In Vitro Sulfate Fixation by Granulation Tissue and Injured Muscle Tissue from Healing Wounds.* (17748)

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It has been reported by Singher and Marinelli(1) and confirmed by Dziewiatkowski(2) that sulfate injected into the intact rat is concentrated *in vivo* in the cartilage, bone marrow and bone matrix of this animal. Some of our quantitative work indicates that this may not be the case for bone marrow free of bony and calcified material(3). By adapting the assay methods used by the workers cited to the conditions met with *in vitro* tissue work, the author(4) has shown that human normal and cancerous tissues maintained *in vitro* will exhibit qualitative differential fixation of the sulfate as insoluble tissue sulfate. This finding has been applied to the diagnosis of chondriogenic tumors. By removing muscle tissue from chick embryos at various stages in their development, and maintaining these tissues in a medium containing sodium sulfate labeled with radioactive sulfur, it was found(3) that embryonic heart, skeletal muscle, and other tissues absorbed sulfate quantitatively from the medium, and fixed the sulfate in the tissues as insoluble organic sulfate. The sulfate fixation capacity of embryonic skeletal muscle reached a maximum value of 1.5 μg of sulfate ion per 100 mg wet tissue between the fourteenth and sixteenth days of embryonic development. After the sixteenth day of the sulfate fixing capacity dropped rapidly, becoming relatively insignificant (*i.e.* less than 0.10 μg per 100 mg tissue) one week or more after hatching.

That the sulfate was bound by the tissue, probably as organic sulfate, is indicated by the fact(3,4) that thorough washing with water did not remove the labeled sulfate absorbed from the medium by the tissues maintained at 38°–39°C. The labeled sulfate was entirely removed from tissues maintained at 0°C. Sulfate fixation is dependent upon the survival of the cells since it was shown that no fixation occurred when KCN was added to the medium. When the tissues were placed in the labeled medium and then immediately heated to 100°C and then cooled before incubation, no sulfate was fixed. Freezing the tissue with dry ice before incubating also destroyed its capacity to fix sulfate. Accidental contamination with bacteria reduced sulfate fixation by the tissues to very low values.

Since most tissues from rapidly growing embryos showed relatively high sulfate fixation values, as compared to muscle tissue from young and adult animals which showed almost no significant sulfate affinity, it was thought that the regenerating tissue from a healing wound in the adult animal might show an affinity for this ion similar to that exhibited by embryonic muscle at some stage in its development. With this in mind a preliminary experiment was conducted on a 4-month-old hen.

It was found that granulation tissue from wounds inflicted from 4 to 11 days previously showed *in vitro* sulfate fixing capacity equal to that of the corresponding muscle (*M. Tensor Fascia Latae*) from the embryonic chick

* This investigation was supported in part by a grant from the American Cancer Society on recommendation by the Committee on Growth of the National Research Council.

The investigation was initiated under a grant from the Nutrition Foundation, Inc., for the study of *in vitro* nutrition of tissues.

The radioactive sulfur employed was obtained from the Oak Ridge National Laboratory on allocation by the United States Atomic Energy Commission for research in cancer and growth.

1. Singher, H. O., and Marinelli, L., *Science*, 1943, v101, 414.

2. Dziewiatkowski, D. D., *J. Biol. Chem.*, 1949, v178, 197.

3. Layton, Laurence L., to be published.

4. Layton, Laurence L., *Cancer*, 1949, v2, 1089.

TABLE I.

Showing the Weight of Sulfate Ion Absorbed from the Medium and Fixed as Insoluble Tissue Sulfate by Tissue Fragments from Various Parts of Healing Wounds. Control samples are from the surrounding normal (unwounded) tissue of the same muscle 5 to 10 mm from the cut edge of the wound.

Chicken No.	Age when wounded, days	"Age" of wound, days	No. of samples	Description of tissue in sample from wound	μg of sulfate ion fixed per 100 mg wet tissue	
					Healing wound tissue	Original tissue from muscle (controls)
1	120	4	3	Pink granulation from center of wound	.70	.06
2	30	7	3	"	1.0	.00
3	30	7	2	"	.90	.03
4	32	4	3	"	.84	.00
5	32	4	3	"	.90	.00
6	32	4	1	"	.97	.06
3	32	4	1	Cut edge of healing wound	.97	—
2	40	6	1	Muscle near but not including cut edge of wound	.25	.04
3	40	6	1	New muscle fascia	.72	.00
4	42	6	3	Greyish granulation tissue from cut edge of wound	1.2	.03
4	42	6	3	Center of new pink hued tissue	1.1	—
4	42	4	1	Edge of wound (tissue grey with fine red spots)	1.26	—
4	42	fresh	2	Fresh blood clots	.04	—
1	130	6	3	Cut edge of wound	1.1	.00
2	44	4	3	"	1.1	—
3	44	4	1	New tissue from adhesion	1.43	—
3	44	4	3	Cut edge of wound	.80	—
4	46	4	1	"	1.96	.06
5	46	4	1	Granular tissue from surface of wound	2.27	.00
5	46	fresh	2	Fresh blood clots	.04	—
4	52	5	3	Pink tissue from center of wound	.80	—
4	52	5	3	New fascia	.44	—
5	52	5	5	Cut edge of healing wound	.94	—

on the seventeenth day of development (*i.e.* approximately 1.0 μg per 100 mg wet tissue). Samples of tissue removed at the time of the initial wounding showed no sulfate absorption, nor did tissue removed from the muscle 5-10 mm from the healing wound. Scar tissue removed from completely healed wounds showed no affinity for sulfate ion.

A series of experiments involving chicks approximately 30 to 50 days of age and one approximately 120 days of age was then conducted. The chickens were wounded by partial section of *M. Tensor Fascia Latae* of both legs. Samples of tissue were removed from the wounds and from the surrounding tissue at irregular intervals over a period of one week, and new wounds inflicted at the time of tissue removal. The excised tissues were weighed, rinsed in the medium, and placed in 2 ml of the author's "X" medium.† After 40 hours in the incubator at 38.5°C the tissues were removed and the amount of sulfate fixed was determined by a method described in detail in another paper(3). Upon inspection of the data presented in the table, it will be seen that new tissue in the growth stages, and the recovering original tissue from the cut edge of the healing wound both show sulfate fixation values several times as great as for freshly excised, unwounded tissue from the same muscle. This relationship is similar to that of growing embryonic muscle tissue to tissue from the corresponding muscle in the chicken several weeks after hatching(3).

In the publication cited(3) the author has suggested that sulfate is required for tissue formation in the embryo, probably being bound in the newly forming connective tissue substance. The fact that sulfate is absorbed and fixed in the regenerating tissue of muscle wounds of young and adult animals is further evidence of the utilization of sulfate in the anabolic processes involved in the synthesis of tissue substance. The fact that high sulfate fixing capacity is exhibited by the recovering original injured tissue at the edges of the new tissue might indicate that the processes involved in outgrowth of fibroblast cells from the original tissue, or the process of repair to the injured original tissue involves the absorption and fixation of sulfate in this tissue. It is possible that sulfate binding capacity may be a limiting factor in the rate of healing or replacement of connective tissue substance.

Experiments are now being conducted with a view to the elucidation of the mechanism of sulfate fixation, and toward the determination of the form in which sulfate is bound in the tissue.

Summary. Granulation tissue and injured muscle tissue from healing wounds were maintained *in vitro* in a medium containing inorganic sulfate labeled with radioactive sulfur, S^{35}. It was found that these regenerating tissues absorbed inorganic sulfate and fixed it in the tissues in an insoluble form. Uninjured muscle tissue from the same animals showed no significant sulfate fixing capacity. It is suggested that sulfate is utilized in the synthesis of the intercellular connective tissue substance, and hence is necessary to wound healing.

Received February 27, 1950. P.S.E.B.M., 1950, v73.

† Tyrode's solution modified to contain 4.8 mg of sulfate ion (as Na_2SO_4) per l. The sulfate was labeled with radioactive sulfur, S^{35}, to give a specific activity of approximately 10^7 counts per mg of sulfate ion, corrected for self absorption to 30 mg of $BaSO_4$, and determined with a Geiger-Muller counter. Sulfate counting standards(3) were prepared from the culture medium in order that counting data could be translated into micrograms of sulfate fixed per 100 mg of wet tissue.