Chemical Acidification of Wounds

An Adjuvant to Healing and the Unfavorable Action of Alkalinity and Ammonia

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FAILURE OR DELAY in wound healing occurs rather commonly in contaminated wounds even in the absence of invasive pathogens. Some granulating wounds not only fail to heal, but occasionally the surface is covered with necrotic slough. In the absence of pathogens, a reason for the healing defect is obscure, and therapy is decided by trial and error. If healing is impaired after an anastomosis of the large bowel, leakage can cause fatal peritonitis. Colonic anastomose normally heal quite slowly, and the development of tensile strength is delayed as compared to the clean anastomosis of stomach and small bowel.[18] Leakage is especially frequent in low colonic anastomosis[7,14] and constitutes a clinical problem. Could there be some single factor which might account for delayed healing of contaminated wounds in a diversity of clinical situations?

One requirement of all healing tissues is an available supply of oxygen. Molecular oxygen is required for the synthesis of collagen and the growth of fibroblasts. Even under normal circumstances, less oxygen is available for healing in wounds than is optimal for tissue needs.[10] Open wounds created in rats by excision of skin heal more rapidly if the rats are placed in an atmosphere of 40–50% oxygen rather than 20% oxygen.[8] Winter and Perins[24] found that epitheliazation of rat wounds was enhanced by exposure of the animal to hyperbaric oxygen. Topical hyperbaric oxygen has been used as an adjuvant to accelerate the healing of bed sores.[5] These experiments confirm that the healing rate of wounds is restrained by the amount of oxygen available and that hyperbaric oxygen effectively accelerates wound healing.

More recently, factors which influence oxygen transport to the tissues has come under scrutiny. The conversion of oxyhemoglobin to reduced hemoglobin is catalized by 2–3 diphosphoglycerate (DPG) and the enzymes which control its release in the human erythrocyte.[1] Erythrocytes deficient in 2–3 DPG will release oxygen only at abnormally low oxygen tensions. Such a defect in the oxygen transport mechanism can produce considerable tissue anoxia. The ability of bank blood to transfer oxygen is considerably impaired in direct relationship to the period it has been stored. This can produce a circumstance where massive transfusions will fail to restore tissue respiration, since the transfused blood no longer carries sufficient oxygen. Similar defects have been found in disease.[13] Any circumstance which significantly impedes the release of oxygen from oxyhemoglobin would impair oxygen transport to the tissues sufficiently to interfere with wound healing and to cause tissue necrosis.

Oxyhemoglobin releases its oxygen more readily in an acid environment. In the classical Bohr effect, the greater affinity of deoxyhemoglobin for protons results in a decreased affinity of hemoglobin for oxygen. Physiologic situations take advantage of the Bohr effect. During exercise, lactic acid and carbon dioxide accumulate in the tissues and facilitate the conversion of oxyhemoglobin to reduced hemoglobin, thereby assuring the maximum release of oxygen for tissue needs and the greatest possi-

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ble A-V oxygen difference. Exercise drops the surface pH of the muscle precipitously.

The adverse effect of alkalinity on tissue oxygen availability can comparably deprive wounds of oxygen by stabilizing oxyhemoglobin. Some bacteria produce ammonia which in itself is necrotizing but which could impair oxygenation of the tissues by raising the pH. Even small changes in pH could induce wide changes in wound oxygen concentration. The effect of pH on the standard oxygen dissociation curve as obtained by Dill is shown (Fig. 1). Although this curve has been more recently refined,[15] its shape, for the most part, remains unchanged. Notice that as the pH decreases, the standard oxyhemoglobin dissociation curve is moved to the right. At low oxygen tensions, a change as small as 0.4 of pH unit will appreciable enhance the release of oxygen from oxyhemoglobin. Hunt[9] has shown that the pO_2 of the early healing wound is about 20mm Hg. The standard dissociation curve at 20 mm shows that oxyhemoglobin constitutes 38% of total hemoglobin at pH 7.6 and 20% at pH 7.0 (Fig. 1). Notice that a shift of pH by only 0.6 releases almost 50% more oxygen. Any factor which causes even a small change in the pH of the healing wound might appreciably alter the available supply of oxygen to the tissue. Conversely, even mild acidification of a wound might substantially hasten healing by enriching the supply of oxygen to the tissues. Such an attractive theoretical premise warrants the confirmation of experimental method.

The present study examined the affect of pH alteration on wound healing and the potential of utilizing the Bohr effect to increase the availability of oxygen to the tissues. The pH of healing surface wounds were measured, and explanations for diviations from the pH of normal plasma were sought. Since alkalinity of the wound would result in an inappropriate Bohr shift and deprive the healing wound of oxygen, factors which influence the alkalinity of surface wounds such as growth of ammonia producing bacteria and CO_2 diffusion from the open surfaces were examined. Since ammonia is in itself a toxic substance, the necrotizing effects of ammonia must be carefully separated from the effect of alkalinity on oxygen transport. The effect of pH on the toxicity of ammonia for cells must be examined in vitro where the oxygen transport system is not involved. Also, the role that ammonia plays in the necrosis produced by urease producing micro organisms should be evaluated in animals immunized to urease. Finally, the comparative effects of various acidifying agents should be studied as to their proficiency.

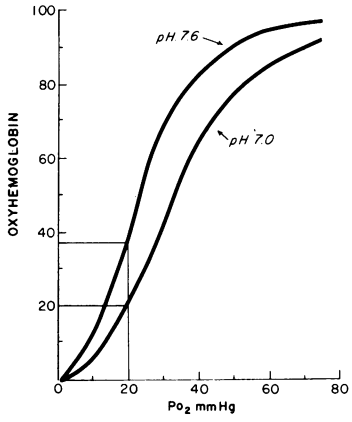

FIG. 1. The percentage of oxyhemoglobin is plotted against partial pressure of oxygen. These are the standard hemoglobin-oxyhemoglobin dissociation curves. Notice that alkalinity shifts the curve to the left which tends to keep oxyhemoglobin fully saturated even at low oxygen tensions. The partial pressure of oxygen usually found in wounds is about 20 mm Hg. A shift of the pH by 0.6 almost doubles the quantity of oxygen released.

Experiments

Since the Dill curve covers only a small pH range, the oxyhemoglobin dissociation was measured over a wider range of temperatures, pH and varying O_2 tensions so that comparisons could be made to wounds.

In another experiment, freshly drawn serum was placed in a petri dish and exposed to the atmosphere. Serum pH was measured periodically to acertain whether loss of CO_2 would cause a shift in hydrogen concentration.

The pH of 137 surface wounds in 46 patients were measured at random using narrow range pH papers (ranges 4–6 6–8 8–10) or a glass electrode. The state of healing of the wound was scored on initial observation. Twenty-seven split thickness skin grafts were tested for pH of wound on initial removal of the dressing. The pH of non-healing areas was correlated to that of healing portions. Wound dressings of these patients were assayed for ammonia by boiling dressings in NaOH and utilizing a simplified test paper to determine the presence of liberated ammonia.

An experiment was set up to perfuse the wound with saline solution at varying pH ranges in a closed system. Acidification was accomplished with lactic acid and alkalinization with sodium bicarbonate. Air was excluded from the system, and the wound was isolated with a heavy plastic membrane (1/16 vinyl) so that diffusion of atmospheric oxygen and loss of CO_2 would not introduce error. The perfusion fluid was measured for pH, temperature and pO_2. The latter was accomplished potentiometrically using a Clarke electrode. A schematic of the apparatus is shown (Fig. 2). A patient's wound being studied in this manner is shown (Fig. 3). A closed system was chosen to preclude the loss of carbon dioxide which might alter wound pH.

Various solutions and gels were tested on wounds to determine how long a single application would maintain an acid pH. A 7% aqueous mixture of Carbopol 934 makes a firm jelly. Carbopol is a polycarboxylated vinyl polymer with a high base binding capacity. An aqueous solution was prepared of polycarboxylic acid which has a molecular weight in the range of 250,000. Polyacrylic acid is synthesized by polymerization of acrylic acid. It also has a high base binding capacity since every

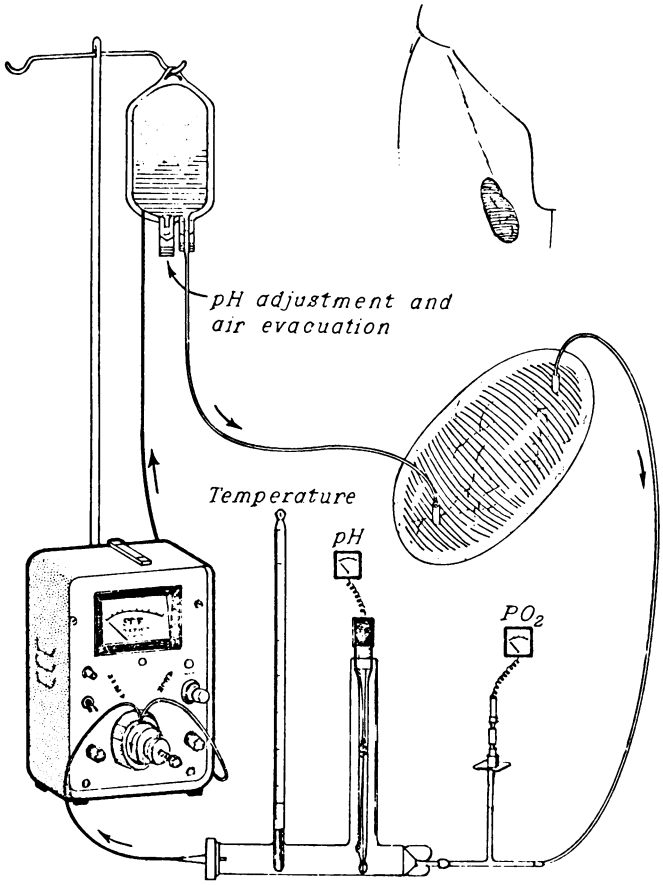

FIG. 2. A wound is sealed from the air by a plastic sheet. An electrolyte solution of varying hydrogen ion concentrations is pumped over the wound. Changes in pH and PO_2 of the irrigant are measured.

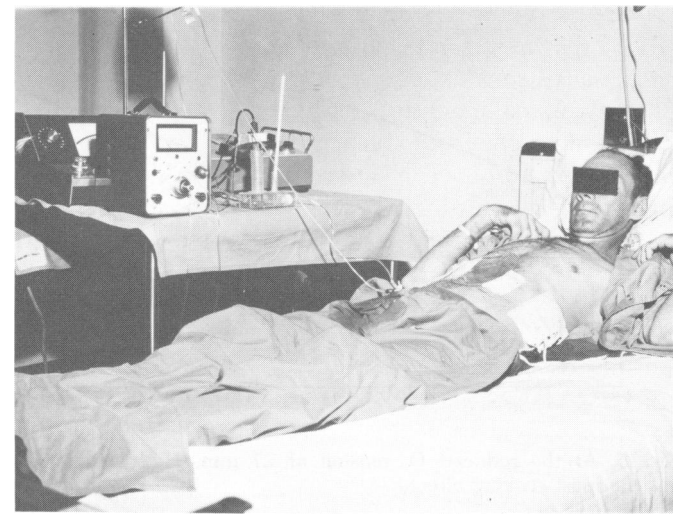

FIG. 3. An experiment in progress. Electrolytis solution is being pumped under a plastic seal on a patient's wound. The perfusate is being tested for temperature, pO_2, and pH.

third carbon is a carboxyl group. A 20% solution of this material makes a clear, viscid liquid which is well tolerated by tissues. A few patients have complained of a mild, stinging sensation. Titration curves were made to compare the base binding capacity for ammonia of 7% carbopol, 20% polyacrylic acid and 1% acetic acid.

The large intestine of a dog was devascularized according to a scheme devised by Cohn.[2] A Clarke electrode was placed under the submucosa (Fig. 4). The bowel content was then acidified by continuous irrigation with 0.5% acetic acid and changes in pO_2 were measured.

To study the toxicity of ammonia on tissues, mice were injected with varying concentrations of ammonia in a normal bicarbonate solution. The following concentrations were used giving .1cc of each concentration. 0.6% NH_4OH, 0.06% NH_4OH, 0.006% NH_4OH. Since ammounia is a hemolytic agent, varying concentrations of NH_4OH were tested *in vitro* against human erythrocytes at varying hydrogen concentrations from pH8 to pH6.5. The following concentrations of NH_4OH were used; 5mg%, 10mg%, 15mg%, 25mg%, 50mg%, 100mg%, 500mg%. Hemolysis was recorded after incubation for 1 hour at 37°C.

Experiments were done on a wild strain of *Proteus morganii* to determine whether urease contributed significantly to the tissue reaction which results from the injection of live bacteria subcutaneously. The bacteria were grown in a standard broth culture and were separated from the culture medium by centrifugation. After washing, a saline suspension of bacteria was prepared so that each cubic centimeter contained 1.2 billion bacteria. The bacteria were injected into the subcutaneous tissues of the back of mice in doses varying from 120 to 240 million bacteria. Four different experiments were

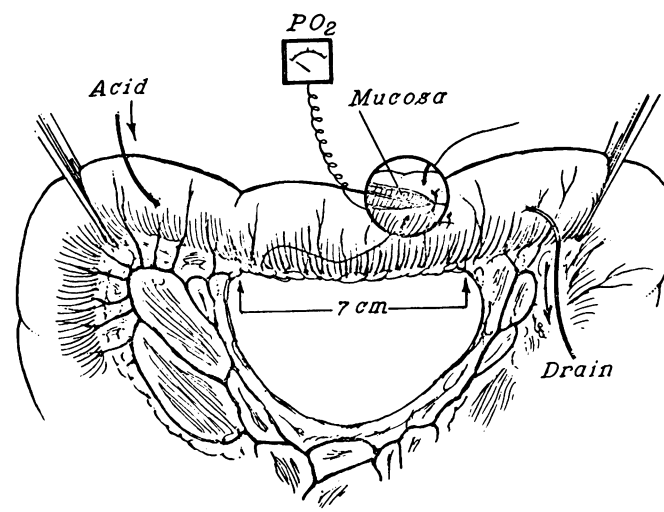

FIG. 4. A 7 cm. section of bowel is devascularized. A pO_2 electrode is carefully placed beneath the submucosa. The effect of changing the intraluminal pH can be correlated to the partial pressure of oxygen beneath the submucosa.

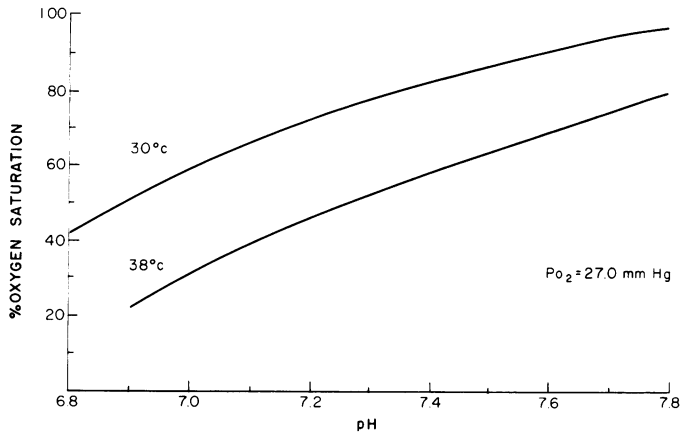

FIG. 5. At the reduced O_2 tension of 27 mm. Hg., acidification has the most striking effect.

done using 10 animals for each experiment. In one experiment the bacteria were killed by acetone which fails to inactivate the urease. In the other experiment the bacteria were heat killed which inactivates urease. In the last test the animals were injected with the live bacteria. In another group of mice, live bacteria were injected into mice which had been immunized against Jack Bean urease. The sera of animals that have been immunized with Jack Bean urease inhibits the ability of urease to convert urea to ammonia both *in vivo* and *in vitro*.[12,19] All the mice were sacrificed after 5 days by reflecting the skin of the back and examining the injection sites. Tissue was taken for microscopic examination.

Results

An expanded effect of pH on oxyhemoglobin and pO_2 at varying temperatures was determined and is charted (Figs. 5 and 6). Notice that the effect of pH on the oxyhemoglobin dissociation curve was not as pronounced at 40.5 mm pO_2 as at 27 mm pO_2. Nevertheless, the oxygen release was more than trebled by one pH unit. At 27 mm Hg and 38°C only 20% of hemoglobin was in the reduced form at pH 7.8. Considering that complete saturation is 95%, only 15% would have been released at a pO_2 of 27mm. At 6.9, 80% of the hemoglobin was in the reduced form or 75% of the hemoglobin had released its oxygen. This is a five-fold increase obtained by a shift of only 0.9pH unit!

TABLE 1. *Presence of Ammonia in 121 of 137 Wounds Examined (89.9%)*

pH Ranges With Ammonia	pH	No Ammonia Present
7	6–7	2
3	7.2	1
5	7.4	1
22	7.6	2
5	7.8	0
79	8–9	10
121		16

The failure of surface wounds to heal was almost invariable correlated with alkaline pH. Wounds which are exposed to air rapidly lose CO_2 and a local respiratory alkalosis is established. (The pH deviates toward a sodium bicarbonate solution.) Serum exposed to air gradually approached pH8. One hundred and twenty-one of 137 wounds (89.9%) contained significant quantities of ammonia on the dressings (Table 1). Only 19 wounds had a pH of 7.4 or below, while 118 wounds had a pH

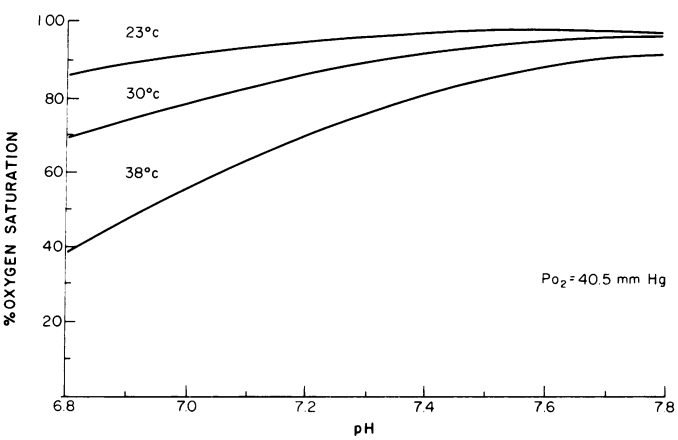

FIG. 6. The variation of the oxyhemoglobin saturation is shown for different pH's and at different temperatures. Notice that the effect is greatest at body temperature. The oxygen saturation drops from 80% to 40% when the pH drops by one unit. This represents almost a threefold increase in the available oxygen.

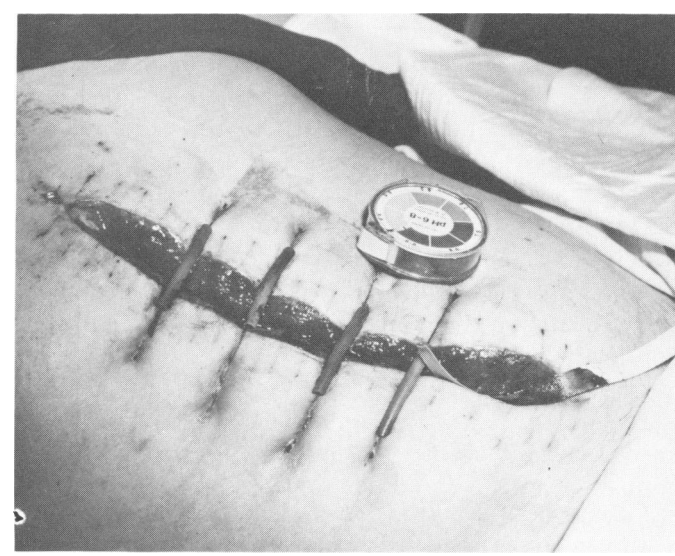

FIG. 7. Narrow range pH (6–8) paper is being used to determine the pH of a patient's wound. Notice the extreme alkalinity. The pH is slightly greater than 8. The dressings contained considerable ammonia. The healthy appearance of the granulation indicates a poor reduction of oxyhemoglobin.

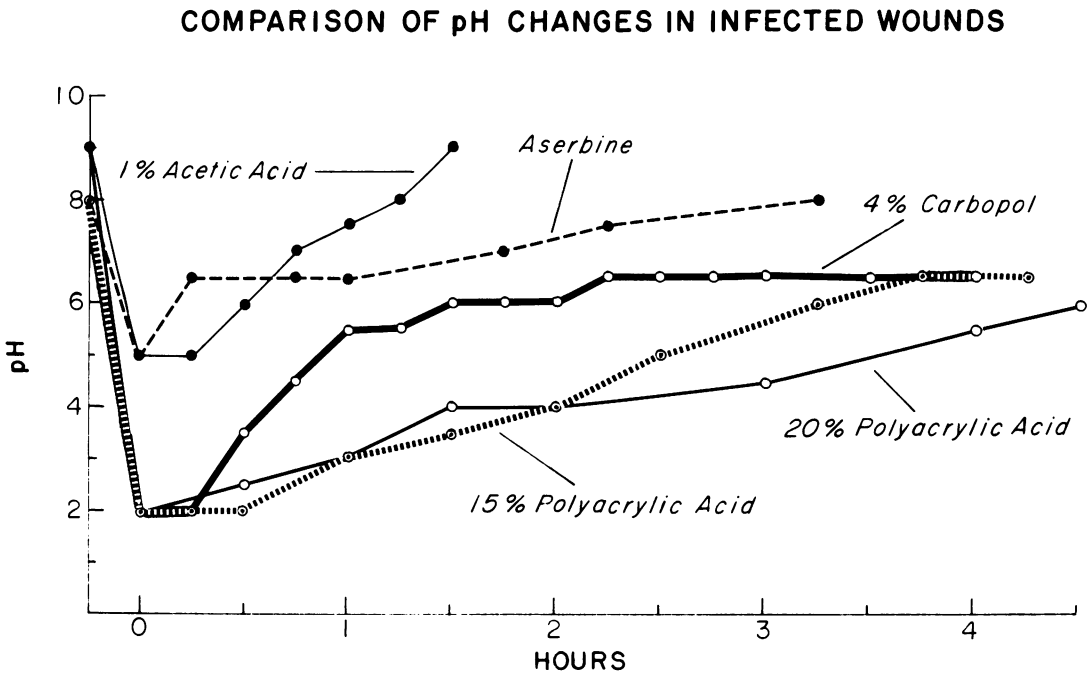

FIG. 8. Open wounds were acidified with a single application of an acid and the duration of acidity observed. Aserbine is a malic acid derivative used in England. Notice the prolonged acidification extending over four hours with the high molecular weight acids which have a high base binding capacity.

of 7.6 or greater. Of 27 skin grafts observed as to healing, the graft failed to take at pH levels created by active ammonia production. Narrow range pH test paper is satisfactory for the chemical determination of the status of the wound hydrogen ion concentration (Fig. 7).

Chemical acidification of the wound always raised the oxygen tension of the solution irrigating the wound. Alkalinization lowered it. The rise in oxygen tension was somewhat below the theoretical prediction indicating that the healing tissue does extract a portion of the newly liberated oxygen. Acidification of 9 occluded surface wounds with 20% polyacrylic acid raised the average value of pO_2 from 32 to 61 as measured by direct oximetry.

Following ligation of the mesenteric vessels in the rabbit, the tissue pO_2 fell from 70 to 20. Instillation of $NaHCO_3$ solution into the bowel lowered this further to 13mm Hg. Acidification of luminal content with acetic acid promptly raised the submucosal pO_2 to 25mm.

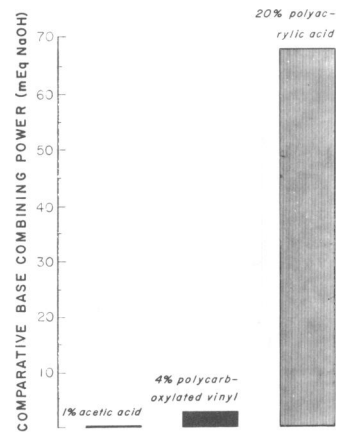

FIG. 9. The ammonia binding capacity of acetic acid, carbopol and polyacrylic acid is compared in the concentratives used clinically.

If chemical acidification of the wound is to have beneficial effects on wound healing, acidity must be constantly maintained. Moist dressings consisting of 1% acetic acid remain acid for only one hour, at which time the pH rises to neutrality or above. Carbopol ointment or 20% polyacrylic acid lowered the wound pH for prolonged period (Fig. 8). The ammonia binding power of acetic acid, carbopol and polyacrylic acid are compared (Fig. 9). The superior ammonia binding capacity of polycarboxylated polymers, and their ability to maintain wound acidity without absorption make them the acidfying agents of choice.

The mice injected with ammonium hydroxide subcutaneously develop skin necrosis over the area of the injection site (Fig. 10). Upon reflection of the skin it can be seen that there is necrosis of deeper tissues. Doses as low as 30 mg% will produce skin necrosis. The injection of 120 million *Proteus morganii* organisms into the subcutaneous tissue of the back produced a slough not unlike that seen with ammonia (Fig. 11). To determine the role played by bacterial urease and ammonia formation in the development of the slough, the bacteria were killed by heat which destroys urease. Acetone kills the bacteria but does not inactivate the urease. The injection of heat-killed bacteria was well tolerated while the injection of acetone killed bacteria produced a slough. These experiments suggest that ammonia formed from urea by the action of bacterial urease contributes significantly to the necrotizing properties. Further confirmation was offered by the failure of lesions to develop in animals immunized to Jack Bean urease. The sera of these animals inhibited ammonia formation by urease

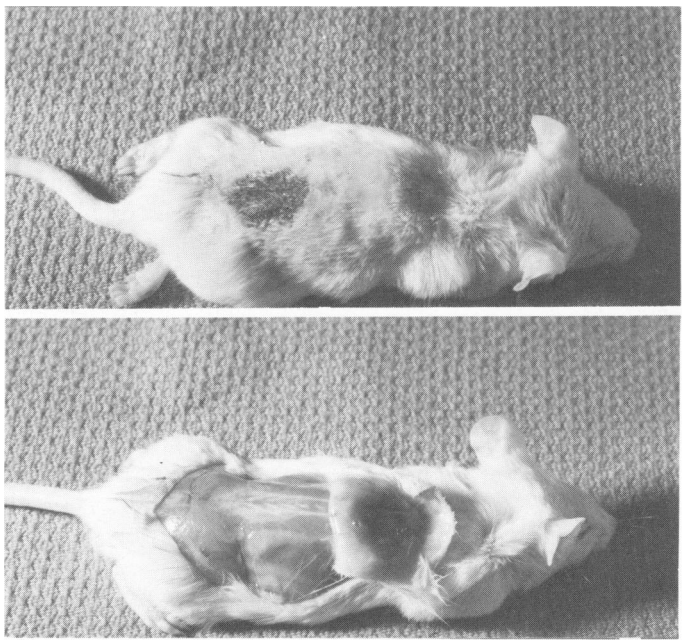

FIG. 10. The extensive necrosis of tissue at the site of the dilute ammonia solution is visible. Reflection of the skin delineates the extent of the lesion.

in vitro. Injection of the bacteria in these animals did not evoke necrosis of tissue (Fig. 12).

The role that pH plays in the histotoxicity of ammonia was tested by the role which pH plays in hemolysis produced by varying concentrations of ammonia. Even concentrations of ammonia which are lower than those encountered clinically can hemolize red cells in alkaline solutions (Fig. 13). In contrast, high concentrations of ammonia do not destroy errythrocytes in an acid medium. The pH of the solution was the critical factor in determining ammonia toxicity and was more significant than the concentration of ammonia itself.

Discussion

An alkaline tissue environment impedes wound healing. Surface wounds tend to become alkaline by loss of carbon dioxide unless the wound is sealed off from the atmosphere. When rats were placed in an environment of 40% oxygen, epithelialization was accelerated. Polyethylene film applied to the wounds also enhanced epitheliazation. In the latter instance, the beneficial effects were ascribed to prevention of dehydration, but polyethylene film also prevents the loss of CO_2 from the wound surface, thus preventing the usual respiratory alkalosis of the surface wound. The acid pH which develops in deep wounds may have a beneficial effect with respect to oxygen transport.

A polycarboxylated vinyl polymer (carbopol 934) proved excellent for chemical acidification of the wound. Since it is a large molecule, gels formed by addition of

FIG. 11. Necrosis is visible at the site of the subcutaneous inoculum of the proteus morganii. The reflected skin shows the extent of the lesion.

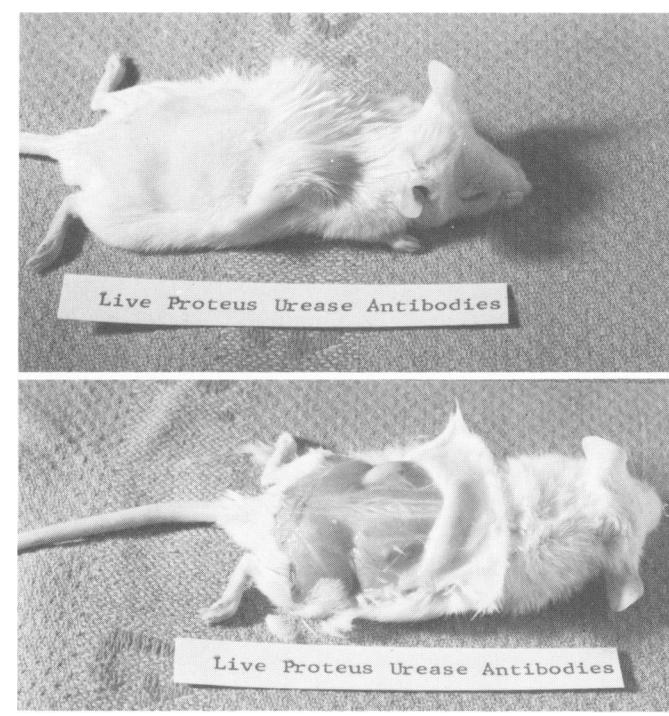

FIG. 12. Injection of proteus bacteria into animals which have been immunized to Jack Bean urease fail to show significant lesions proving that ammonia formation must contribute significantly to the slough which occurs in non ammunized animals.

water are not hypertonic. Polyacrylic acid with a molecular weight of 250,000 is also ideal for topical acidification. These substances have high base binding capacity per unit weight (Fig. 9) and maintain wound acidity for prolonged periods (Fig. 8). Even after they are fully neutralized, they act like ion exchange resins and bind ammonia by exchanging a Na^+ for an $NH4^+$. Most important is that small molecules such as acetic acid are rapidly absorbed into the circulation. Absorption has even caused acidosis during sulfamylon therapy of burns,[4] and sulfamylon is a large molecule when compared to acetic and lactic acids. Chemicals used for wound acidification must have high molecular weights and high base binding capacity. They must not be degraded by bacteria or tissue enzymes on the wound surface. Both carbopol and polyacrylic acid fulfill these fundamental requirements.

Wound acidification in addition to being an adjuvent to healing, controls pseudomonas aerogerosa infections which prolong healing and are often resistant to therapy. Even acidification with acetic acid promptly eliminates pseudomonas aerogenosa from most surface wounds.[16]

Cold influences the oxyhemoglobin dissociation curve in a manner identical to alkalinization.[3] Low temperatures push the dissociation curve to the left (Fig. 14). Gimbel[6] found that lowering the skin temperature impeded the epithelization of the donor site of a split thickness skin graft. Epithelization was accelerated by heat up to 41°C at which point there was an abrupt transition to tissue destruction. The temperature end point is quite sharp. At cold temperatures of 5°–10°C the skin takes on a bright red color similar to that produced at 45°C at which temperature vasodilitation occurs. Since the skin vessels are contracted by cold, cyanosis might be expected; but, at temperatures under 10°C oxyhemoglobin undergoes little dissociation even at low oxygen tensions.[11] The healthy cherry red appearance of very cold skin, does not indicate that the tissue is well oxygenated, but, rather, that the oxyhemoglobin has not been desaturated of oxygen by passage through tissue. Similarly, the alkaline wound may exhibit healthy cherry red granulations primarily because oxyhemoglobin cannot liberate its oxygen in an alkaline medium.

Ammonia is a highly toxic substance.[21] Its presence in the blood in micro quantities has been observed to produce convulsions and death in cirrhotics. A slight shift toward alkalinity greatly enhances the histotoxicity of ammonia. Ammonia penetrates cells and is toxic only in the form of NH_3. The ratio of NH_3 to NH_4^+ is dependent upon the hydrogen ion concentration (Fig. 15). Notice that the pK (pH at which half of the ammonia is

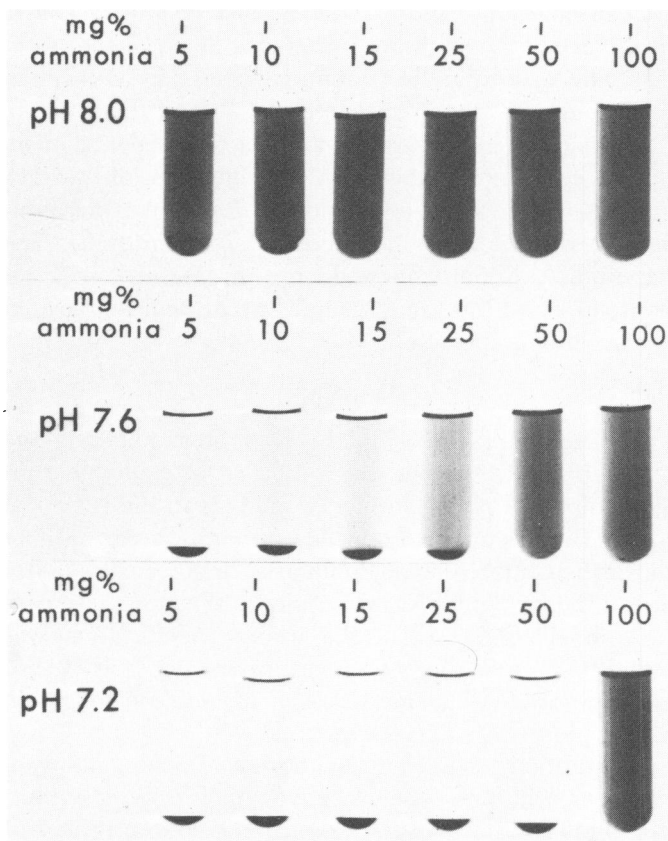

FIG. 13. The hemolysis of red blood cells by ammonia is demonstrated. Notice that even high concentrations of ammonia are nontoxic for erythrocytes at an acid pH.

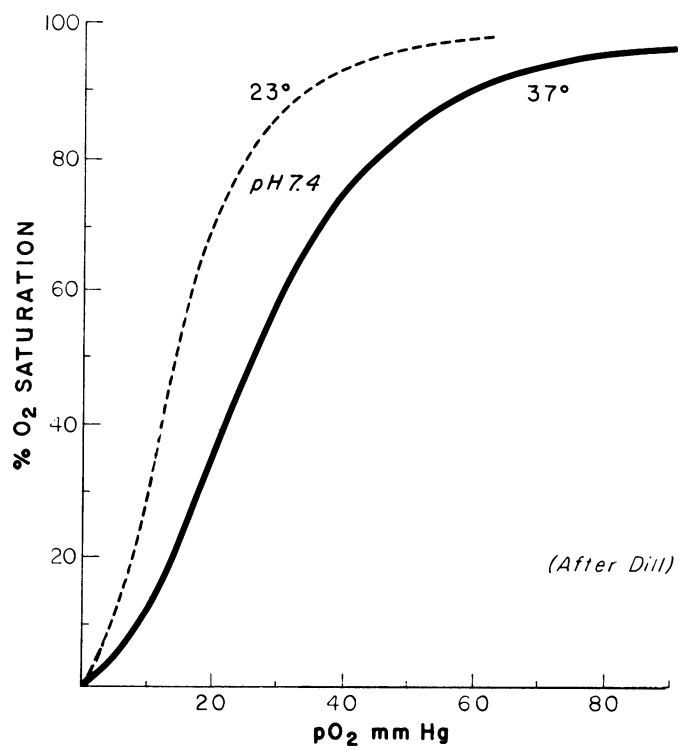

FIG. 14. Compare the effect of pH in Fig. 1. to reduce temperatures. Low temperatures shift the curve to the left in a manner similar to that seen with a high pH.

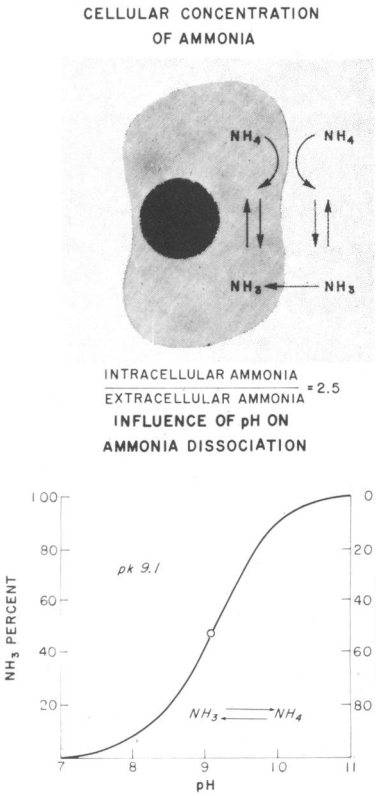

FIG. 15. The cell is freely permeable to NH_3 but not to NH_4^+. Therefore intracellular penetration of the cell membrane is determined by the acidity. Notice that at acid values, all of the ammonia is in the form of NH_4^+. The increase in NH_3 is sizable above pH of 7.8.

in the form of NH_3) is 9.1. As the acidity increases, less and less is in the form of NH_3. Th cell membrane is permeable only to NH_3 and not NH_4^+. Some of the ammonia which diffuses into the erythrocyte as NH_3 is trapped within the cell by the formation of ammonium ion. Thus, the ratio of intracellular ammonia tends to rise above that found in extracellular fluid. The surface wound has a pH of 8 which is on the steep portion of the ammonia dissociation curve where small changes in pH produce great alteration in the concentration of NH_3. In contrast, the normal body pH is 7.3 which is at the foot of the NH_3 NH_4^+ dissociation curve. These facts offer a ready explanation for the histotoxicity of ammonia on surface wounds.

The ubiquitous presence of urease-producing organisms accounts for the high incidence of ammonia on surface wounds. Although most physicians associate urea splitting with the genus proteus, many bacteria produce urease. The ammonia found on surface wounds and in intestinal wounds is the result of urease acting on urea derived from the blood urea nitrogen. Urea is so diffusable that it is present in extracellular fluid in the same concentration in all lean body tissue. If all the urea in extracellular fluid were split to ammonia, clinically significant quantities of ammonia would be found in the wound (about 25mg%). Conversion of urea to ammonia in the wound brings about further diffusion of urea nitrogen from the blood into the wound thus perpetuating the presence of ammonia.

This situation in the wound is similar to what Walser[23] has described for the colon. Water turnover in the human colon is appreciable.[17] Urea diffuses into the colon with water. Therefore, there is a large urea turnover in the colon. This urea is immediately converted into ammonia. The conversion is so rapid that urea is not a constituent of fresh feces.[25] The newly-formed urea is absorbed into the portal circulation where it is reconverted to urea by the liver. One fifth of total body urea is so transformed into ammonia each day.[23] This formation of ammonia in the colon is of such a magnitude that it must adversely influence the healing of colonic anastomoses. Evidence for this will be presented in subsequent publications. Visek immunized animals with Jack Bean urease. Immunized animals survived lethal facitial colitis produced by X-radiation. Control animals died and were unable to maintain the integrity of the colonic mucosa while surviving immunized animals healed their colonic lesions.[20] The growth-promoting effects of antibiotics in domestic farm animals may possibly be explained by their ability to suppress urease-producing organisms.[22] Acidification of colonic contents not only raises the mucosal pO_2 but beneficially influences the healing of colonic anastomoses, by reducing the histotoxic effects of the ever present colonic ammonia. Some of the beneficial effects ascribed to continuous irrigation with Kanamycin[2] may be due in part to the acidity of the solution, dilution of bacterial urease, and bactericidal action on urease splitting organisms. What happens on surface wounds with respect to ammonia formation is accentuated in the colon wounds and in fecal peritonitis. The importance of urease-producing organisms in fecal peritonitis and intestinal wound healing will be covered in a separate publication.

Summary

Surface wounds developed a respiratory alkalosis due to escape of CO_2 from the wound surface into the air. Therefore, the pH of surface wounds is in the region of pH 8. This alkalinity produced an inappropriate shift of the oxyhemoglobin-hemoglobin dissociation curve and reduced the available oxygen supply to the tissues. In contrast to alkalinization of the wound, acidification was found to increase the pO_2 of surface wounds by virtue of an appropriate shift in the oxyhemoglobin-hemoglobin dissociation curve (Bohr effect).

In addition, significant quantities of ammonia were found present on most surface wounds further contributing to the alkalinity of the wound. The concentrations of ammonia found in surface wounds was histotoxic. This histotoxicity is pH dependent. Ammonia is nontoxic in an acid medium.

A number of chemicals suitable for clinical acidification of wounds were investigated. Prerequisites are a large molecular size to prevent absorption and high base binding capacity. Acetic acid solutions do not maintain acidity for periods longer than one hour. Polyacrylic acid and polycarboxylated vinyl resins proved most proficient for prolonged chemical acidification of clinical wounds.

The histotoxicity of ammonia provokes the slough found in infections caused by some potent urease producing organisms. This slough could be prevented by immunization to Jack Bean urease. Chemical acidification of wounds is most effective in minimizing the toxicity of the ammonia formed by urease producing organisms.

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