Tricarboxylic Acid Cycle in Wound Healing by Granulation.* (30362)

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No systematic studies of carbohydrate metabolism in wound healing have been carried out, particularly with respect to glucose breakdown. Earlier investigations were concerned with the synthesis of polysaccharides as part of the ground substance of connective tissue(1,2). Woessner and Boucek(3) found the presence of aldolase, enolase and lactic dehydrogenase. Of the enzymes of Tricarboxylic Acid Cycle, they demonstrated the activity of the condensing enzyme and succinic and malic dehydrogenases. The presence of glucose-6-phosphate dehydrogenase and the 6-phosphogluconic dehydrogenase suggests that part of the glucose enters the pentose phosphate pathway. The same authors showed later(4) that during the process of granulation, obtained by subcutaneously implanted polyvinyl sponges in rats, the activity of lactic dehydrogenase steadily rises from the fourth day (earliest investigated material), reaches its peak on the 21-22nd day, then slowly decreases. Malic dehydrogenase follows a similar pattern, except that the curve of its activity is more bell-shaped as compared to that of lactic dehydrogenase. From these results the authors concluded that the Embden-Meyerhof pathway and Tricarboxylic Acid Cycle exist in the granulation tissue. A large amount of this work was done on 24-27-day-old stages(3); however, according to our experience and that of other observers, at these stages, the most active morphogenetic changes are almost concluded and though there are still some very limited foci of inflammation and of fibroblasts, the wound consists primarily of connective tissue. In the work to be described, we studied glucose metabolism at 3 different stages: 5-6th, 10th, and 20-22nd days after operation. We preferred to work with open wounds rather than those produced by implantation of polyvinyl sponges or stainless steel wires, because the latter techniques present a series of handicaps which yielded in our hands a not quite satisfactory experimental material. One of the reasons was the fact also noticed by Edwards *et al*(5), who worked out this technique, that around 30 or more days, the fatty connective tissue replaces the fibrous one. This phenomenon was also observed by Boucek *et al*(6) and Noble *et al*(7), who found that when this technique is used, the granulation process slowly leads to degeneration. In our histological preparations the tendency towards fatty degeneration was never observed. On the contrary, the open wound healed well and formed a perfect scar.

The process of wound healing consists of a large number of morphogenetic changes such as formation of fibroblasts and fibrous tissue produced by the former, transformation of monocytes to histocytes, mass movement of cells, etc. Parallel to these processes a series of endergonic metabolic processes takes place, such as synthesis of proteins and other substances, which require considerable amounts of energy in form of adenosine triphosphate (ATP). Therefore it seemed of particular interest and importance to investigate the activity of Tricarboxylic Acid Cycle in the processes of wound healing by granulation. The aim was not to demonstrate the presence or absence of a certain enzyme or enzymes, but to investigate the entire metabolic pattern.

Material and methods. Healthy white rabbits, male and female, of 2-3 kg weight were anesthetized with Nembutal and skin areas of 8 × 10 cm excised on the back of the animal. The wounds were covered with gauze spread with Furacin to prevent infection and carefully bandaged. Our experience showed that Furacin had no inhibitory effects upon the glycolytic enzymes. After 5, 10, and 20 postoperative days, the animals were again anesthetized and the granulation tissue formed was carefully shaved off with a bent

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razor blade, immersed immediately in ice-cold physiological NaCl solution, washed carefully several times and either homogenized in 0.15 M KCl solution or cut into slices of uniform thickness. Small pieces of granulation tissue were taken at random for microscopic study, fixed in Bouin solution, embedded in paraffin, and cut into 10 μ-thin sections. For nuclear staining Heidenhain haematoxylin was used and the protoplasm counterstained with picric acid. Thus a histological check was permanently carried out.

Activity of Tricarboxylic Acid Cycle was measured in Barcroft-Warburg manometers at 38°C by the increase of oxygen uptake in the presence of various intermediates of the cycle(8). The substrates and co-factors were added either directly to the flask or tipped in from the side arm after gassing and temperature equilibration. Throughout the experiments, Krebs-Ringer buffer at pH 7.4 was used and the substrates, as sodium salts, adjusted to this pH. The co-factors were prepared directly in the buffer. Amounts of added substrates and co-factors are given in each Table. As gas phase, pure oxygen was passed for 5-6 minutes. The 20% homogenates were prepared in all glass Pyrex hand homogenizers.

Wet weight of the slices in each cup (70-100 mg) was determined and dry weights calculated from the percentage wet weight/dry weight of samples, which were taken for this purpose and dried overnight in an oven at 102°C.

Results. Oxygen uptake of 10-day slices of granulation tissue was first measured (Table I). Glucose in concentrations 0.01 and 0.02 M did not increase the rate of oxygen consumption as compared to the control; addition of fructose-1,6-diphosphate was also without effect. Except for succinate and malate, addition of the intermediates of the Tricarboxylic Acid Cycle also had no influence. These results suggested that majority of the substrates do not penetrate the cell membranes. To check this possibility, identical experiments were repeated on homogenates. It was found (Table II) that the oxygen uptake of homogenates not fortified with cofactors, gave very low figures, even with succinate as substrate, which usually shows considerably higher Q_{O_2} values. As was expected, the addition of ATP, DPN, (or DPNH respectively), cytochrome C, Coenzyme A and reduced glutathione in amounts indicated in Table III, increased markedly the oxygen uptake as compared to that of the controls. In cases of citrate and α-ketoglutarate, the increase was about 4.5-fold. These positive results, obtained with fortified homogenates, led us to carry out experiments with slices in the presence of co-factors. Table IV shows that they significantly influenced the oxidations. Differences between the blank and the added substrates were not as marked as in case of homogenates, but this is consistent with the differences in thermodynamic conditions of intact cells as compared to those of homogenates, and with the regulatory mechanisms of enzyme activities present in the intact cells. Except for malate, the oxygen uptake of all the intermediates in-

TABLE I. Effect of Added Intermediates on Respiration of 10-Day-Old Rabbit Granulation Slices. (Parentheses: No. of experiments.)

Substrate	Molar conc. of substr.	Q_{O_2}
None	—	2.2 (18)
Glucose	.02	2.3 (6)
Glucose-6-phosphate	.01	2.2 (6)
Fruct.-1,6-diphosphate	.005	2.3 (8)
Pyruvate	.01	2.3 (12)
Oxalacetate	.01	2.8 (12)
Citrate	.01	2.6 (12)
α-Ketoglutarate	.01	1.6 (12)
Succinate	.01	4.5 (12)
Fumarate	.01	2.1 (12)
Malate	.01	3.9 (12)

TABLE II. Effect of Added Intermediates on Respiration of Homogenates of 10-Day-Old Granulation Tissue. (Parentheses: No. of experiments.)

Substrate	Molar conc. of substr.	Q_{O_2}
None	—	1.0 (15)
Glucose	.01	.5 (6)
Glucose-6-phosphate	.005	1.4 (6)
Fruct.-1,6-diphosphate	.005	1.3 (6)
Pyruvate	.01	1.0 (7)
Oxalacetate	.01	1.6 (10)
Citrate	.01	.7 (9)
α-Ketoglutarate	.01	1.5 (7)
Succinate	.01	.7 (8)
Fumarate	.01	1.1 (8)
Malate	.01	1.0 (8)

creased about 40% over those without co-factors. These results indicated that the investigations can be carried out with slices, which reflect better the actual metabolism of the cell than homogenates, where thermodynamic equilibrium is disrupted and has to be artificially reconstructed. We therefore measured the influence of addition of intermediates on 6-, 10-, and 20-day granulation slices, in the presence of added co-factors (Table V). On the 6th day, the Q_{O_2} of the control was 1.30, and with pyruvate, citrate, α-ketoglutarate and fumarate, Q_{O_2} values were 2.14, 2.44, 2.25, and 2.19 respectively, whereas succinate gave 4.21, malate 3.06, and oxalacetate 3.59. On the 10th day, the Q_{O_2} of control was 2.10 and addition of pyruvate, citrate, fumarate, and oxalacetate did not appreciably increase the rate of oxygen uptake: 2.46, 2.76, 2.21, and 2.82, respectively. Succinate and malate gave Q_{O_2} of 4.62 and 3.90 respectively. On the 20th day, the value for control was identical with that on the 10th day and except for fumarate, there was a very definite increase with all the intermediates, the highest value being obtained by succinate: 6.22.

TABLE III. Influence of Intermediates of Krebs Cycle upon Oxygen Uptake of Homogenates of 10-Day-Old Rabbit Granulation Tissue Without and With Added Co-Factors. (Parentheses: No. of experiments.) Composition of digest: 0.5 ml 20% homogenate prepared in 0.15 M KCl; substrates (as sodium salts): 30 μmol in Krebs-Ringer phosphate buffer at pH 7.4. Total contents of Cremer flask: 2.6 ml digest + 0.4 ml 15% KOH in center well. Co-factors added: 5 μmol ATP, 2 μmoles DPN or TPN, 0.05 ml of 1% cytochrome C, 1.3 μmol Coenzyme A, 20 μmoles reduced glutathione, NaF: 30 μmoles.

Substrate	Without co-factors	With co-factors
None	1.0 (15)	2.1 (18)
Pyruvate	1.0 (7)	3.4 (12)
Citrate	.7 (9)	3.2 (12)
α-Ketoglutarate (+ malonate)	.7 (8)	3.2 (12)
Succinate	1.5 (7)	4.0 (12)
Fumarate	1.1 (8)	2.6 (12)
Malate	1.0 (8)	2.7 (12)
Oxalacetate	1.6 (10)	5.6 (12)

TABLE IV. Effect of Added Intermediates of Krebs Cycle and Co-Factors upon Respiration of 10-Day-Old Granulation Tissue Slices. Composition of the medium, as in Table III, except DPN and TPN. (Parentheses: No. of experiments.)

Substrate	Q_{O_2} Without co-factor	Q_{O_2} With co-factor
None	2.10 (16)	2.03 (16)
Pyruvate	2.46 (20)	3.41 (12)
Citrate	2.76 (20)	3.28 (12)
α-Ketoglutarate	3.43 (25)	5.35 (12)
Succinate	4.63 (20)	6.29 (17)
Fumarate	2.21 (20)	3.05 (18)
Malate	3.90 (20)	3.52 (13)
Oxalacetate	2.82 (29)	5.24 (10)

Discussion. From our experiments it follows that the granulation tissue has a well established Tricarboxylic Acid Cycle. However, Florey's statement about inflammation (9) can be directly applied to the wound healing, namely, that it is not a state, but a process. As such, it consists of different types of cells at different stages. Though these cells are generally grouped together (like those involved in inflammation or aglomerates of fibroblasts) they are present in various proportions in each slice. Thus, after the production of a wound, an aseptic inflammation sets in, gradually fibroblasts are formed and final-

TABLE V. Effect of Intermediates of TCA Cycle upon Oxygen Uptake of 6-, 10-, and 20-Day Granulation Tissue (Rabbit) Slices. Substrates added in amount of 30 μmoles in Krebs-Ringer phosphate buffer at pH 7.4.

Substrates	Q_{O_2} 6th day	Q_{O_2} 10th day	Q_{O_2} 20th day
None	1.30 ± .19*(8)	2.10 ± .21*(16)	2.08 ± .31*(14)
Pyruvate	2.14 ± .29 (8)	2.46 ± .21 (20)	3.28 ± .69 (8)
Citrate	2.44 ± .26 (7)	2.76 ± .38 (20)	3.06 ± .17 (7)
α-Ketoglutarate	2.25 ± .24 (6)	3.43 ± .60 (25)	3.50 ± .26 (8)
Succinate	4.21 ± .25 (7)	4.62 ± .49 (20)	6.22 ±1.92 (14)
Fumarate	2.19 ± .14 (7)	2.21 ± .27 (20)	2.28 ± .23 (6)
Malate	3.06 ± .32 (7)	3.90 ± .43 (20)	3.56 ± .26 (7)
Oxalacetate	3.59 ± .17 (16)	2.82 ± .48 (29)	3.21 ± .37 (7)

* Standard deviation.

TABLE VI. Influence of Glucose upon Formation of Lactic Acid Under Aerobic and Anaerobic Conditions (10-Day-Old Granulation Tissue)—Krebs-Ringer Phosphate Buffer (pH 7.2) 30 μmoles Substrate.

Substrate	Q_{O_2}	Lactate (μmoles/100 mg wet wt/hr)	$Q^{N_2}_{CO_2}$	Lactate (μmoles/100 mg wet wt/hr)
None	2.10 (16)*	traces	3.18 (16)	.24
Glucose (30 μmoles)	2.25 (22)	.64	2.82 (12)	.62

* No. of estimations.

ly mucopolysaccharides and collagen are synthesized to produce the connective tissue. Histological analysis showed that each of the investigated stages contained all 3 elements but in different proportions. On the 6-day stage, the inflammation was prevalent, though some areas of fibroblasts and a small area of connective tissue were already formed. On the 10th day, the prevalent picture was that of fibroblasts and on the 20th day, primarily the connective tissue was formed, leaving only a small and limited foci of inflammatory area and of fibroblasts. If therefore an attempt is made to correlate the biochemical findings with morphological structure, the quantitative relations of these different areas have to be taken into account, since each such area, whether inflammation, fibroblasts or connective tissue, has its own characteristic metabolic pattern.

An interesting phenomenon observed throughout our work is that addition of glucose did not increase oxygen uptake. A closer investigation of these results gave the impression that the Pasteur effect might be absent in granulation tissue particularly at the 10-day stage: anaerobic and aerobic formation of lactic acid with glucose as substrate was identical (Table VI), 0.63 μmole against 0.64 μmole. A similar result was obtained with glucose-6-phosphate. The influence of fructose-6-phosphate was investigated only anaerobically, but here also the amount of lactic acid formed was equal to that formed from glucose-6-phosphate (0.53 μmole against 0.56 μmole). These results suggested the presence of some unusual metabolic activities within the Embden-Meyerhof pathway, investigation of which will be published separately.

Summary. The existence of all the enzymes of Tricarboxylic Acid Cycle in granulation tissue was demonstrated by the increase in oxygen consumption in the presence of appropriate intermediates. No effect was observed with added glucose and values for aerobic and anaerobic lactic acid production by 10-day-old granulation tissue were identical. These results suggest the absence of Pasteur effect at this stage of wound healing.

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