animals fed a high carbohydrate ration.

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Healing of Urinary Bladder Wounds. Uptake of S^{35}-Sulfate in Acid Mucopolysaccharides.* (32218)

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Wound healing processes involve biochemical and morphological changes usually in the connective-tissue elements of the tissue. The importance of collagenous fibers for tensile strength is well-documented. The fibers and cells are embedded in the amorphous ground substance, containing acid mucopolysaccharides, electrolytes, hormones, and many other substances. The acid mucopolysaccharides are polysaccharides containing hexosamine and uronic acid. They are present as macromolecules, linked to proteins. Even if the exact mode of action is unknown, they undoubtedly participate in biologic processes such as inflammation, tissue repair and ageing.

The aim of this study was to examine the synthesis of sulfomucopolysaccharides in healing linear wounds in the urinary bladder of rabbits, by measuring the uptake of $S^{35}O_4$. The results of biochemical and morphological studies on mucopolysaccharides and collagen in the same wounds have previously been reported(1,2).

Material and methods. Fifty-five male albino rabbits weighing about 2.5 kg were kept on an adequate laboratory diet for at least one week before operation. Five animals were not operated, but were killed at the time of operation and served as controls. The animals were anesthetized by intravenous injection of 60 mg Nembutal® supplemented by ether inhalation. The abdomen was opened through a low midline incision. The urinary bladder was emptied by puncture and aspiration. An incision through all layers was made in the midline of the anterior wall from the top to the neck. The bladder was closed in 2 layers with continuous sutures of 4-0 nontraumatic silk. In the first layer the suture was passed through the entire bladder wall near the wound edge. The second suture was seroserous. The abdominal wall was closed in 2 layers with silk sutures.

The operated animals were divided into 11 groups, sacrificed 2 to 28 days after the operation (Table I). They were killed by intravenous injection of 300 mg nembutal. Forty-eight hours before sacrifice the rabbits were given an intraperitoneal injection of 1.5 mCi $S^{35}O_4$ in aqueous solution. The original carrier free S^{35}-labelled sodium sulfate obtained from the Radiochemical Centre, Amersham, England, was diluted by adding 0.04% sodium sulfate to a final activity of 1 mCi per ml. The wounds were removed, and, after drying and defatting, the tissue was homogenized in 0.5 N NaOH. The uptake of $S^{35}O_4$ was determined by the method of Moltke(3) as modified by Marckmann(4). After wet ashing of 2.5 ml homogenate with fuming nitric acid in a sand bath, the $S^{35}O_4$ was precipitated as barium sulfate. The samples were counted at infinite layer thickness with a Geiger-Müller tube to a statistical error below 3%.

In 2 animals the ureters were ligated through an abdominal incision before the injection of radiosulfate. The animals were

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TABLE I. Uptake of $S^{35}O_4$ in Healing Wounds.

Days after wounding	No. of animals	C.p.m./10 mg dried defatted tissue[a] Wound	W/B-ratio[c]	C.p.m./10 μg AMP[b]
0	5	109 ± 10	1.00	90 ± 14
2	7	172 ± 24	.93 ± .08	*62 ± 6
3	4	*216 ± 30	1.16 ± .10	71 ± 8
4	5	143 ± 13	1.04 ± .05	*46 ± 5
5	4	***287 ± 25	*1.33 ± .07	86 ± 6
6	4	178 ± 31	*1.34 ± .09	52 ± 4
7	4	144 ± 10	*1.27 ± .06	68 ± 5
9	4	164 ± 16	1.07 ± .17	85 ± 7
11	4	130 ± 5	1.08 ± .02	66 ± 3
14	4	134 ± 10	1.20 ± .08	93 ± 15
21	5	164 ± 16	*1.29 ± .08	110 ± 15
28	3	121 ± 23	1.13 ± .15	96 ± 18

Figures represent mean values and standard error of mean.

a. Total uptake of $Na_2S^{35}O_4$ given in counts per min per 10 mg dried defatted tissue with correction for physical decay and for varying body wt of the animals.

b. Specific activity of the acid mucopolysaccharides calculated as counts per min per 10 μg uronic acid (carbazole method).

c. Wound to bladder (posterior wall) ratio.

Significantly different from controls at:

* 5% level of probability
*** 0.1% " " "

killed 48 hours later and the bladders removed for analysis.

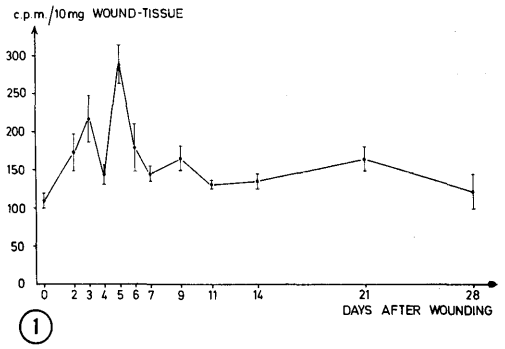
Electrophoresis and autoradiography. Twenty mg dried defatted tissue from control animals was ground and mixed with 0.2 ml 0.5 N NaOH. After extraction for 24 hours at room temperature 2 microliters of the supernatant were applied to cellulose acetate strips. Electrophoresis was carried out for 5 hours in lithium acetate buffer at pH 7.5 with subsequent staining of the acid mucopolysaccharides with Alcian blue as described by Foster and Pearce(5). Hyaluronic acid, heparin and chondroitin sulfate were used as reference standards. After drying the strips were placed in contact with a Kodak X-ray film for 2 months to produce autoradiograms.

Results. The uptake of $S^{35}O_4$ in the wounds was increased from the second day after operation during the entire examination period, being significantly elevated on the third and fifth days (Table I, Fig. 1). The ratios between activity in wounds and posterior bladder wall were calculated for each animal. From Fig. 2 it appears that the values showed an almost rectilinear increase from a value a little below 1.00 on the second day until a maximum was reached on the sixth day.

The activity in the bladders of rabbits in which the renal excretion of radioactive sulfur was blocked was 567 and 1021 counts per minute per 10 mg dried defatted tissues, *i.e.*, 5 to 10 times higher than the normal control values.

The uptake of radiosulfate was compared with the results of the biochemical analysis of mucopolysaccharide content(2). The specific acivity of the acid mucopolysaccharides was calculated on basis of the content of uronic acid (Fig. 3), determined by the carbazole method of Dische(6). Table I shows that the specific activity was decreased from the second day after wounding. The normal level was not reached until the 14th day.

The electrophoretic analysis (Fig. 4)



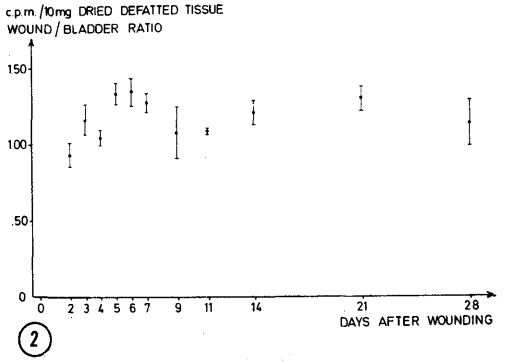


FIG. 1. Total uptake of $S^{35}O_4$ given in counts per min per 10 mg dried defatted tissue. Standard error of mean (s.e.m.) indicated by vertical lines.

FIG. 2. Ratios between total uptake of $S^{35}O_4$ in wounds and posterior bladder wall. S.e.m. indicated by vertical lines.

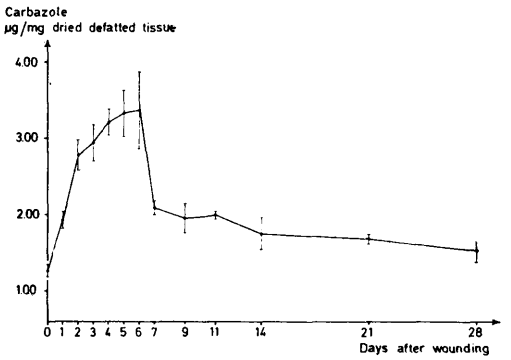


FIG. 3. Content of uronic acid (determined by the carbazole method) in healing wounds. S.e.m. indicated by vertical lines.

showed 3 fractions, one with a mobility like hyaluronic acid, another with a mobility like chondroitin sulfate and one intermediary fraction. Besides, there was an intensely stained band at the line of application corresponding to a non-migrating mucopolysaccharide fraction. Autoradiograms showed radiosulfate to be located in the chondroitin sulfate and the non-migrating fractions.

Discussion. Forty-eight hours after intraperitoneal injection of $S^{35}O_4$ practically all demonstrable radioactivity is in sulfomucopolysaccharides(7). The activity can therefore be used as an indication of the synthesis of this fraction of acid mucopolysaccharides. Biochemical analysis of the wound tissue(2) revealed a rapid increase in water content with a maximum on the second day accompanied by an increase in hexosamine content (Fig. 5). This, together with the significant increase in uptake of radiosulfate from the third day after wounding, reaching a maximum on the fifth day, indicate that wound healing processes in the urinary bladder correspond to those found in other tissues. The sequences in regenerative processes are always 1) watery edema, 2) mucinous edema, and 3) collagen cicatrization (3,8).

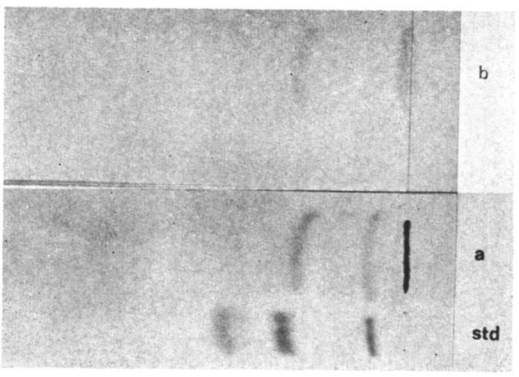


FIG. 4. Electrophoretic separation of acid mucopolysaccharides in extract of urinary bladder tissue Std: Reference standards. Bands represent from the right: Hyaluronic acid, chondroitin sulfate and heparin. a: Acid mucopolysaccharides in tissue extract. b: Autoradiogram of a.

The specific activity of acid mucopolysaccharides was calculated on basis of uronic acid content. The decrease during the first 11 days in spite of an increased total uptake of radiosulfate, demonstrates that relatively more of the increased content of acid mucopolysaccharides in this period is due to non-sulfated than sulfated mucopolysaccharides.

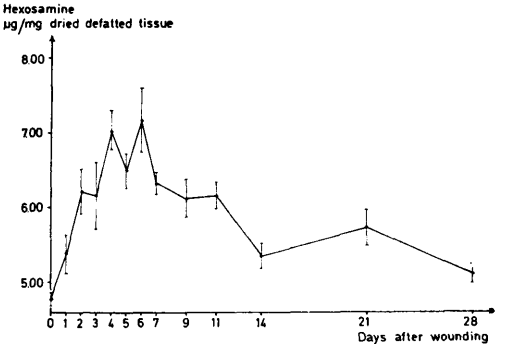


FIG. 5. Total content of mucopolysaccharides in healing wounds. Hexosamine values are determined by the Elson and Morgan procedure(10). S.e.m. indicated by vertical lines.

The non-migrating sulfomucopolysaccharides on the electrophoresis strips are considered to be linked to proteins. The intermediary band with a mobility between hyaluronic acid and chondroitin sulfate may be due to heparitin sulfate(9). The band was so faint that it possibly could contain radiosulfate without producing any visible blackening of the X-ray film.

Summary. The uptake of $S^{35}O_4$ in healing linear urinary bladder wounds of male rabbits was investigated and compared with biochemical studies on the content of acid mucopolysaccharides. The total uptake of radiosulfate was increased from the second day after wounding through the entire examination period of 28 days. Maximum uptake

was demonstrated 5 days after operation. The specific activity of the acid mucopolysaccharides was lowered during the first 11 days. This indicated that the increased content of acid mucopolysaccharides in this period was predominantly due to non-sulfated mucopolysaccharides.

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A Novel *in vitro* Assay for Anti-Inflammatory Agents Based on Stabilization of Erythrocytes. (32219)

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In vitro studies by de Duve *et al* suggested that several compounds including cortisone might act to regulate the release of enzymes from lysosomes(1). Subsequent work confirmed their prediction and further suggested that glucocorticoids may exert pharmacologic effects by preventing the disruption of the enveloping membranes of lysosomes (2-6).

Only indirect evidence supports the hypothesis that certain diseases of connective tissues may be due to an abnormal fragility of lysosomes(6). Investigators suggested that gold salts (used in the treatment of rheumatoid arthritis) may act in inflammatory states by inhibition of lysosomal enzymes of phagocytic cells in the inflamed synovial tissue(7). Furthermore, acute and chronic arthritis can be produced in rabbits by repeated injections of streptolysin S, a lysosome-disruptive agent(8). However, the role of lysosomes in the etiology of rheumatic diseases remains to be established.

Several agents capable of releasing hydrolytic enzymes from lysosomes also injure erythrocytes. *In vivo* exposure to detergents (9), lecithinase(10), excess Vitamin A (11), and ultraviolet irradiation(12) can disrupt red cell membranes. These agents and procedures also disrupt lysosome membranes (2,13-15). Similarities between the action of streptolysin S and streptolysin O on red cells and lysosomes also suggest that the membranes bounding erythrocytes and lysosomes have common properties(5).

While this paper was in preparation, Miller and Smith(16) demonstrated that acetylsalicylic acid stabilizes lysosomal membranes *in vitro*. When this report was written, we could find no evidence to show that nonsteroidal anti-inflammatory agents might stabilize red cell membranes.

A new *in vitro* technique for the rapid screening of potential anti-inflammatory compounds, based on their ability to inhibit heat-induced hemolysis of red blood cells, forms the basis of this report.

Methods. Anesthetized (sodium pentobarbital, 30 mg/kg i.v.) mongrel dogs of either sex weighing 10-14 kg were used. Whole blood (usually 100-150 ml per dog) was collected by catheterization of an external carotid artery using heparin to prevent clotting. The blood was centrifuged at 650 $\times$ *g* for 15 minutes between 0-5°C. The volume of erythrocytes (RBC) was measured and reconstituted as a 40% (v/v) suspension with cold (0-5°C) M/15 sodium phosphate buffer, pH 7.4. The