The Healing of Rat Skin Wounds *

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SEVERAL YEARS ago, we began a systematic reappraisal of the healing of rat skin incisions. This was occasioned by some observations which made us suspect that such wounds healed considerably more slowly than is ordinarily thought. It is often stated in standard textbooks of surgery and pathology, and in a recent symposium on wound healing, that the great bulk of collagen formation and gain in strength of healing skin incisions occurs during the second week, and that the wound is approaching its maximal strength by the end of the second or third week.[5, 8, 12, 13] Our experiments do not support this view; we have found, by histologic examinations and breaking and tensile strength measurements of healing paravertebral skin wounds, that the rate of collagen formation and gain in strength is just as fast from Day 21 to 42 as it is from Day 7 to 21. The rate of gain of strength of these wounds then declines fairly rapidly during the next 4 weeks and then stops. At the end of a year, the wound is only about 80 per cent as strong as comparable unwounded skin. Histologic observations have shown that collagen is being laid down at a rapid rate during the period of maximal rate of gain of tensile strength; thereafter, there is a slight but progressive increase in fiber caliber and compactness up to one year.

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Methods and Results

Walter Reed pathogen-free male rats (descendants of Wistar Strain) were used in these studies. The animals weighed between 220 and 250 grams on arrival at our laboratory and were housed individually in stainless steel 2-mesh wire-bottom cages in an air-conditioned room (76 ± 2° F.) during the study. They were fed D & G * rat biscuits and water *ad libitum* and were weighed three mornings a week, at operation and at sacrifice. The rats were observed for at least 2 weeks prior to operation.

The operation consisted of 5-cm. paravertebral wounds made under sterile conditions; sutures were removed on the seventh postoperative day, the animals were

* See Discussion.

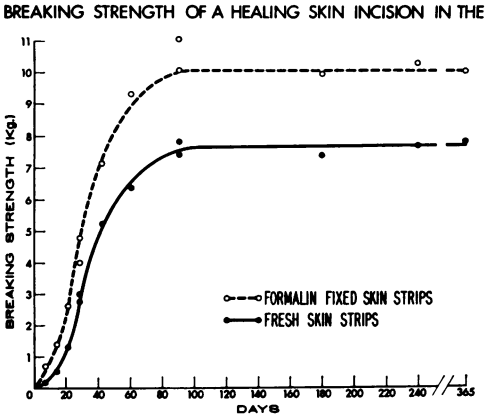

FIG. 1. Simple plot of breaking strength of a healing skin incision in rat as a function of healing time.

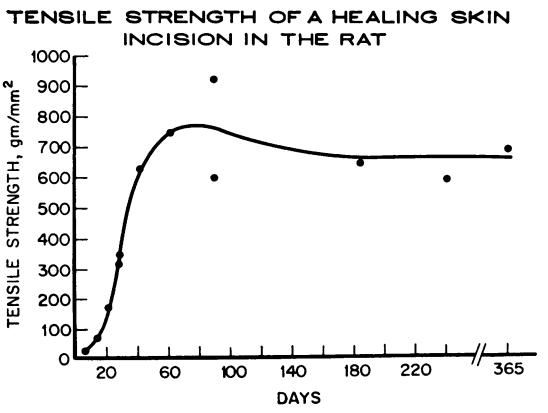

FIG. 2. Tensile strength of a healing skin incision as a function of time.

sacrificed at the predetermined times, the wounds were excised, sampled, tested for tensile strength and thickness, and prepared for histologic study. These technics are all described in detail elsewhere.[1, 4, 18]

Groups of rats were sacrificed beginning on the fifth postoperative day, and continuing until day 365. The increase in breaking strength proceeds at a rapid and fairly constant rate from the 14th day to the 42nd day, slows down rapidly during the next 4 weeks, and the wound approaches its maximal strength at about 3 months. There is a very slight gain in wound strength during the next 9 months. The entire curve is sigmoid in form (Table 1, Fig. 1). Figure 2 shows tensile strength gain over the same period; tensile strength is breaking strength per unit cross sectional area.[19, 22]

The variations in breaking strength of the wounds of the various rats in each group is

TABLE 1. *The Breaking and Tensile Strength of Healing Skin Wounds of Rats*

Wound Age Days	No. Rats	Sacrifice Body Wt.* Gm.	Fresh Breaking Strength of DSI** Gm.	Fresh Thickness** mm.	Fresh Tensile Strength** Grams/sq. mm.	Formalin Fixed Breaking Strength of DSI** Grams
7	10	295 ± 12	210 ± 18	1.35 ± .02	26 ± 4	635 ± 48
14	10	323 ± 17	515 ± 59	1.42 ± .03	64 ± 7	1420 ± 74
21	10	343 ± 26	1350 ± 92	1.38 ± .03	163 ± 13	2740 ± 100
28	10	368 ± 23	2990 ± 168	1.46 ± .04	343 ± 22	4000 ± 127
28	22	349 ± 16	2730 ± 114	1.47 ± .03	313 ± 18	4880 ± 160
42	22	371 ± 15	5260 ± 206	1.48 ± .03	626 ± 27	7225 ± 318
61	21	421 ± 32	6395 ± 252	1.45 ± .04	746 ± 22	9244 ± 358
90	21	477 ± 27	7475 ± 215	1.70 ± .05	600 ± 28	11189 ± 408
90	23	462 ± 42	7850 ± 235	1.45 ± .03	923 ± 41	10199 ± 308
184	21	512 ± 32	7420 ± 274	2.16 ± .05	646 ± 33	10052 ± 278
240	22	572 ± 42	7790 ± 165	2.22 ± .06	584 ± 30	10620 ± 312
365	22	576 ± 62	7890 ± 248	1.91 ± .05	690 ± 28	9290 ± 342

* Mean ± Standard Deviation.

** Mean ± Standard Error.

All differences in successive values of both breaking strength and tensile strength are statistically significant through day 42.

reflected in the standard error of the mean for each group (Table 1). This varied from 9 per cent of the mean at Day 7 to 5 per cent after Day 28 for the fresh wounds; for the formalin fixed samples these values were 7 and 4 per cent. This amount of variation is well within the range expected for a wide variety of biologic parameters among laboratory animals.

As is evident in Table 1, the rats gained weight during the year of study, and we thought it was important to study the changes in skin thickness and skin composition which occur as rats grow. We have shown elsewhere [4] how these factors may affect the breaking strength of healing wounds independently of the rate of healing. A number of male rats, born less than 7 days apart, were received soon after weaning and put randomly into one of six groups of 10 rats each. Five of these groups were sacrificed when the rats of the respective group weighed, on the average, 100, 200, 300, 400 and 500 grams. The group which was to have been sacrificed at an average weight of 600 grams unfortunately had dwindled to 3 rats so this group was not studied, but another somewhat less uniform group was substituted.

The breaking strength of the unwounded paravertebral skin of these rats increased as the weight of the rats increased (Fig. 3). Breaking strength and tensile strength curves did not exactly parallel each other

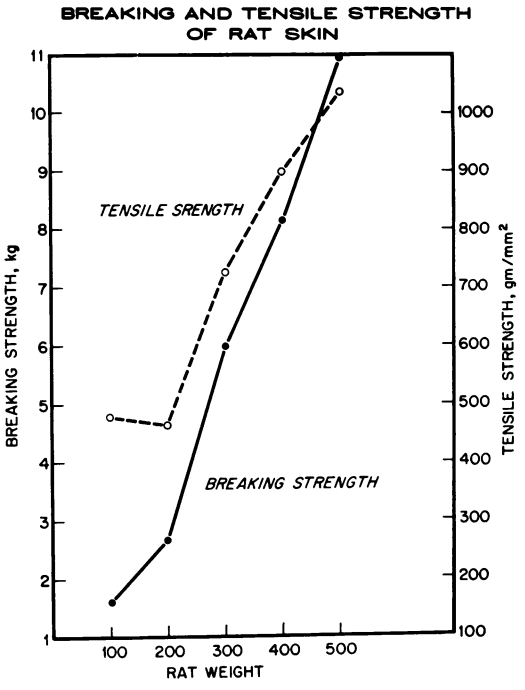

FIG. 3. Breaking and tensile strength of unwounded rat skin as a function of rat weight.

(Fig. 3); we thought that this might reflect changes in composition of the skin as the rats grew (Table 2). The change in breaking strength of the unwounded skin strip correlated poorly with its water content, weight, and concentration of hydroxyproline per gram of skin strip (Fig. 4); the best correlation was with the hydroxyproline content of the entire skin strip (Fig. 5).

TABLE 2. *Changes in Composition and Strength of Unwounded Rat Skin*

No. animals	10	10	10	10	10
Age (days)	45	56	73	94	121
Body weight (Gm.)	101 ± 1	201 ± 1	299 ± 2	403 ± 2	507 ± 3
Wet wt. of skin strip (Gm.)	1.27 ± 0.08	1.63 ± 0.10*	2.35 ± 0.16*	2.43 ± 0.14	
Per cent water	72.0 ± 0.6	68.0 ± 0.5*	66.0 ± 0.5	65.0 ± 0.4	64.5 ± 0.5
Ho-proline of strip (mg.)	11.2 ± 2.1	14.8 ± 3.2	28.0 ± 2.9	40.6 ± 4.2*	
Ho-proline of strip (% of wet wt.)	0.88 ± 0.16	0.91 ± 0.18	1.19 ± 0.22	1.67 ± 0.34*	
Per cent fat of strip		15.1 ± 4.1	14.2 ± 3.5	9.5 ± 3.8	
Breaking strength (Gm.)	1590 ± 99	2680 ± 194*	5980 ± 285*	8145 ± 332*	10953 ± 552
Strip thickness (mm.)	0.56 ± 0.01	0.96 ± 0.04*	1.40 ± 0.19*	1.52 ± 0.22	1.76 ± 0.28
Tensile strength (Gm./sq. mm.)	479 ± 33	467 ± 35	728 ± 53*	900 ± 64*	1037 ± 67

± Indicates standard error
* Indicates that difference between successive values is statistically significant ($P < 0.01$)

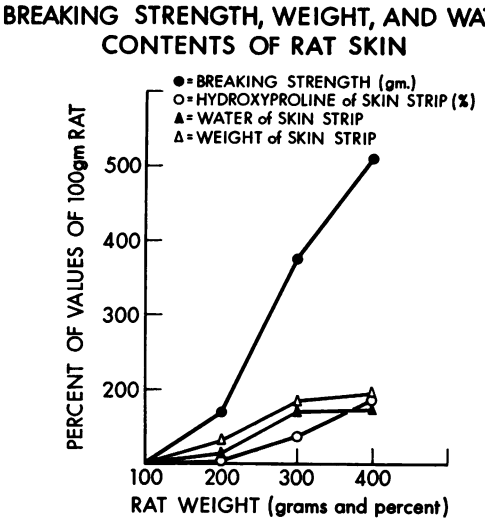

FIG. 4. Breaking strength, weight and water contents of unwounded rat skin as a function of rat weight.

The change in tensile strength correlated with hydroxyproline concentration. We believe that the apparent difference in the shapes of the breaking strength and tensile strength curves in the period 90–180 days post-operatively reflects difficulties in measuring wound thickness (see Discussion).

We normalized the breaking strength values of the healing paravertebral incisions by dividing the mean wound values by the mean breaking strength of *unwounded* skin of rats of the same weight and sex (male) of the respective groups (Fig. 6). When this is done the shape of the curve of gain in breaking strength is not changed very much, but the rate of gain during the first 8 weeks is somewhat slower than suggested by the unnormalized values. It is apparent that even at the end of 1 year, the wound is only about 80 per cent as strong as comparable normal unwounded skin.

The shape of the curve of gain in tensile strength of the wounds tested after fixation in 10 per cent neutral formalin for 11 weeks was similar to that of the "fresh" wounds, but the formalin-fixed wounds were always stronger than their fresh counterparts (Fig. 1). This difference varied at different times during the healing process, the per cent variation being greatest early, dropping sharply during the first 6 weeks, and then leveling off at about 35 per cent thereafter (Fig. 7).

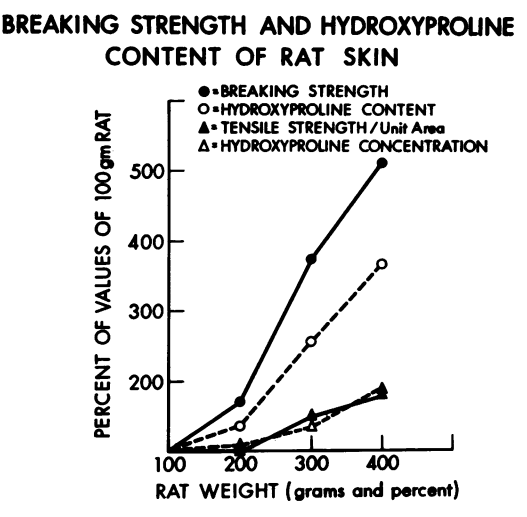

FIG. 5. Breaking strength and hydroxyproline content of unwounded rat skin as a function of rat weight.

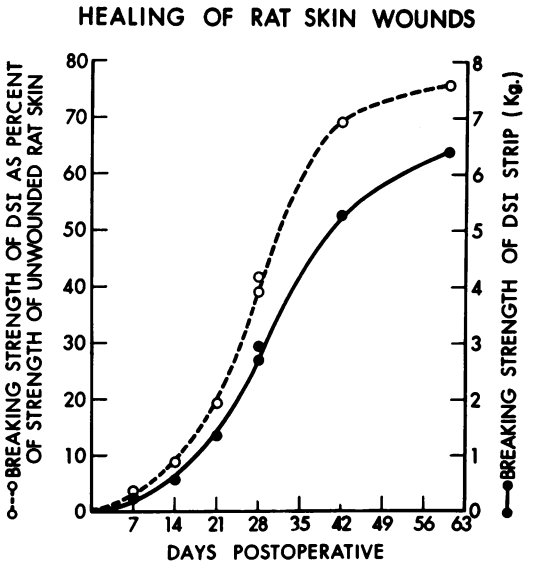

FIG. 6. Increase in breaking strength of a healing wound shown absolutely and as per cent of strength of comparable unwounded skin.

Microscopic Examinations and Findings

The center strip of wound tissue selected for microscopic study was stapled to cardboard and fixed in 10% buffered formalin. The specimen was prepared for sectioning using routine histologic methods and an automatic tissue processor. Hematoxylin and eosin, Rinehart's modification of Hale's colloidal iron, Verhoeff's elastic, Wilder's silver, PAS-colloidal iron, toluidine blue and alcian blue stains were applied. Collagen fiber deposition was best demonstrated with the Rinehart method in which fibers stain red due to their affinity for acid fuchsin. In the descriptions of collagen fibers that follow, this stain is implied unless otherwise stated. It should be emphasized that the services of a skilled and careful technician are essential. Faulty fixation or processing of tissue not only affects cellular detail but also that of the collagen fibers. All the above stains, except possibly the hematoxylin and eosin, require careful preparation. Frequent use was made of positive control material. A somewhat arbitrary use of the terms, fibril and fiber, was adopted in this study. We are aware of the fact that the finest caliber, hair-like fibrils seen under oil in the light microscope are, in turn, composed of bundles of smaller filaments with characteristic axial periodicity visible in the electron microscope. In our wound descriptions the term fibril was used until the caliber approached that of the pre-existing dermal collagen fibers. Then the latter term was substituted. A calibrated oculomicrometer disc was used for evaluation of fiber size with full appreciation of the limitations of such measurements on formalin fixed dehydrated tissue. A comparison microscope proved of great assistance in evaluating slight differences in fiber caliber and compactness.

Five-day wounds. At this stage the wound margins were only loosely held together. Manipulation during the technical preparation for microscopic examination often resulted in stretching, folding or distortion. Microscopically, the wound was covered with fibrin which overlay multiple layers of squamous epithelium and consisted of cellular granulation tissue in which the H & E-stained section revealed numerous fibroblasts, endothelial cells, undifferentiated adventitial cells, histiocytes, and lymphocytes (Fig. 8A, B). Less numerous were eosinophils, polymorphonuclear leucocytes and, in the margins, mast cells. The stroma contained pink proteinaceous material, probably fibrin. Young fibroblasts were numerous. The nuclei were large, pale and vesicular, usually oval in shape and sometimes lobulated. Nuclear chromatin was delicate and occasionally small nucleoli were apparent. The cytoplasm was neutrophilic, moderate in amount and loosely arranged. The cytoplasmic borders were often poorly outlined. It was impossible to differentiate the nuclei of young fibroblasts from those of young endothelial or adventitial cells on a morphologic basis alone. The latter two cell types were identified by their relation to newly formed vascular channels. In the older fibroblasts in the uninvolved dermis the nuclei were smaller, spindle or rod shaped and hyperchromatic. The cytoplasm was absent or scanty and eosinophilic. With the Rinehart technic only rare fine pale pink or orange colored fibrils were apparent in the wound, often mixed with coarse red fragments or droplets of degenerating pre-existing dermal collagen. Extravasated protein (fibrin) in the stroma stained yellow except in those areas with affinity for iron. Rare fragmented black fibrils were visible in the wound in the section stained by the Verhoeff method for elastic tissue. With the silver stain, fine black or brown newly formed fibrils were visible in the wound. The stroma of about half of the wounds showed affinity for iron demonstrated as Prussian blue, but sometimes masked in areas rich in fibrin which stained yellow. In alcian blue-stained sections the wound stroma was bright blue. Metachromasia of the wound stroma to a violet color could usually be demonstrated with toluidine blue and there was abundant intra- and extracellular PAS positive magenta colored material. In a sample of ten wounds the width varied from 0.1 to 1.5 mm. as measured with an oculomicrometer. In six out of ten, the wound extended from the base of a deep epidermal pocket. The pocket often contained exudate mixed with keratinized material. In four out of ten, the wound configuration seemed abnormally wide and shallow. This might have been the result of stretching during the mounting and

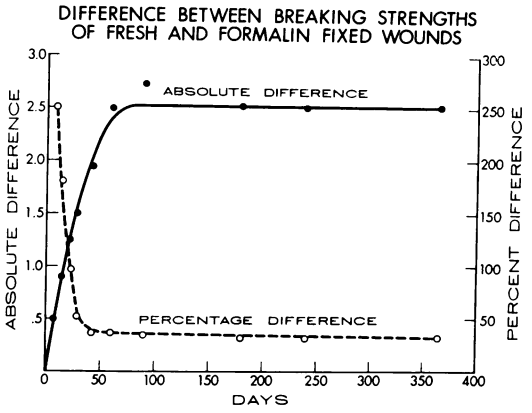

FIG. 7. Absolute and percentage differences between breaking strengths of fresh and formalin-fixed wounds as a function of time of healing.

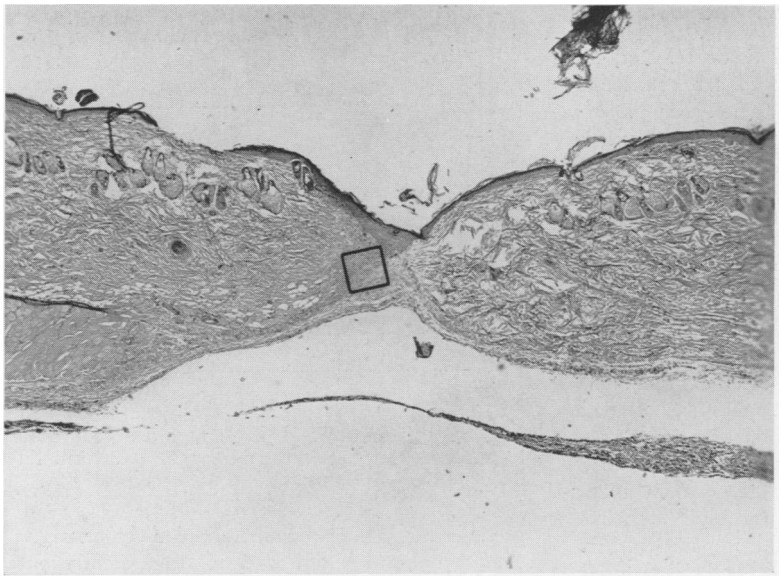

FIG. 8A. 5-day paravertebral skin wound, H & E stain, × 23. Note restoration of surface epithelium over wound which extends down from base of depressed area or pocket.

stapling of the specimen to cardboard preliminary to fixation, since at this age, the wound had little fibrillar support. The adjacent skin in five wounds was in the anagen phase of the cycle and in the catagen phase in the other five. Measurement of the skin and subcutaneous fat down to the panniculus carnosus muscle was made at a point 5.0 mm. away from the wound. It revealed a range in thickness from 1.25 to 1.90 mm. in these ten specimens.

*Fourteen-day wounds.** At this stage the cellular reaction was less than in the 5-day wounds (Fig. 9A) and collagen deposition was abundant. The new epidermis was slightly thicker than normal. The wound stroma showed little or no af-

* Although wounds were also studied critically at 7, 10, 17, 28, 32, 56, 63, 71, 78, 90, 215 and 240 days, the microscopic descriptions are omitted for the sake of brevity.

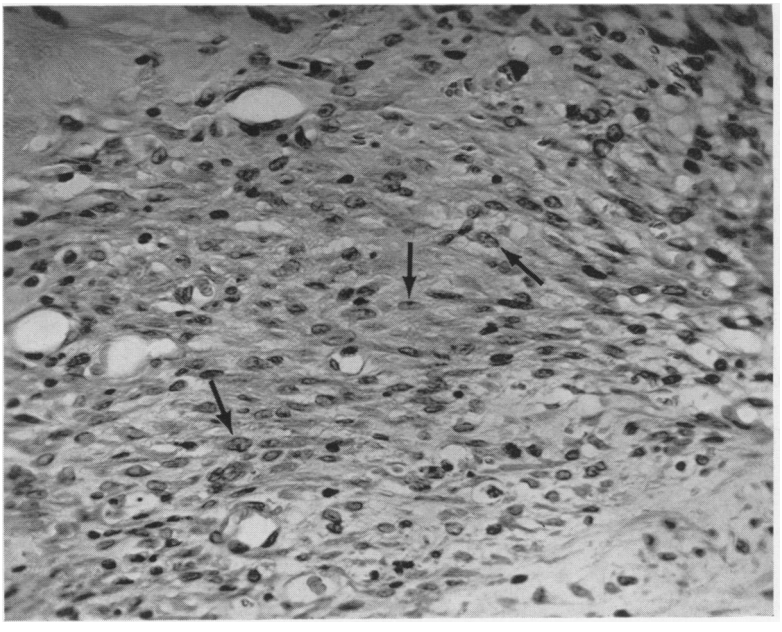

FIG. 8B. Higher magnification of inset area in Fig. 8A, × 396. Note endothelial cells lining newly formed capillaries, undifferentiated adventitial cells along capillary walls, fibroblasts (arrows) with oval or lobulated nuclei, delicate chromatin and occasional nucleoli and lymphocytes with small round hyperchromatic nuclei.

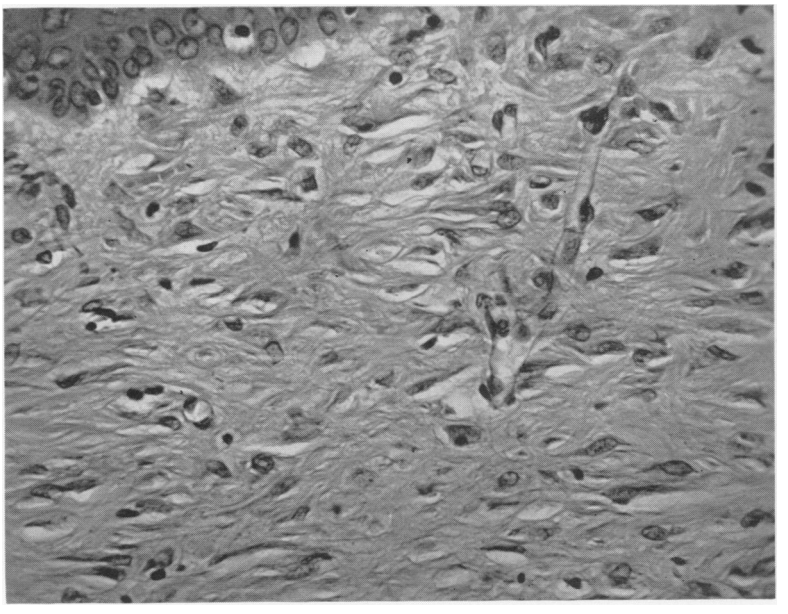

FIG. 9A. 14-day paravertebral skin wound, H & E stain, × 396. Note decreased cellularity compared with that in the 5-day wound (Fig. 8A).

finity for iron. However, slight metachromasia was still demonstrable in most wounds with the toluidine blue stain. Alcian blue-stained sections also usually revealed a bright blue wound stroma. Red fibrils were numerous in the Rinehart-stained-section with a maximum diameter of about 3 μ; 1–2 μ was representative (Fig. 9B). For the first time fibrils were arranged in loose fascicles with a maximum diameter of 10μ; 5 μ was representative. Elastic fibrils were absent. PAS positive fibrils were numerous but the stroma only faintly positive. In silver-stained sections the larger fascicles stained brown or chocolate color as contrasted with the black of the finer fibrils. In a test of variability of wound fiber collagen, two wounds of the first ten in this group were selected as representative. A subsequent additional group of 14 was then compared with these representative wounds. In

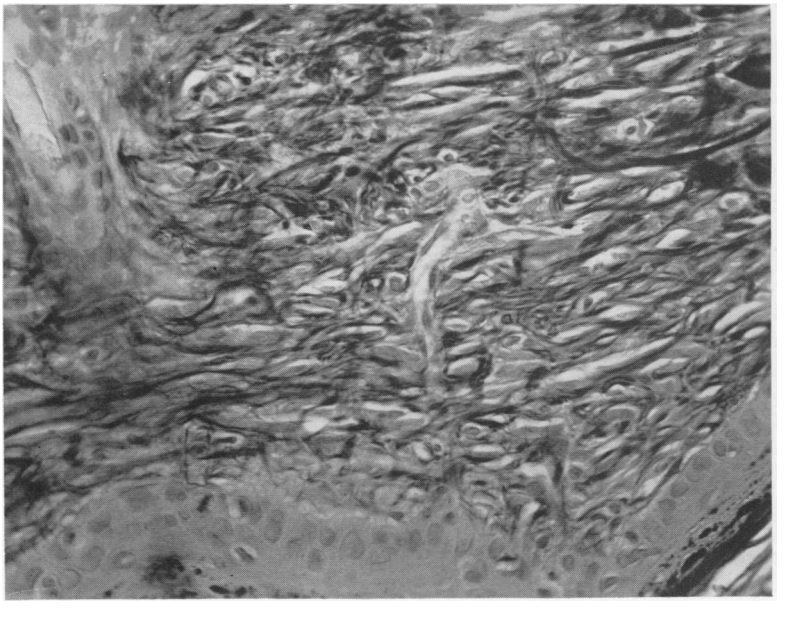

FIG. 9B. 14-day paravertebral skin wound subepidermal area, Rinehart stain, × 396. Note abundance of greyish black fibrils, red in original stained section.

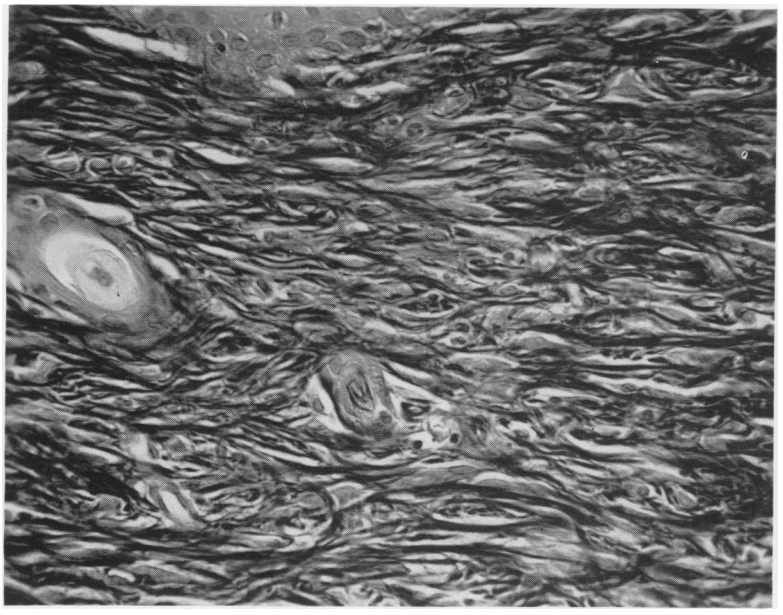

FIG. 10. 21-day paravertebral skin wound, subepidermal area, Rinehart stain × 396. Interlacing aggregates of collagen fibrils.

four of the 14, the collagen fibrils were considered coarser and more mature; in eight they were similar and in two the fibrils appeared looser and finer than in the "standards." Young fibroblasts and vascular channels were more numerous within the wound than in the uninvolved derma. In a random sample of ten, the wound width varied from 0.1 to 0.5 mm. Five wounds involved skin appendages. In three wounds the incision extended from the base of a deep epidermal pocket. The skin was in the anagen phase of the cycle in nine and in catagen in the other. The skin and subcutaneous tissue varied in thickness from 1.0 to 2.0 mm.

Twenty-one-day wounds. At this stage of maturation the wound was only slightly more cellular than the nearby uninvolved dermis. Within the wound the collagen fibrils were arranged in loose interlacing fascicles staining pink to red. Such fascicles, although much less compact, began to approximate the caliber of the smaller fibers in the pre-existing dermal collagen. Fascicles or fibers were visible with a maximum diameter of 15 μ, but 5–10 μ was representative. Maximum sized compact fibrils measured 4 μ (Fig. 10). The fibrils were only faintly PAS positive. Young fibroblasts were still somewhat more numerous than mature forms, this ratio being greater than in the uninvolved derma which was also less vascular. Of ten specimens selected at random the wound width varied from 0.25 to 0.5 mm. Three extended into, but not completely through, the panniculus carnosus. In six the wound involved an appendage or showed within its confines a focal foreign body type granuloma. No wound showed affinity of the stroma for colloidal iron. However, in six, faint bright blue foci could be identified in the wound stroma in the alcian blue stained sections. In another, focal metachromasia was identified in the toluidine blue stained section. The skin was in the anagen phase of the cycle in six, in catagen in three and in telogen in the other. The skin and subcutaneous tissue varied in thickness from 1.0 to 1.9 mm.

Forty-two-day wounds. At this stage of maturation the wound continued to be relatively acellular and collagen deposition was extensive within it. The fibers were numerous, deep red, large and compact (Fig. 11). They were still not as large or discrete as in the pre-existing intact dermal collagen nearby. Aggregates up to 15 μ were apparent, but 10 μ was representative; typical compact forms measured 5 μ. Although the fibers were more compact than in younger wounds, their fibrillar character was still more easily recognized than in the nearby intact collagen. In the latter fibers, fibrils could be recognized only in certain planes of section or at the ends of the fibers. Furthermore, the clefts between fibers were not as prominent and restoration of the felt-work design of the normal dermal collagen was not attained. Thus, the wound outline was relatively easy to trace. Vascularity was about the same as in the uninvolved dermis. Young fibroblasts appeared somewhat more numerous than mature forms but the ratio approached that of the uninvolved derma. In a random sample of ten wounds of this age, the wound

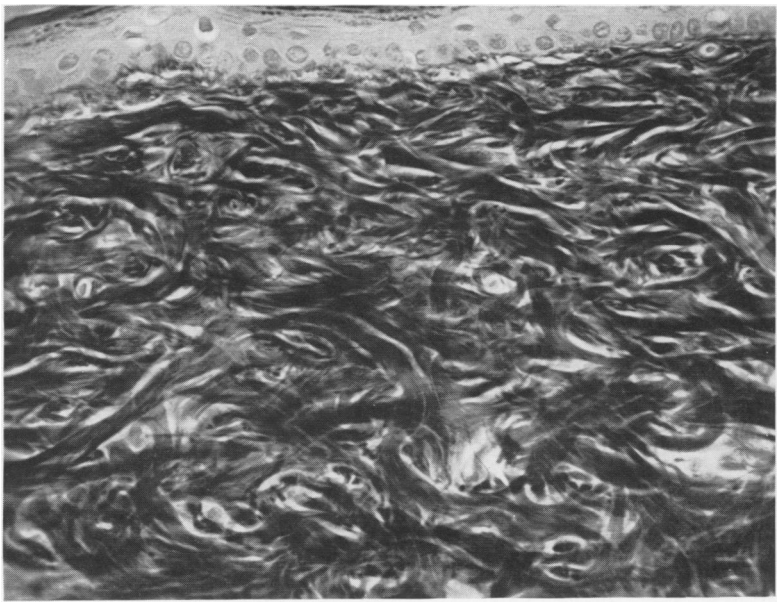

FIG. 11. 42-day paravertebral skin wound, subepidermal area, Rinehart stain, × 396. Coarser and more compact fibers than at 21 days (Fig. 10).

width varied between 0.1 and 0.4 mm. In two, the panniculus carnosus muscle was barely involved or incompletely incised by the wound. In three, the wound had involved skin appendages. At this stage the stroma or ground substance of the wound showed no affinity for colloidal iron. The toluidine blue stained sections were also negative for metachromasia. Rare small bright blue foci could still be identified in the wound stroma in the alcian blue-stained sections. The skin was in the anagen phase of the cycle in three, in catagen in four and telogen in three specimens. The skin and subcutaneous tissue varied in thickness from 1.4 to 1.75 mm.

Six-month wounds. Seven were available for microscopic study. Comparison with the average or typical wounds of the 42-day group revealed about the same fiber size, compactness and num-

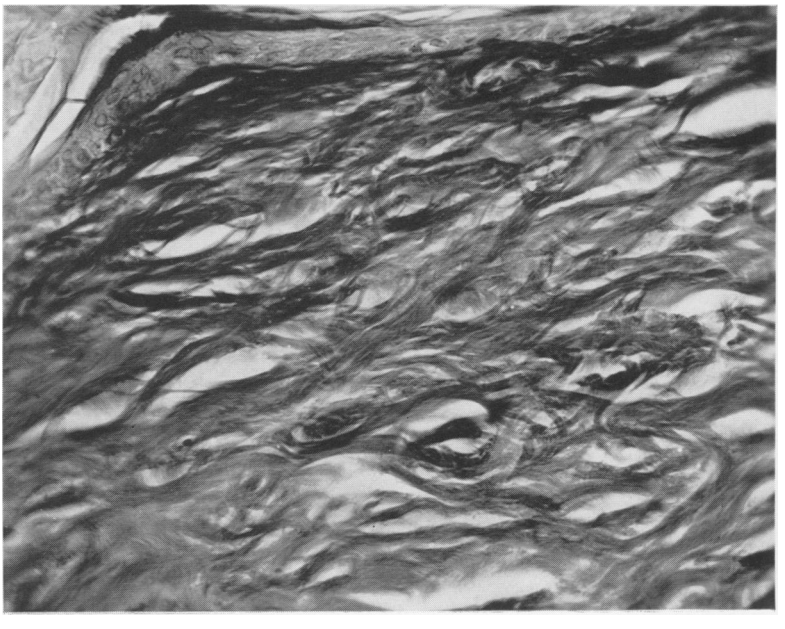

FIG. 12. 6-month-old paravertebral skin wound, subepidermal area, Rinehart stain, × 396.

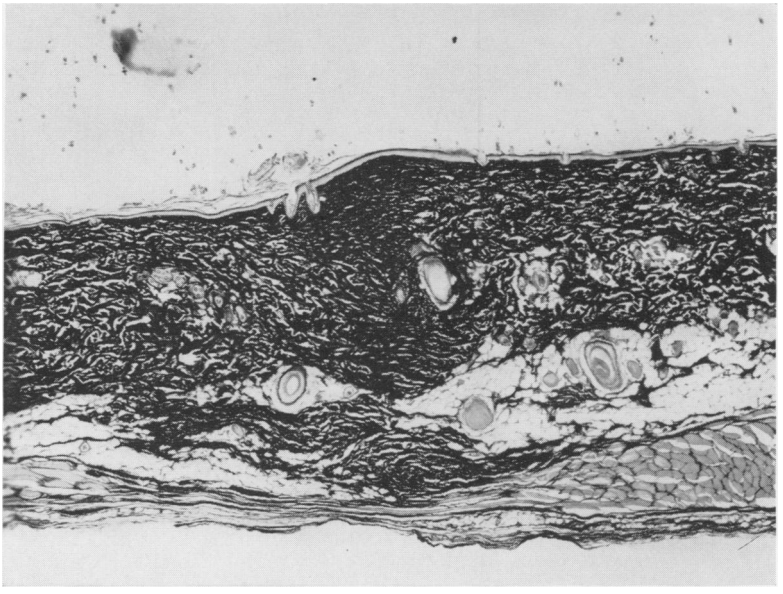

FIG. 13A. 1-year paravertebral skin wound, Rinehart stain, × 23.

ber in three, similar caliber but more compact in two and larger caliber in the remaining two. The latter were similar to the one 42-day wound with exceptionally large collagen fibers but in one the fibers were not as compact. Representative aggregates measured between 10 and 15 μ; the compact fibers between 5 and 10 μ. The wound width varied from 0.50 to 1.0 mm. Skin appendages were involved in two wounds. The panniculus carnosus was incompletely incised in one. Three wounds were in the telogen phase and four were in catagen. The thickness of skin and subcutaneous tissue down to the panniculus carnosus varied between 1.75 to 2.25 mm. The cellular population was sparse and consisted mainly of mature fibrocytes. Mast cells and endothelial elements were apparent also, the former in the deepest portions of the wound (Fig. 12).

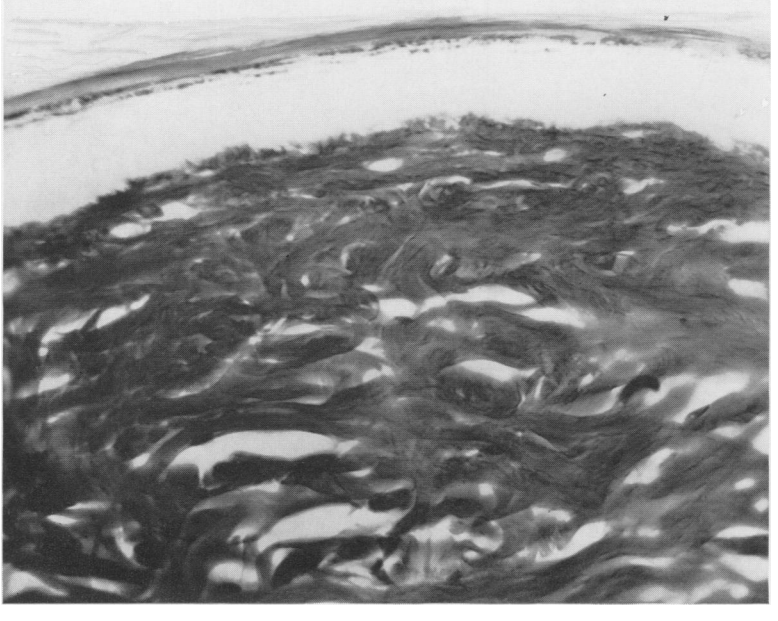

FIG. 13B. 1-year paravertebral skin wound, subepidermal area, Rinehart stain, × 396. Slightly coarser and more compact fibers than in Fig. 11.

365-day wounds. Seven were available for microscopic examination. Comparison with the average wounds of the 42-day group revealed increased fiber caliber and compactness in all the older wounds (Fig. 13A, B). Aggregates measured between 10 and 15 μ and the compact forms about 10 μ. The wound width varied between 0.37 and 1.0 mm. Skin appendages were involved in four. The panniculus carnosus was completely incised in all. Five wounds were in catagen and two in the telogen phase. The thickness of skin and subcutaneous tissue down to the panniculus carnosus varied between 1.37 to 2.75 mm. The cellular population was sparse, mainly mature fibrocytes and endothelial elements. Rare mast cells were visible, usually in the deepest part of the wound.

Discussion

Wounds of all ages created an impression of individuality on a microscopic level as specific as a finger print. Our findings of the epidermal pockets in the healing incisions are similar to Hartwell's [15] and Gillman's [11] observations of the downward growth and migration of epithelial cells for a short distance into the underlying connective tissue in contact with the cut dermal edges; the union of epithelial cells from opposite sides of the wound is accomplished beneath the surface of the skin. However, we feel that this can also be produced by variations in approximation of the wound margins. Healing, as judged by collagen fiber caliber and density, was usually most advanced in the portion of the wound just under the epidermis. In young wounds of 14 days and less duration, the lower half of the wound often contained small fragments and droplets of degenerating pre-existing or preformed collagen. Short curly and haphazardly arranged fibrils were mixed among the fragments and droplets. Rare short, newly formed fibers were also visible in the 14-day wounds. By contrast, the upper half of the same wound often revealed longer fibrils with parallel orientation to the epidermal surface and fewer droplets or fragments of pre-existing collagen injured during wounding. In older wounds the fibers were usually coarsest and most compact there. In addition, fiber arrangement approaching the three dimensional interwoven configuration of normal dermal collagen was often attained there only. The distribution in the deeper part of the wound was more irregular and disoriented so that it was not difficult to identify wounds even as old as 365 days under the microscope.

Gillman and associates [11] have previously reported that fibrosis of the incised dermis is apparently accomplished by the downward growth of connective tissue and new vessels from the subepithelial connective tissue reaction and upward growth of connective tissue from the subcutaneous fat and fascia. Also relevant to this observation is the finding by Weiss [24] that the basement lamella of amphibian skin has a highly ordered pattern and that, after wounding, it is reconstituted first directly beneath the epithelium and then progressively at deeper and deeper levels.

Elastic fibers played no detectable role in wound healing.

As a rule, the shorter the interval between wounds of varying ages, the less impressive and less consistent the differences in microscopic appearance. Thus, it was easy to distinguish between wounds 42 days old and those 14 days and younger in all wounds in these groups, but with shorter intervals, individual exceptions were observed with increasing frequency. Critical examination of groups of wounds of known ages in the comparison microscope showed slight but progressive increase in fiber number, caliber and compactness up to 365 days, but after 42 days it was impossible to estimate the ages of a group of wounds by "blind" readings when the only information submitted was that the wounds were older than 42 days and up to 365 days old. Such differentiation, though, could be made by breaking strength measurements up to 90 days after wounding.

The generally held view that the great bulk of collagen formation and gain in

tensile strength of healing skin incisions is over in the first 2 or 3 weeks, and that the wound reaches its maximal strength at the end of this time, stems in large measure from the classical paper of Howes, Sooy and Harvey (1929).[17] However, this view fails to take into account the paper of Howes, Harvey and Hewitt (1939).[16] We believe that this is due in large measure to their separation of the healing process into two phases: the period of fibroplasia and the period of scar formation. In their view, the period of fibroplasia occurred during the first 14 days postoperatively and the *wound* reached maximal strength at that time. After a brief period during which the strength of the wound remained unchanged, the second phase, or period of scar formation, began; the breaking strength of the wound gradually increased and purportedly reached the strength of unwounded skin in about 60 days.

This separation of the healing process into two phases has, in our opinion, led to confusion and misinterpretation of the rates at which skin wounds heal. For example, Douglas [5] has concluded that experimental incisional wounds of the dorsal aponeurosis in rabbits attain maximal breaking strength values at a rate significantly slower than skin incisions; this comparison was based on the thought that skin wounds reach maximal strength in a few weeks. In fact, when the data of the present experiments are considered along with those of Howes, Harvey and Hewitt, it is apparent that the fascial and dermal wounds heal at similar rates. In a like vein, Gaylis *et al.*[9] reported a study of healing of arterial autografts in dogs and found maximum tensile strength after 5 to 6 weeks. They also felt that the healing velocity was much slower than in skin wounds as reported by Howes, Sooy, and Harvey, but the arterial autografts had in fact healed at about the same rate or somewhat more rapidly than dermal wounds.

Howes, Harvey, and Hewitt had pointed out that when the wound had reached maximal strength at the end of 2 weeks, it was only 13 per cent as strong as unwounded rat skin. This was ascribed to the fact that the newly formed connective tissue was loose, vascular, and composed of fine parallel fibers with numerous fibroblasts in contrast to the well-differentiated, heavy, and compact collagenous fibers of the original corium. They ascribed the secondary rise in strength, which began about the 20th day to an organizing differentiation of the primary tissue produced by the fibroplasia.

When we replotted the data of Howes, Harvey, and Hewitt, omitting the points for which data on only a few rats were obtained, the curve for gain of tensile strength is remarkably similar to that which we have obtained and does *not* demonstrate any break in the curve. It seems clear, then, that the two phases Howes, Harvey, and Hewitt described are not separated in time by a "plateau"; in all likelihood, almost as soon as the first collagen is laid down, reorganization and remodelling begin. From the quantitative point of view, we interpret our data as showing that increasing amounts of collagen are laid down during at least the first 6 to 7 weeks, rather than the 2 to 4 weeks as suggested by Howes *et al.*[16] We base our conclusion on 1) the histologic evidence of increasing stainable collagen fibers during this time and 2) the effects of formalin fixation on the breaking strength of the wounds.

Formalin fixation of a wound of any age increases its breaking strength. The absolute increase in strength increases with increasing age of the wound until approximately the 7th postoperative week, after which this difference remains constant. This sharp breaking point corresponds with the sudden decline, also about the 6th to 7th postoperative week, in the rate of collagen increase as judged histologically and in the

gain of wound strength. The *percentage* difference in breaking strength between fresh and formalin fixed wounds also shows a sharp break point at about the 7th week, but this curve is the mirror image of the *absolute* difference curve; that is, there is a rapidly *declining* percentage effect of formalin until the 7th postoperative week and then a leveling off. These data demonstrate that the effect of formalin, mediated through methylene bridges between NH groups, is greater on an *immature wound* than on a mature one. It is likely that the earlier, sparser, and finer collagen fibrils lend themselves to a *greater* number of cross-links per macromolecule after formalin treatment than do the later, more compact, and coarser fibrils, which have presumably already undergone a certain amount of cross-linking by hydrogen bonding spontaneously. Dunphy and Jackson [7] have also advanced the idea that reparative collagen undergoes increasing cross-linkages as the wound heals.

A similar effect was seen by us in wound specimens from rats given 25 per cent D_2O.[21] This treatment resulted in an abnormal healing pattern. The wounds showed focal porosity and focal decrease in fiber caliber and density. Tensile strength of these wounds was 40 per cent below control levels, a difference greater than we would have expected from the degree of histologic difference. This suggests a qualitative change in the collagen of the D_2O animals. This view is supported by the finding that formalin fixation increased tensile strength of the D_2O wounds disproportionately compared to the H_2O controls, so that the tensile strength deficiency of the D_2O wounds was entirely eliminated. The effect seems analogous to that mentioned above, that is, fine or immature collagen, however produced, is cross-linked to a greater extent by formalin than older and coarser fibrils.

Another point heretofore unmentioned, to our knowledge, in discussions of experimental wound healing of the rat, is the effect of the progressive growth of the rats on the unwounded dermal and wound collagen. The ratio of young to mature fibroblasts or fibrocytes within the wound was compared to that of the nearby uninvolved derma. After 42 days, the ratio seemed to be the same. However, this does not indicate a cessation of fibroblastic function. The rats continued to grow throughout these year long experiments, although at a somewhat slower rate during that latter six months. Obviously, the skin also participated in this growth. From our measurements (Table 2) it increased in thickness and composition as well as in total area. Fibroblastic function must have been continuous, therefore, to supply the increased bulk of dermal collagen. It would be important in a future study to compare the rate of maturation of collagen fibers in the wounds of adult animals in a species with stable body weights.

Howes, Harvey and Hewitt related the breaking strength of the wounds to their cross-sectional areas. We have discussed the necessity for, but difficulties in, measuring wound area.[18] As far as we know, all methods used to date to measure wound thickness lack the level of reliability of the breaking strength measurement. It is our thought that the slight difference in shapes of the breaking and tensile strength curve around 90 to 180 days reflect differences in wound thickness measurements. Howes and his colleagues also tested normal unwounded rat skin for its strength, and reported that the healing skin scars reached the strength of unwounded skin in about 60 days. Our data shows, however, that even at the end of a year, the healed wounds were only about 80 per cent as strong as unwounded skin. We have shown the marked changes in thickness and strength of the skins of rats of different ages. The breaking strengths of unwounded

skin from rats of different ages correlated poorly with the skin thickness, water concentration, weight, and concentrations of hydroxyproline per grams of skin strip; the best correlation was with the hydroxyproline content of the skin strips. The change in tensile strength correlated with hydroxyproline concentrations. This is consistent with the generally accepted view that collagen is primarily responsible for the strength of tissues.[14]

We have already pointed out that the histologic appearance of the wound which had healed for a year was still detectably different from that of the unwounded skin, a fact which may explain why the one year old wound was still significantly weaker than comparable unwounded skin. Douglas has related the healing of fascial wounds to that of unwounded fascia of the same rabbits. There, too, the wounds never reached (within a year) the strength of the unwounded fascia. In general, the gain in strength of the rabbit fascial wound and rat skin wounds are similar in the first months, but Douglas interprets his data as showing a constant gain of breaking strength of the fascial wounds throughout the first year (as judged from a plot of the breaking strength against the log of the postoperative time). Our data of rat skin wounds do not show this; there is a relatively constant gain of wound strength as judged by this sort of plot for the first 3 months, but it then slows down remarkably. We doubt that there is a fundamental difference in the healing of fascia and skin wounds. Dunphy [6] has also recently pointed out the long duration of the healing in skin and fascia.

In a previous publication [10] we pointed out a number of microscopic variables which would have affected the healing process of midline laparotomy wounds in the rat. These wounds were all made by the same operator in as "standard" a manner as possible. At that time, we were concerned because of our failure to obtain reproducible breaking strength values in wounds which we felt were comparable. In the present experiment, we eliminated some of the variables by making paravertebral incisions which were restricted to the skin and superficial muscle. However, microscopic variables could still be identified despite our precautions. In a few wounds the incisions had not extended completely through the panniculus carnosus. In some, the wound margins were not as closely approximated by the sutures as in the majority. In others, the wounds were everted at their upper ends at the time of operation with resultant formation of epidermal pockets apparent on microscopic examination. Thus, it would appear that some variation in wound depth and configuration is inevitable.

Finally, there were variations in the phase of the skin cycle as well as in skin thickness. Hair and skin cycles of 30 days duration have been described in the rat.[2, 3, 20, 23] The term anagen is used for the growing phase of about 18 days. It is characterized, histologically, by the thickening of the epidermis and dermis, lengthening of hair follicles and an increase in fatty tissue. The skin shows beginning involution in catagen, the next phase, which has a short duration of 2 or 3 days. In catagen, the dermis is intermediate in appearance between the thick skin of anagen and the thin resting structure of telogen with its sparse fat and short scattered appendages. Telogen lasts about 10 days. It was not possible to correlate individual variations in degree of healing at any stage with the phase of the skin cycle. Up to 32 days postoperatively, the wounds showed a predominance of the anagen phase. It has been stated that plucking of hair will start a new cycle in the rat, rabbit, and mouse.[23] This apparently is not true of clipping and shaving preliminary to operation as was done on our animals, since they showed considerable intragroup variation postoperatively.

Unless careful attention is paid to the various steps of the histologic technic, including the automatic tissue processor, errors may occur which affect collagen fiber detail and stainability; improper temperature control of the paraffin, overloading the processor with too many specimens, use of a new supply of paraffin with a different melting point and neglect of the diminishing concentration of the dehydration alcohols and clearing agents following repeated use are a few examples. Furthermore, preparation of a new supply of staining solutions may be followed by disturbing changes in the shade of collagen fibers and in the affinity of the ground substance for the freshly prepared dye. Some of these changes would not be considered serious under ordinary conditions of examination. For example, a surgical pathologist could make a diagnosis on such material. But their effect on collagen fiber caliber, density and outline reduced the "sensitivity" of a comparative study with groups prepared at different times, or made such a comparison impossible. Ideally, a wound from each of the 17 groups reported in the study should be included in each "run" and processed and stained with the others under identical technical conditions. We have found that the automatic tissue processor (Technicon) can handle a maximum of 20 specimens satisfactorily in a single tiered instrument.

Summary

1. Experimental dermal wounds in rats showed progressive microscopic changes in number, caliber and density of the collagen fibers up to 1 year.

2. The rate of change varied; it was much more rapid up to 42 days than afterward, but still detectable after that time when cellular activity and vascularity in the wound had subsided to that of the adjacent uninvolved derma.

3. The breaking strength of wounds increases rapidly and almost linearly, from about the 14th to the 42nd postoperative day; this rate of increase then declines during the next 4 weeks, and the wound attains almost maximal strength in 3 months. The entire curve is sigmoid. We conclude that wound collagen increases for 6 to 7 weeks after the wound.

4. Tensile strength gain probably parallels the breaking strength gain over the healing period, but uncertainties in the wound thickness measurements make the exact tensile strength curve less precise than the breaking strength curve.

5. Formalin fixation increases wound breaking strength, but has a proportionately much greater effect on early wounds (up to 6 or 7 weeks) than later ones. This corresponds exactly to the period of increasing wound collagen, suggesting that immature and submaximally crosslinked collagen is most affected by formalin, itself a crosslinking agent.

6. The breaking strength of unwounded skin from animals of different ages varies almost linearly with the weight of the animals; it correlates poorly with skin thickness, water concentration, weight and hydroxyproline concentration in the skin strip; the best correlation was with the hydroxyproline content of the strip. This is consistent with the view that collagen is primarily responsible for the strength of connective tissue.

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