection at one point in the periphery that fitted into a notch in the skin.

Heating to a temperature of 50°C. for 10 minutes should have killed the tissue,
under the conditions. Such heat regularly results in an eschar after 2 days, when
applied to the shaved body of rats by way of a glass disc through which hot water
circulates. But it was only after the lapse of about a week that the heated grafts
became evidently necrotic. Until then they maintained the aspect of life, being
pliable though definitely swollen and somewhat more opaque than control grafts
on the other side of the same animal. They continued to be pallid, however,
long after healthy grafts had begun to show the flush of a renewed circulation;
and eventually they dried, remaining adherent over the advancing edge of the
skin as it encroached on the defect beneath them.

If the appearance of the heated grafts was for a long time much
like that of surviving tissue this was never true of their reaction.
From the moment that they were sewn back in place they were always
frankly alkaline to phenol red, as alkaline to appearance as the sur-
rounding normal tissues and occasionally somewhat more so, being
then of a more purple hue (Fig. 2).

Repeated rapid freezing and thawing, unlike heating, failed to render
a graft more opaque than ordinary, and when first sewn in place
it had precisely the appearance of the control graft on the other side of
the same animal. Yet freezing and thawing is known to kill mamma-
lian cells of many sorts; and I have repeatedly utilized it to destroy
the cells of a transmissible chicken sarcoma without injury to the
filterable agent responsible for the growth. It did cause eventual
failure of the skin grafts. These retained their appearance of life
for a week or 10 days, though, and often developed what appeared
to be a surface union with the surrounding tissues. They never
vascularized, however, but remaining pallid, and, becoming gradually
thinner and parchment-like, they dried up. Unlike heated grafts
they were never in the least edematous. From first to last the
frozen and thawed skin stained red with phthalein, often a more
purple-red than the animal.

It was, as has been stated, a part of the plan of the experiments to
injure the implanted skin only to the extent necessary to insure a
failure to “take.” For, obviously, greater tissue changes would have
lessened the chance that the graft would remain placed and in condi-
tion to take the stain, and have complicated the interpretation of the
results. That the heating was close to the critical amount appears
from the fact that a graft submitted to only 48°C. for 7 minutes
stained *apricot orange*, as does a healthy graft, immediately after it has been replaced in position, *coral red* 24 hours later, and it only attained to the *jasper red* of the surrounding body surface on the 2nd day after operation. The long persistence of the frozen and thawed grafts and their temporary union with the surroundings also bespoke the minimum damage compatible with the purpose in hand.

The experiments support the view that the orange staining of the grafts destined to “take” is referable to acid products arising out of the life processes taking place within them, and that the ruddy hue of grafts doomed to fail results from an impairment of such processes.

*The Altered Permeability of Injured Grafts.*

In not a few instances the avascular implants injured by freezing colored up more rapidly with phthalein than did control implants on the opposite side of the animal (Fig. 1). The latter, though, were somewhat edematous which might well have interfered with the fluid exchange; while furthermore the orange color of the phenol red penetrating into them was not so readily to be perceived as the purply rose in skin that had been frozen and thawed. For these reasons the difference in the rate at which the grafts stained could not be certainly ascribed to a loss of semipermeability of the injured tissue; and it was necessary to resort to special experiments, now to be described, to determine whether such a loss had occurred. The injured grafts retained phenol red for quite as long a period after decolorization of the host (Fig. 3) as did their healthy fellows—a fact which would indicate that the acidity developing in the latter did not lead to any unusual fixation of the dye upon them, like that encountered by Kendall in another relation (15).

For the tests of relative cell permeability in healthy and injured implants erythrolitmin has been employed. The color of animals vitally stained with this indicator is due in the main to an intracellular storage of it; and through local changes in the hue one is enabled to perceive at once when the barrier of semipermeability normally maintained by the cells is broken down. The highly diffusible phenol red is not segregated within cytoplasm, under ordinary conditions at least, and hence it could not be utilized for the work.
Rats and mice repeatedly injected with litmus, or with erythrolitmin, the effective constituent of Kahlbaum's cube litmus as at present available, have much of the indicator segregated within the cells of the subcutaneous tissue, though there is also some diffuse distribution of it throughout the intercellular material. The diffusely distributed litmus is always in the alkaline or blue form, whereas the segregated is red (16). As result of a combination of the two sorts of staining the living animal appears violet-pink for some weeks after injection. Everywhere throughout the corium one finds microscopically that the cells are crowded with pink or red globules, while the intercellular fabric has an even, light blue tint. Injury to the individual cells results in an immediate color change of the globules from red to blue, and the litmus diffuses from them secondarily, staining the cytoplasm and nucleus blue (17). A similar secondary staining under such circumstances with stored vital dyes having no indicator properties has long been familiar to cytologists; and it is plainly referable to a loss of the normal semipermeability. The change from red to blue of the litmus-containing intracellular granules which occurs prior to diffusion of the dye from them is traceable to a like event, the acidity that had prevailed within the segregated material being lost as the alkaline tissue fluids penetrate to it through the cytoplasm. Any gross trauma to the skin of the stained and living animal results at once in a change in the color of the region affected from violet-pink to a sharp blue.

For the purpose of the present tests mice were three times injected into the peritoneal cavity at intervals of a day or more with 0.35 cc. of a 2 per cent solution of erythrolitmin in 0.9 per cent NaCl. Between 1 and 2 weeks later skin grafting was carried out, according to the accustomed technic; but the graft was frozen and thawed three or four times before it was sewn into place. In several animals a control graft of the same size was removed from the other side and replaced at once.

When taken for freezing the graft had the same color as the rest of the integument—a hue approximating the light vinaceous lilac of Ridgway (Fig. 5). But immediately that it had been frozen and thawed it changed to a brilliant blue, Blanc's blue or Yale blue (Fig. 5); and this hue it retained as long as it continued to fill the skin defect. This it did for the period usual with such frozen and thawed material, remaining flexible, and translucent for a week or more, during which period a smooth union with the neighboring skin often appeared to take place.

Despite the most careful handling the healthy graft placed on the other side of the animal also changed somewhat from the hue it had prior to separation. Wherever it was held with forceps or thrust through in the course of the suturing it became blue, as did also the normal skin so treated; and in addition, after it was sewn back, it was noted to be somewhat bluer throughout than its surroundings were (Fig. 5). Next day this unusual color was still as pronounced as at first, and

1 The erythrolitmin was prepared by a modification of the older methods, which will be described by Dr. P. D. McMaster in a forthcoming paper.
only toward the end of the 1st week, as the graft became established, did the hue gradually revert to the "normal."

The expectation had been that the blue erythrolitmin liberated in the frozen and thawed graft by death of the cells would be carried off little by little in consequence of the local fluid interchange, and that in consequence the color would become much lighter. But, as just mentioned, even the relatively slight amount of blue pigment liberated in the healthy graft persisted as such in it for several days. Erythrolitmin has an affinity for intercellular substances, which remain blue for months after the coloration has disappeared elsewhere (18); and it is highly colloidal, passing to and from the tissues with difficulty. In the light of these facts there is no need to invoke chemical alterations in the fabric of the several times frozen graft to explain its enduring blue color.

The observations demonstrate that the cells of grafts frozen and thawed repeatedly lose their semipermeability. That the change takes place everywhere and all at once may be doubted however: for not only do the grafts retain the aspect of life for many days but their color by transmitted light, prior to implantation after the injury, is ruddy here and there, showing that some of the erythrolitmin still persists in the red form. Only by reflected light is the hue a deep, clear blue.

Permeability of Skin Grafts for Carbon Dioxide.

The increased permeability of the damaged, avascular skin graft has been shown in another way, namely by submitting the body of a mouse carrying it and a healthy graft to an atmosphere of carbon dioxide. The gas penetrates the injured skin with immense rapidity, rendering it acid.

It was Lavoisier himself who first showed that carbon dioxide passes out of the intact skin of mammals; and his observations have been often repeated, with variations. When the skin is hyperemic and moist a not inconsiderable gaseous interchange may take place through it, as much as 4 per cent of the total CO$_2$ being eliminated in this way (19). To the present no experiments seem to have been made on the penetration of CO$_2$ from without. Skin grafts in situ are ideal for a study of the phenomenon since their avascular condition creates an opportunity for the gas to accumulate within them, as it cannot to any considerable degree within the normal integument.

For the purpose of the tests there has been utilized the funnel gas chamber devised for a study of the changes in pH occurring in raw tissue surfaces (20). The CO$_2$ was let in at $D$ in the apparatus as figured in the paper referred to, and
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it escaped, in some part, through the other tube E, which had now been provided with a connection leading off to the floor. The mouse lay on top of two layers of rubber dam which in turn were spread upon an electrically heated pad. The animal had been stained with phenol red as usual and rendered quiet by the intraperitoneal injection of 0.15-0.2 cc. of 20 per cent urethane shortly before the observations were begun. These latter were only undertaken 2 days or more after the body surface of the animal had been shaved and the grafts placed, in order to rule out the possibility of any entrance of the CO₂ through surface abrasions. The hair had been removed from about the neck of the animal with a sodium sulfide solution; and a round hole in the rubber dam closing the gap at the margin of the funnel fitted the neck snugly. The edges of the dam were everywhere attached to the funnel by adhesive except below, where the rubber extended along the pad in a broad apron ending 6 to 10 cm. away from the head of the mouse. In order to rule out any possibility that the animal might inhale CO₂ a second piece of rubber dam was placed about its neck, over the first, to block off the gas chamber more completely; and the head was thrust into a small funnel through which a continuous gentle stream of air was blown against the nostrils. Tests with smoke after the experiments showed that some CO₂ escaped from beneath the funnel here and there, as well as through the proper outlet for it, but that none whatever could have been inhaled. A thermometer was introduced into the gas chamber through separate piercings in the layers of rubber dam, with the bulb lying between the groin of the mouse and the heating pad. The temperatures ranged between 36° and 38°C.

When an atmosphere consisting entirely of CO₂ was to be used the gas was led in from an ordinary Kipp generator after passage through a wash-bottle. The water in this latter never contained more than a trace of HCl, and there would seem to be no possibility that the effects on the skin surface of the mouse can have been due to another cause than carbon dioxide. The rapidity with which they disappeared when the surface was once again exposed to air also bespeaks the action of the gas. In some special experiments a mixture from a compression cylinder, containing 10.35 per cent CO₂ and approximately 20 per cent O₂ and 70 per cent N₂ was employed. Dr. C. A. L. Binger kindly determined the percentages.

When comparisons were to be carried out with the Ridgway color standards the stopper was briefly removed from the top of the gas chamber so that the inspection could be made without the intervention of a glass wall. Access to the body of the mouse could also be had in this way without changing the gas. To replace the latter with air a tube was thrust in and the chamber emptied almost instantaneously with the aid of the laboratory vacuum.

On running pure carbon dioxide into the chamber a change could be noted practically at once in the color of the frozen and thawed, avascular graft. It began to turn from eugenia red through apricot orange to a brilliant untempered orange, reaching this hue within 8 or 10 minutes (Fig. 4). Further exposure to
CO₂ did not alter the color for the good reason that the acid end of the range of phenol red had been attained. The control graft on the opposite side of the animal was apricot orange or apricot buff to begin with; and during the brief period in which the frozen and thawed tissue was running the gamut from purply red to orange it altered slightly, to zinc orange; but it obviously lagged in changing color as compared with the injured tissue and was still definitely less orange than the latter after 15 minutes, the maximum period of the observations (Fig. 4). On the other hand when once again exposed to the air it kept its unusually intense orange color long after the frozen and thawed skin had once again become eugenia red. This happened within about 15 minutes.

During the time that the grafts were undergoing these changes a slight but definite alteration was to be noted in the color of the body surface generally. It turned from red toward yellow, that is to say from eugenia red to jasper red in the case of animals approximating the first mentioned hue, and from jasper red to carrot red in some other individuals. There is, by the way, not a little variation in the surface hue of normal animals stained with phenol red, as the mention of these differing initial colors will attest. Variations in the normal pH of the blood, similar in magnitude to those here indicated by the phthalein have, of course, long been recognized to exist.

In order to determine whether the general change in color of the animals subjected to CO₂ was referable to absorption of this gas by way of the grafts or to a passage of it through the undisturbed skin everywhere, normal mice were shaved from fore legs to hind and 2 days later were exposed to pure CO₂. There ensued changes in the general hue identical with those just described. At the end of three-quarters of an hour they were no more marked than after 15 minutes. They were indicative of an apparent fall in pH from about 7.6 or 7.5 to 7.4 and 7.3 respectively. The rapidity with which the color reverted to the “normal” when the mouse was once again exposed to air was startling. Within 3 minutes the change had been completed. The surface acidosis described evidently resulted from a continuous passage of CO₂ through the skin, one not entirely compensated for locally, in the surface regions at least, by circulatory and respiratory readjustments. In a number of animals the rate and amplitude of the breathing were followed throughout the experiment. Changes accompanied the exposure to CO₂, and doubtless analyses of the expired air would have shown that no inconsiderable quantity of the gas, in addition to that resulting from body processes, was being given off through the lungs.

Water never condensed within the funnel chamber out of the CO₂; and the skin of the mice was as dry to the feel as ordinary. The findings certainly cannot be laid to the presence of an abnormal amount of moisture on the skin surface. Moistening the grafts led, as was to have been expected, to a more rapid penetration of CO₂ into them. To demonstrate this drops of distilled water were placed here and there on the skin and on the grafts before the gas was run in. They stood high and hemispherical, like dewdrops; and after various periods were
removed. It was possible to do this with no other alteration of conditions within the chamber than were involved by the introduction from above of a long-handled forceps carrying a piece of filter paper to blot up the fluid. At times when the injured graft was becoming acid in consequence of the exposure to CO₂ but had not yet attained to the hue of orange a narrow circle of this hue could be briefly seen after the application of the filter paper, a circle corresponding in situation to the edge of the drop, the place at which the layer of water had been thinnest. When now the graft was reexposed to air the regions still moist turned purple first. Healthy grafts gave less outspoken findings; and only occasionally were slight differences of the general nature of those just described to be seen on the intact skin.

The experiments brought out a number of facts. Carbon dioxide penetrates the intact skin of the shaved mouse so rapidly as to cause some change in the surface hue of animals stained with vital red; it renders vigorous skin grafts somewhat more acid than they already are as the result of ischemia; and it penetrates injured grafts with an astonishing rapidity, rendering them pronouncedly acid. How acid the injured tissue can become is not yet certain, for no indicator other than phenol red has been employed.

Even in vigorous skin grafts there is much cell degeneration and death. The epithelium in particular is soon reduced to a thin layer of living cells (21). When the tissue has been injured experimentally the retrograde changes must be still greater; and one might think of the heated, or frozen and thawed, graft as a mere raw dead surface exposed to the air, did not its texture and the absence of seepage or drying belie the view. Doubtless the alkaline reaction of grafts thus injured is due in some part to an escape of carbon dioxide from them. The experiments involving exposure for a long time to a gas mixture containing 10.35 per cent CO₂ possess significance in this connection. Exposure to such a proportion of CO₂, approximately twice that in alveolar air, leads to an alteration in the hue of injured grafts,—there is a change from eugenia red (or purpler) to carrot red; but the alteration is slight, as compared with the change to apricot orange promptly undergone by stained pieces of healthy skin when grafted in an atmosphere of air. Exposure to 10.35 per cent CO₂ does not result in any definite color change in the mouse’s body surface generally, and leads to only dubious ones in healthy grafts already in situ.
The Association of Tissue Acidosis and Edema.

Healthy skin grafts are regularly somewhat edematous during the first few days after implantation, at the time that is to say when the local reaction is acid as compared with that of the rest of the integument. A similar association of edema with tissue acidosis is not infrequent under other circumstances,—prevailing opinion and potentiometric determinations to the contrary notwithstanding. It can be observed to exist about surface abrasions in animals vitally stained with phenol red, as well as at other points of local inflammation, the phthalein in the swollen areas being orange as compared with the red of that in the normal skin nearby. And a rapid local development of edema and acidosis takes place when a tube through which water circulates at 50-52°C. is applied to the skin of an anesthetized rat stained with phenol red. Under such circumstances the edema and a change from red to orange of the phthalein contained in the region involved by it both become pronounced within 15 minutes.

In contrast to the swelling and acidity manifested by healthy grafts one sees in frozen and thawed grafts not the least edema, and a reaction which is definitely alkaline. The facts are not to be taken, however, as furnishing support to the hypothesis that acidosis determines edema. Edema develops, yes, in the living and acid skin graft, but it progressively disappears during the days immediately after the grafting, whereas the acidosis does not diminish. Furthermore edema is regularly to be met in grafts that have been injured by heat, although the reaction of the injured tissue is pronouncedly alkaline. In a succeeding paper observations are reported which would suggest that under the conditions of widespread and enduring tissue acidosis brought about with hydrochloric acid no important water retention occurs.

DISCUSSION.

The foregoing observations provide data on the conditions which prevail in skin grafts and determine their survival; but it is in a broader relation that they have principal claim to attention,—namely, in relation to the happenings within ischemic tissues as a class. Despite the general recognition that interference with the blood supply
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of a living tissue results in the local formation of acid substances the
corollary that ischemic tissues must endure for some time in an acid
milieu if they are to survive seems not to have had the attention that
it merits. As the present work shows, the more vigorous the cells
the more acid do they render the tissue when its blood supply is inter-
fered with; and the tissue survives despite this acidity. Skin has a
relatively slight metabolic activity, and some part of the carbon
dioxide accumulating in avascular grafts of it must escape to the air
or into the body of the animal, while a continuous, if slow, fluid in-
terchange with the neighboring tissues acts also to reduce the local
acidity. Yet notably acid the tissue is, nevertheless. This being the
case what must one suppose the reaction to be within a leg severed and
reunited without suture of the vessels (Halsted), or in an arm surviving
despite a clot in the axillary artery? Can one doubt the development
of a more pronounced acidity in these ischemic members?

That cells of some sorts will survive and proliferate in vitro in a frankly acid
medium is a fact sufficiently attested (22). But the occurrence of proliferation
renders the case somewhat different from that of tissue surviving within the body
under acid conditions. For it might well be that the individual elements of a
culture tolerate the condition of acidity but poorly, and that the strain survives
only because its elements continue to divide, furnishing fresh entities more rapidly
than the injurious medium kills them oft. An analogy to this state of affairs is to
be found in the growth of certain tumors composed of cells surviving for so short
a time that retrogression of the mass would inevitably ensue were not the rate of
wastage more than outstripped by that of proliferation. The condition is one
familiar to every student of neoplasms. It is often plainly evident when “Chicken
Tumor I,”—a transmissible sarcoma,—is cultivated in vitro.

No attempt has been made in the present work to determine more
than approximately the degree of acidity developing in the grafted skin
of the mouse. Manifestly the knowledge could have only a special
interest; for one would expect a much greater acidity to develop in
tissues of high metabolic activity. To determine the relative reaction
within ischemic portions, living and dead, of organs which appear to
be frankly acid under normal conditions,—the liver and pancreas for
example,—will be an interesting task for the future.

Current generalizations on the changes which take place within
tissues dying in the body are largely based upon studies of material
autolyzing in the mass *in vitro*; and perhaps they can be safely applied to large masses autolyzing *in vivo*. The interchange of material with the body round about will have little significance for the immediate fate of large infarcts and accumulations of pus despite the unusual permeability of dead tissue. But when the necrotic mass is small the factor of interchange assumes great importance, as the experiments with the skin grafts show. The occurrences subsequent to the injury of small cell aggregates cannot be explained on the basis that a local accumulation of acids determines autolysis or atrophy (23), for the sufficient reason that the local reaction will tend toward alkalinity rather than toward the acidity of vigorous tissue surviving an ischemia. It follows that the chemical changes which take place in small necroses must differ in some respects from those occurring in large masses of dead tissue. A single set of generalizations as concerns autolytic processes will not cover both instances.

While the life processes of vigorous tissue suddenly rendered bloodless act to create a milieu that would seem prejudicial to survival, the alterations that take place in injured tissue would appear superficially to favor this event. To judge from the observations on skin the lessened cell activity consequent upon injury results in a smaller accumulation of acid material,—save for that referable to the trauma itself (as in muscle); and the increased permeability of the damaged tissue results in a more rapid escape from it of carbon dioxide and of the other substances causing acidity. But needless to say the rough correspondence thus brought about between the reaction of the injured graft and the normal tissue surrounding it is a superficial phenomenon, not the sign of a good state of affairs but of one which masks profound cytological derangements.

The eventual drying of grafts which fail to “take” is not due to any failure to obtain fluid from underneath, as the experiments have shown. Rather must one think of it as consequent on an abnormal loss of fluid from the surface, itself a manifestation of the increased permeability which can be demonstrated by exposure of the tissue to carbon dioxide. There is good reason to suppose that the reaction of the tissue involved in skin lesions often deviates from the normal owing to the influence of the factors dealt with in the present work.

The observation that edema and tissue acidosis sometimes coexist
would at first sight seem difficult to reconcile with the fact that edema fluids as obtained for potentiometric examination are regularly alkaline (24). But the contradiction is merely apparent. Local acidosis and edema occur together only when the fluid accumulation is not very pronounced and the metabolic activities of the tissue are either abnormally heightened,—as during inflammation,—or are taking place under conditions which permit of an accumulation of acid products,—as in skin grafts. There is little doubt that the development of edema can act to maintain alkalinity, the profuse alkaline fluid deriving from the blood having effect to drown out, so to speak, what might otherwise be a local acidosis. I have never found a very pronounced inflammatory edema that did not yield a fluid alkaline to phenol red, although inflammation as such conduces to local acidosis. And Henning (25) who injected n/10 HCl into the leg muscles of guinea pigs, observed that the initial acidity was supplanted after 24 hours by a pronounced local edema and alkalinity. It is conceivable that sometimes during the development of an inflammatory edema there may be such an escape of blood protein into the tissues as will suffice to influence phenol red, with result in a greater alteration in the color of the phthalein than the actual acidosis would warrant. No such happening can be invoked to explain the case of edematous skin grafts, however.

Some comment is necessary on the difference between the hue of the skin surface of the vitally stained mouse, as seen by reflected light, and the color of the corium—the skin component principally stained with phenol red—when viewed by transmitted light. The purply red of the former corresponds with pH 7.5 to 7.6, as ascertained by the wedge method described in the present paper; whereas the connective tissue of the corium, when examined separately under oil, has the yellow-pink of about pH 7.2 (26). The reasons for this difference—one which persists when the blood vessels have been flushed out—are largely to be found in the differing optical conditions, but also in some part in the suffusion of the tissue with an alkaline lymph, heavily charged with phthalein, and in consequence ruddy. When a flap of the oiled skin surface of a deeply stained mouse is compressed between slides so that the interstitial fluid is forced out of it for a moment its color is altered from yellowish red to orange.
The observations here set forth may, perhaps, be thought of as the first steps in an analysis of the physical factors which act to determine the fate of engrafted tissues. It is habit to suppose that this fate depends on the ability to survive temporary ischemia, on absence of infection, prompt vascularization and, in the case of iso-grafts, on a tolerance by the host of the strange tissue, and by the graft of its alien surroundings. So of course it does. But the first requisite for survival, namely the ability to survive ischemia is directly referable to physical conditions within the graft, as is also, doubtless, the development of the vasculature that will eventually rescue the tissue from its precarious state.

Gesell has recently brought forward (27) a theory of respiratory control based on the assumption that changes in the hydrogen ion concentration of the respiratory center are the responsible influence rather than changes within the blood. The happenings within skin grafts furnish a suggestive analogue to what goes on within the center according to the view of this author.

SUMMARY.

By means of vital staining with indicators a study has been made of the changes in reaction and in certain other attributes of a tissue abruptly rendered ischemic. Grafts of mouse skin have been employed as test material. It has been found that almost at once after implantation vigorous grafts become notably acid as compared with the normal skin and that they survive and "take" despite the acid condition, which remains at a maximum for several days. Weak or injured grafts on the contrary tend to be as alkaline as their surroundings, if not more so. Through experiments directed to the purpose reasons for this difference have been found in the lessened metabolic activities of the cells of the injured skin, and in an increased permeability which leads to a generalized suffusion of the damaged tissue with the alkaline lymph and an abnormally rapid escape of carbon dioxide from it. The influence of these factors to determine the reaction of tissues dying within the body has not been sufficiently taken into account in considering the chemical changes that occur after cell death, and some revision of current views regarding these as they affect small necrotic masses would seem called for.
Carbon dioxide penetrates so readily into the living skin as to cause some local increase in the hydrogen ion concentration within cutaneous regions exposed to an atmosphere of it, even when the local circulation and the ventilation by way of the lungs have not been interfered with. It penetrates injured skin with especially great ease. Tissue acidosis and edema not infrequently occur together; but no relationship between them of cause and effect has been made out.

BIBLIOGRAPHY.

EXPLANATION OF PLATE 32.

FIGS. 1, 2, and 3. Right and left sides of a mouse, to show the course of the vital staining of grafts with phenol red. Period: any time during the first 3 days after implantation.

FIG. 1. The staining 15 minutes after an intraperitoneal injection of the indicator. The healthy graft shows no color as yet, whereas the one that was frozen and thawed has become pink. The animal is already deeply colored.

FIG. 2. 45 to 60 minutes after the injection. The grafts are now intensely stained, the hue of the healthy one being indicative of a condition of relative acidity, whereas that which has been injured would seem to be slightly more alkaline than the normal tissue round about, judging from its color. The mouse itself is beginning to decolorize.

FIG. 3. 3 hours after the injection. The animal is now completely decolorized but there is still much phenol red within the grafts and the same differences are visible in them as before.

FIG. 4. Effect on the phenol red coloration of exposure of the body surface to an atmosphere of carbon dioxide for 15 minutes. The injured graft is now no longer alkaline but frankly acid, as evidenced by its hue; and the healthy graft while slightly more acid than ordinary is not as acid as the injured graft. The color of the body surface generally is indicative of a slight change in the direction of acidity.

FIG. 5. Right and left sides of a mouse stained with erythrolitmin, to show the alterations in color of engrafted skin and of skin that has been purposely damaged as well as engrafted. The healthy graft is slightly bluer than the body surface generally, a fact which attests to some injury to its cells with a loss of the normal semipermeability; and the graft which was frozen and thawed is deep blue,—evidence that the cells have become freely permeable to the alkaline body fluids.
Healthy grafts

15 minutes

45-60 minutes

180 minutes

Exposed to CO₂

Stained with erythrolitmin

(Rous: Relative reaction within living tissues, VI.)