Energy Metabolism of Experimental Wounds at Various Oxygen Environments

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Energy metabolism of healing tissue was studied in experimental wounds of rats chronically breathing 11% O_2, air or 55% O_2. Increasing oxygen supply elevated both P_{O_2} and P_{CO_2} in the wound tissue. At the early phases of healing hypoxic wounds contained less DNA than normoxic or hyperoxic tissues. In hypoxia the accumulation of wound collagen was clearly retarded. Furthermore, tissue taken from wounds healing in hypoxic environments and tested *ex vivo* in air showed decreased capacity for glucose utilization, lactate production and oxygen consumption. Concentrations of AMP, ADP and ATP in repair tissue increased as healing progressed. The more oxygen available the higher the amounts of ADP and ATP. The AMP content was not affected by changes in local oxygen tension. These results support the earlier concept that the supply of oxygen in healing tissue may be rate-limiting. Reduction of available oxygen either by systemic hypoxia or by increased diffusion distance impedes healing.

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AN ADEQUATE SUPPLY of oxygen is of vital importance for healing wounds since fibroblasts require oxygen for their energy production and collagen cannot be synthetized in the absence of molecular oxygen.[7,16] In dead space wounds the rate of collagen accumulation has been shown to be, over a wide range, directly proportional to the amount of oxygen delivered to the wound.[8,12] The ratio RNA/DNA of wound cells is known to increase during chronic hyperoxia.[12] This suggests that fibroblasts can increase their synthetic apparatus if added oxygen is available.

Earlier work from this laboratory demonstrated that hyperoxia shifts the wound metabolism from anaerobic towards aerobic glycolysis and thereby activates the citric acid cycle.[21] This work has now been continued by testing the effects of hypoxia and hyperoxia on the concentrations of adenosine phosphates in an experimental wound model, the thin cellulose sponge implant. Wound tissue gases were measured by means of a Silastic tonometer embedded in the implant. Oxygen-induced changes in the amounts of wound collagen were compared with corresponding earlier data obtained from wounds with a larger dead space.[12] Furthermore, tissues taken from wounds healing in various oxygen environments were tested ex vivo in air for the capacity for glucose utilization, lactate production, and oxygen consumption.

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Methods

Viscose cellulose sponge (Kongfoss/Fabrikker, Bygdo, Oslo, Norway) was used as a matrix for the growth of granulation tissue.[22] The sponge was cut into thin sheets measuring 2.5 mm by 14 mm by 20 mm and weighing about 35 mg. This type of an implant was used in most experiments. For studies of tissue gas tensions the same material was cut into sheets of 2.5 mm by 14 mm by 70 mm, and a loop of gas-permeable Silastic tube, 15 cm long, with an outside diameter of 1.5 mm and an inside diameter of 1.2 mm, was implanted into the sponge by means of a wide-bore needle leaving the tube ends outside the sponge. These tubes are made for shunt systems used in the treatment of hydrocephalus (Atrial Catheter-J, B-190, Extracorporeal Medical Specialties Inc., King of Prussia, Pennsylvania) and are available in sterile packings.

All sponges were sterilized by boiling for 30 min in physiological saline solution prior to implantation. A low dorsal midline incision, 3 cm long, was made in male Wistar rats weighing 170-190 g and two sponge sheets of the smaller size were implanted under the skin, one on each flank. Sponge sheets containing the Silastic tube were implanted (only one per animal) longitudinally in the dorsal midline. The skin wound was closed with silk sutures. Implantations were performed under ether anesthesia.

Altogether 150 rats were used. They were divided into three equal groups and housed in air-tight transparent Perspex containers, volume about 23 l, 8 to 10 rats in each container. The first group was exposed to 11% O_2, the second group to 55% O_2, and the control group to air.

Gases were supplied to the containers at atmospheric pressure and the flow rates were adjusted to 5 l per minute. The 55% oxygen mixture was obtained by passing pure oxygen through a gas mixer (Puritan,® Puritan-Bennet Corporation, Kansas City, Missouri). The 11% oxygen mixture was obtained by passing nitrogen through a similar mixer. The gas concentrations inside the chambers were checked 3 times a week. The CO_2 level remained always below 0.5%. The temperature was about 23 C and the relative humidity varied between 70 and 85%. Drinking water and food pellets were supplied *ad lib*.

All the measurements in implants were performed on the 6th, 10th or 15th day after implantation. Determinations of oxygen and carbon dioxide tensions in the repair tissue were carried out by the method of Niinikoski and Hunt[13] as modified by Kivisaari and Niinikoski.[9] During the measurements the rats were lightly anesthetized with intraperitoneally injected Nembutal and received their breathing gas through a head tent. The ends of the Silastic tube were exposed through a 1 cm incision and the tube was filled with hypoxic saline solution (P_{O_2}, 0 to 8 mm Hg) for two minutes. During this period P_{O_2} and P_{CO_2} equilibrations of 95 and 85% were achieved between the saline and the granulation tissue surrounding the Silastic tonometer. After the 2 min period the equilibrated fluid was sampled into an Astrup glass capillary tube by filling the tonometer with another dose of hypoxic saline from a 1 ml syringe. The ends of the glass capillary were sealed with wax and the capillary was stored on crushed ice. After all samples were collected the ends of the capillaries were cut off and the tubes were mounted one at a time into a microsample injector (Radiometer, Copenhagen, Denmark). The fluid in the capillary was injected into a thermostated cuvette (37 C) containing either a Clark oxygen electrode or a Severinghaus carbon dioxide electrode. The electrodes were connected to a gas monitor (Radiometer, Type PHM 71, Copenhagen, Denmark).

Zero adjustment of the O_2 electrode was obtained with gaseous nitrogen and the calibration took place with aerated saline (P_{O_2} 150 mm Hg) using the capillary sampling technique. The CO_2 electrode was also calibrated by the capillary sampling technique using saline solutions of two known CO_2 tensions (26 and 63 mm Hg).

The measurements of tissue gas tensions were made under clean but not strictly sterile conditions. On the occasions that the same wound was tested more than once, the ends of the Silastic tube were reimplanted under the skin and the skin was reclosed between tests.

For the assay of DNA, collagen hydroxyproline and adenosine phosphates in repair tissue, the rats were sacrified by a blow on the neck, the implants were dissected out and immersed immediately in liquid nitrogen. With this procedure the implants were frozen within one minute after the death of the animal. The implants were stored at −80 C for two hours and then homogenized in 7 ml of ice-cold 0.5 M perchloric acid in an Ultra-Turrax TP 18/2 N homogenizer (Janke & Kunkel KG, Staufen, Germany). The homogenates were centrifuged at 15,000 G at 4 C for 30 min. Supernatants and sediments were separated and stored at −80 C for analyses.

The sediment was mixed with 10 ml of 0.5 M perchloric acid and heated at 90 C for 40 min. The mixture was centrifuged at 20,000 G at 4 C for 30 min. The amounts of DNA and collagen hydroxyproline in the implant were determined from the supernatant. DNA was determined by the diphenylamine reaction as described by Burton.[4] Hydroxyproline was assayed by the method of Woessner[23] after hydrolysis in 6 M hydrochloric acid.

The supernatant obtained after the initial extraction with cold perchloric acid was used for determinations of adenosine phosphates. AMP, ADP and ATP were assayed by standard enzymatic methods (C.P. Boehringer & Soehne, GmbH, Germany; Biochemical Test Combination) using a Perkin-Elmer UV-VIS Spectrophotometer 139.[1,2] The measurements were performed at the wavelength of 340 nm.

For determinations of glucose consumption and lactic acid production implants were dissected out and rinsed in physiological saline solution to remove blood. Each implant was minced with scissors into pieces not exceeding 0.4 mm in diameter and subsequently incubated in 5 ml of Krebs-Ringer-HEPES medium supplemented with glucose (22.4 mM)[19] in a metabolic shaker (Compenstat, Gallenkamp & Co., London, England). The medium was in contact with air as the oxygen source. After a preincubation period of 30 min, 1 ml of the medium was removed for estimations of glucose and lactate.[3,6] The flasks were then covered with laboratory film to prevent evaporation and after an incubation of 60 min another 1 ml aliquot was taken for the assay of glucose and lactate. The rest of the incubation mixture was used for the determination of DNA. Glucose consumption and lactate production were calculated per milligram of DNA from concentration differences between pre- and postincubation samples.

For measurements of oxygen consumption by the granulation tissue the implants were quickly removed from rats and immersed in ice-cold Krebs-Ringer-HEPES medium supplemented with glucose (22.4 mM). Each implant was divided in three equal pieces which were minced separately with scissors as described above. Oxygen consumption rates of samples, each consisting of one third of an implant, were then measured in a Biological Oxygen Monitor® (Model 53, Yellow Springs Instrument Co., Yellow Springs, Ohio). The monitor has a polarographic O_2 electrode that fits air-tight into a glass chamber, where the sample was placed in 5 ml of Krebs-Ringer-HEPES medium containing glucose. At 37 C and ambient barometric pressure (P_{O_2} 150 mm Hg) 1 ml of this medium contains 5.0 μl of oxygen. Oxygen consumption in the chamber was detected as a fall of P_{O_2} in the incubation medium and the slope was monitored by a recorder (Servogor, Goerz

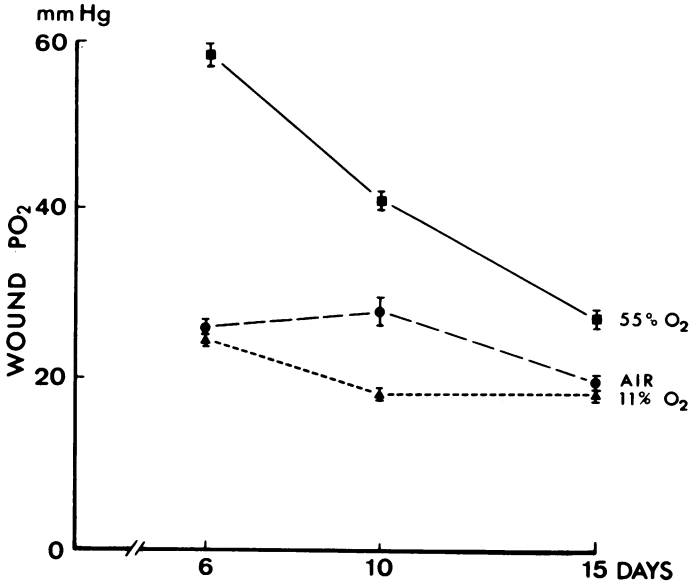

FIG. 1. Effect of changes in inspired oxygen tension on wound P_{O_2}. For 10-day values $P<0.001$; analysis of variance. In Figs. 1 to 7 each value represents the mean ± the standard error of the mean of 4 to 8 animals.

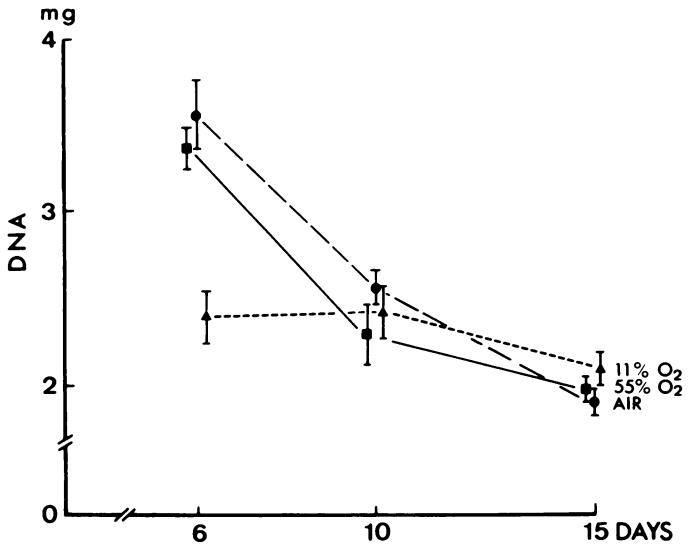

FIG. 3. Effect of changes in inspired oxygen tension on the amount of wound DNA.

Electro GmbH, Vienna, Austria). The decline in oxygen tension was linear from 150 to 100 mm Hg and observations of O_2 consumption rates were made at this area and calculated per milligram of DNA in the sample. DNA was determined from the incubation mixture after precipitation with ethanol.

Results

Figure 1 represents a summation of P_{O_2} measurements in implants of all ages. In general, the highest wound oxygen tensions were observed in rats breathing 55% O_2 and the lowest tensions in rats breathing 11% O_2. However, on days 6 and 15 the tissue P_{O_2} showed very little difference between normoxic and hypoxic animals.

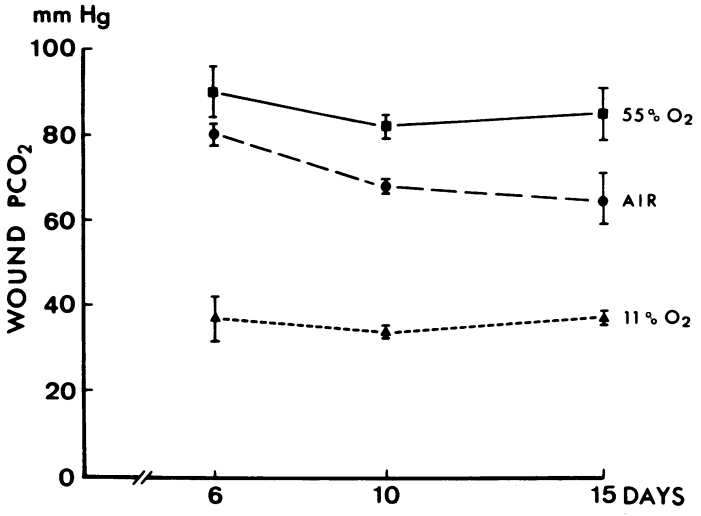

FIG. 2. Effect of changes in inspired oxygen tension on wound P_{CO_2}. For values of each observation day $P<0.01$; analysis of variance.

The higher the inhaled oxygen concentration the higher the wound P_{CO_2} (Fig. 2). The mean tissue P_{CO_2} varied between 82 and 90 mm Hg in 55% O_2, between 65 and 80 mm Hg in air and between 34 and 37 mm Hg in 11% O_2.

Six days after implantation the amount of wound DNA in the hypoxic group was approximately 30% lower than the values in normoxic and hyperoxic animals (Fig. 3; $P<0.001$; t-test). On the days 10 and 15 these differences were no longer observed.

The amounts of collagen hydroxyproline rose gradually in all groups as healing progressed (Fig. 4). The sharpest rise took place between days 6 and 10. On the 15th day the values in hypoxia remained significantly below the control level ($P<0.05$).

In general, the amounts of adenosine phosphates increased gradually as healing progressed. The AMP concentration (Fig. 5) was not affected by changes in oxygen supply. On days 10 and 15 the highest wound ADP concentrations were observed in rats breathing 55% O_2 and the lowest concentrations in rats breathing 11% O_2 (Fig. 6). However, the only statistically significant difference in ADP levels was on the day 15, between normoxic and hypoxic wounds ($P<0.01$). The results of ATP determinations (Fig. 7) were comparable with the changes of ADP. The highest wound ATP concentrations were observed in 55% O_2 on day 10 ($P<0.001$ vs air group). In hypoxia the ATP levels remained clearly below the controls ($P<0.01$ for 15-day wounds).

The highest capacity for glucose consumption by the repair tissue was observed on the 10th day in all groups (Table 1). At this phase animals kept in hypoxia showed significantly retarded capacity for utilization of glucose under the standard test conditions ($P<0.05$). The ex vivo rates of lactate production (Table 1) were comparable with the rates of glucose consumption. In implants removed

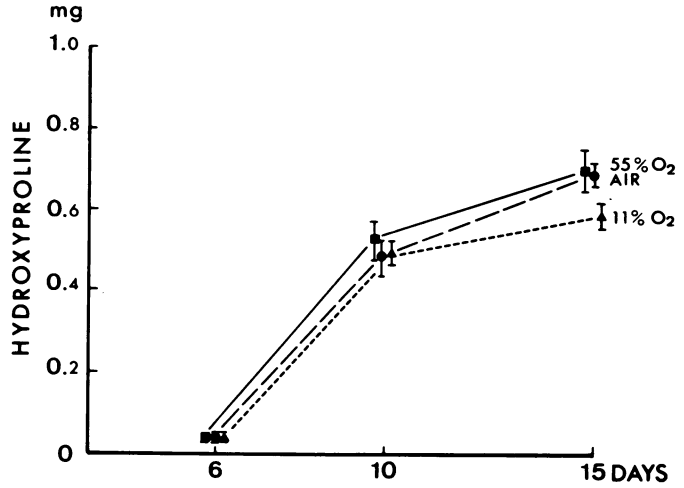

FIG. 4. Effect of changes in inspired oxygen tension on the amount of wound collagen hydroxyproline.

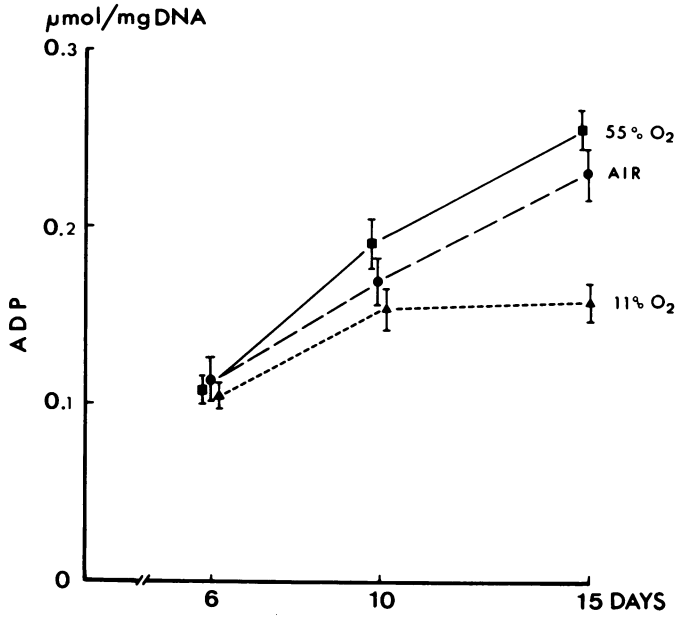

FIG. 6. Effect of changes in inspired oxygen tension on the concentration of wound ADP.

from hypoxic rats on day 10 the lactate production was 55% below the control level ($P<0.001$). The capacity for oxygen consumption (Table 1) behaved similarly in control and hyperoxic groups, increasing sharply between days 6 and 10 and remaining unchanged thereafter. In tissues taken from hypoxic animals oxygen utilization increased more slowly ($P<0.05$ for 10-day implants) and reached the control level first on the 15th day.

Discussion

Oxygen kinetics in healing wounds gained increasing attention after it was shown that repair processes respond to changes in oxygen supply.[7,8,12] In several types of wounds increased oxygen tensions enchance healing and, conversely, reduction in available oxygen inhibits tissue repair.[10,11,15,18,20] In experimental wounds containing a large dead space the accumulation of collagen is roughly proportional to the arterial blood P_{O_2} at nontoxic ambient oxygen tensions.[8,12]

Studies with cylindrical cellulose sponge implants indicated that the Pasteur effect pertains to healing wounds.[21] Hyperoxia shifts the wound metabolism towards aerobic glycolysis and activates the citric acid cycle. The activity of succinate dehydrogenase, a linking enzyme between citric acid cycle and electron transfer chain, also increases with increasing oxygen supply.

The large size of cellulose sponge implants used in studies of wound healing creates a considerable dead space.[8,12,22] The granulation tissue grows slowly from the

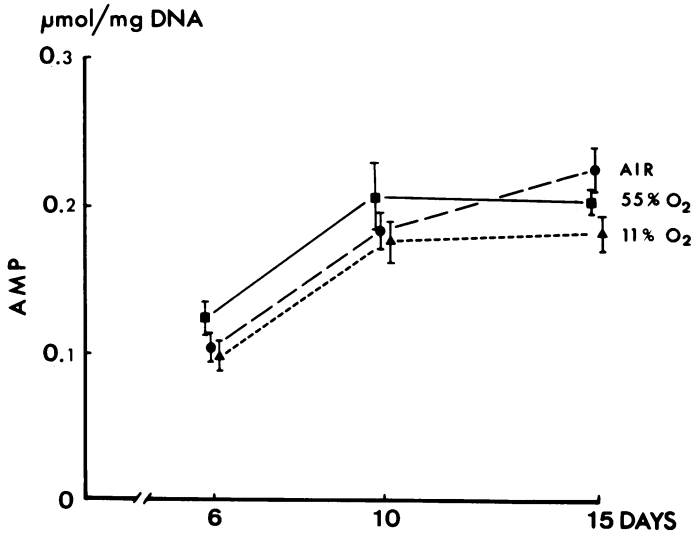

FIG. 5. Effect of changes in inspired oxygen tension on the concentration of wound AMP.

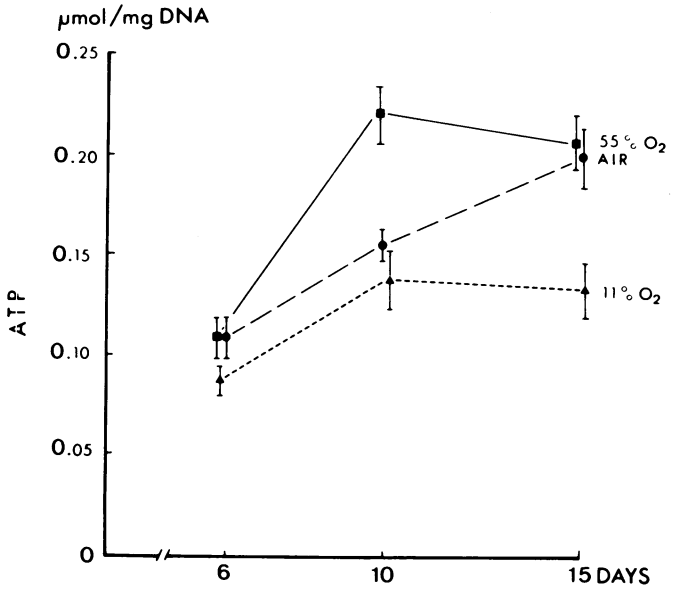

FIG. 7. Effect of changes in inspired oxygen tension on the concentration of wound ATP.

TABLE 1. *Glucose Utilization, Lactate Production and Oxygen Consumption in Tissues Taken From Wounds Healing in Various Oxygen Environments and Tested ex vivo in Air*

Group	Glucose consumption μmol/mg DNA/hr			Lactate production μmol/mg DNA/hr			Oxygen consumption μl/mg DNA/hr		
	6 dys	10 dys	15 dys	6 dys	10 dys	15 dys	6 dys	10 dys	15 dys
11% O_2	0.41 ±0.17	0.83 ±0.16	0.73 ±0.12	0.69 ±0.11	2.00 ±0.39	3.19 ±0.30	56 ±21	89 ± 22	159 ± 3
Air	0.63 ±0.12	1.74 ±0.18	0.80 ±0.17	1.53 ±0.11	4.28 ±0.32	3.38 ±0.17	86 ±17	178 ± 17	174 ± 14
55% O_2	0.62 ±0.19	1.39 ±0.21	0.76 ±0.19	1.04 ±0.12	4.62 ±0.33	2.80 ±0.09	66 ±17	139 ± 10	183 ± 18

Each figure represents the mean ± S.E.M. of 8 wounds

periphery of the implant towards the central dead space and the sequences of healing overlap each other. Thus the outer layers usually contain mature collagen fibers whereas in more central parts the cellular inflammatory reaction is still progressing. In the present model the matrix was only 2.5 mm thick and the tissue ingrowth occurred mainly from two opposite directions. This ensured that there was more granulation tissue of the same phase in different parts of the implant.

It has been demonstrated that when arterial P_{O_2} is elevated the mean capillary P_{O_2} rises, more oxygen reaches wound tissue and the capillary-tissue oxygen gradient increases.[14,17] The present findings of tissue oxygen tensions fully agree with this concept. In thin implants the average tissue P_{O_2} clearly exceeded that reported in dead space wounds.[8,14,21] This is probably due to decreased diffusion distance for oxygen from the supplying capillary. The wound P_{O_2} in rats breathing 55% O_2 gradually decreased from the 6th day onwards (Fig. 1). This might result from increasing oxygen consumption, diminishing vascularization of the sponge or impaired oxygen diffusion through the developing collagenous stroma.

The increase in wound P_{CO_2} with increasing respiratory oxygen tension was a constant finding on each observation day (Fig. 2). The high tissue P_{CO_2} values in hyperoxia could be ascribed to enhanced production of carbon dioxide, oxygen-induced vasoconstriction, hypoventilation, decreased carbon dioxide-carrying capacity of hemoglobin or changes in vascular pattern. Reverse phenomena may account for the decreased wound P_{CO_2} in hypoxic rats.

Early cellular invasion and proliferation resulted in rapid accumulation of DNA in implants of normoxic and hyperoxic rats (Fig. 3). Hypoxia appeared to inhibit cellular proliferation. Between days 6 and 10 the proliferative phase subsided and the amount of collagen increased sharply.

The amounts of collagen hydroxyproline showed very little variation between the different groups (Fig. 4). Only the 15-day values of hypoxic rats were clearly below the control level. This suggests that the local oxygen tension was generally sufficient for adequate collagen synthesis even in hypoxic animals in which the lowest mean P_{O_2} was 18 mm Hg. The findings agree with earlier measurements of wound oxygen tensions according to which collagen synthesis appears to diminish as oxygen tension drops below a critical level at about 15 mm Hg.[17] In large dead space wounds the rate of collagen accumulation has been directly proportional to arterial blood P_{O_2} probably because their oxygen tensions remain below 15 mm Hg under normal

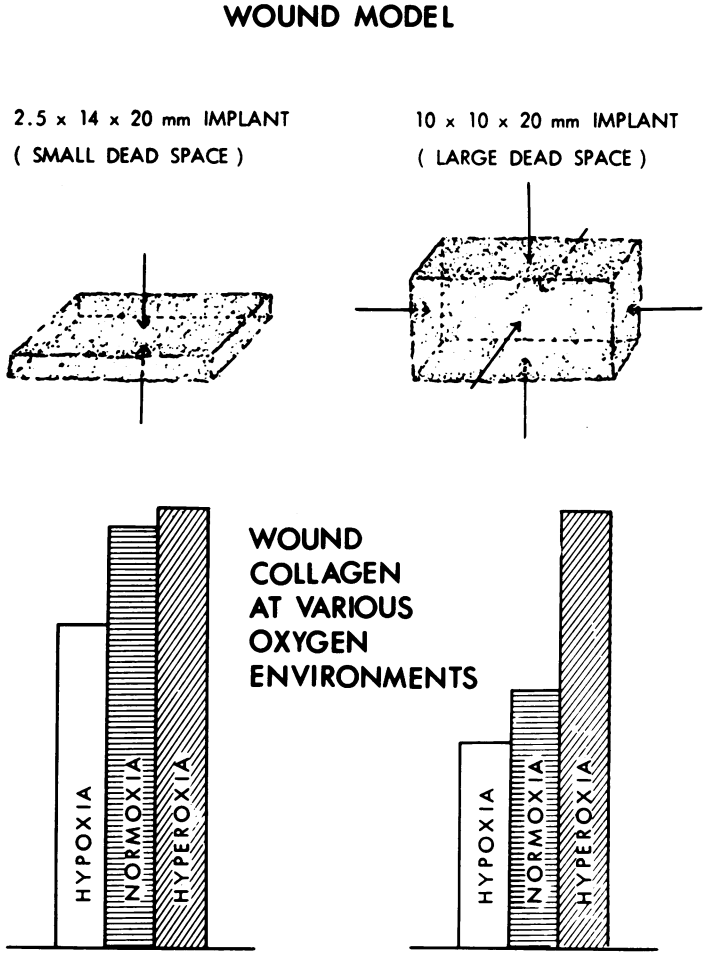

FIG. 8. Scheme summarizing the influence of implant size on collagen accumulation at various oxygen environments. Data of thick implants are from an earlier work.[12]

conditions and even in moderate hyperoxemia.[8] Obviously, vascular network fills the thin sponge implant within a few days. This reduces tissue hypoxia and decreases the need for additional oxygen. In large dead space wounds the situation is different (Fig. 8); to achieve optimal healing the supply of oxygen must be markedly increased.

Cellular respiration is controlled by substrate level, oxygen supply, ADP concentration and by the capacity of respiratory chain.[5] If oxygen is lacking the energy metabolism uses more substrates, the supply of which may become rate-limiting. Oxygen economizes substrate use and, if the supply of oxygen is sufficient, the control of respiratory rate is shifted to ADP or the respiratory chain. The present data suggest that the effect of oxygen on wound energy metabolism is not limited to saving of substrates since the amounts of ADP increased with increasing oxygen tension (Fig. 6). Succinate dehydrogenase of healing wounds is elevated in hyperoxia denoting an increased capacity of electron transfer chain.[21]

The concentrations of ATP in repair tissue also responded to changes of local oxygen tension (Fig. 7). Since hyperoxia abolished several limiting factors in energy production the high concentration of ATP in hyperoxic wounds is not due to reduced consumption of chemical energy but corresponds to a new level at which both energy production and use are elevated. Presumably the effects of hypoxia are the converse.

The experiments on the capacity for glucose and oxygen consumption and lactate production were carried out *in vitro* with air as the oxygen source. Thus the P_{O_2} of the medium was 150 mm Hg, and therefore, the wound tissues, accustomed to artificial atmospheres, were now being tested at an oxygen tension far higher than they had ever experienced before. Although the diameter of the tissue slices was less than 0.4 mm, these slices are large enough to necessitate large oxygen gradients even in tissue incubated in air. Therefore, there has to be some reservation about the quantitative changes measured by this method. It is possible that the various oxygen environments had produced sufficient changes in the tissue to influence the results in a manner that cannot be predicted.

In general the capacity for glucose utilization, lactate production and oxygen consumption was decreased in tissues taken from hypoxic animals (Table 1). Since all the incubations were carried out at the same atmosphere it is suggested that in hypoxic wounds the low P_{O_2} is not necessarily the only limiting factor in wound energy production. Low ADP concentrations and depressed succinic dehydrogenase levels in hypoxic wounds[21] further support this concept.

It is concluded that reduction of tissue oxygen supply by systemic hypoxia has a detrimental effect on wound energy metabolism. Measurements of wound oxygen tensions agree with earlier findings according to which retarded healing occurs as the P_{O_2} declines below a critical level of 15 to 20 mm Hg. Increase of P_{O_2} over this critical level did not accelerate healing.

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