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A LIGHT MICROSCOPICAL STUDY OF HEALING INCISED DERMAL WOUNDS IN RATS, WITH SPECIAL REFERENCE TO EOSINOPHIL LEUCOCYTES AND TO THE COLLAGENOUS FIBRES OF THE PERIWOUND AREAS

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Summary.—Incised wounding of rat skin had a significant effect on the number of eosinophils both within the scar and in the adjacent areas. The numbers increased, after the inflammatory phase, to a peak between the 7th and 12th–14th days after wounding and remained elevated for the rest of the 30-day experimental period. In the areas contiguous to wounds, the dense bundles of collagenous fibres characteristic of normal dermis became progressively more dispersed from the 3rd to the 14th day, and then gradually re-aggregated into bundles. The fact that eosinophils, which are known to be collagenolytic, increase in numbers and change in morphology at the same time as newly formed scar collagen is remodelled and fibres around wounds are aggregated indicates a role for these cells in connective tissue metabolism. It is suggested that the dispersion of the collagenous fibres in the areas around wounds which occurs before the rise in eosinophil numbers could be related to the known increase in sulphated glycosaminoglycans around incised wounds.

THIS study was prompted by a chance observation (Bassett, 1965) that eosinophil leucocytes were present in healing dermal wounds in rats and mice.

None of the available reviews on wound healing (*e.g.* Arey, 1936; Ross, 1968; Schilling, 1968; McMinn, 1969; Dunphy and Van Winkle, 1969; Hunt, 1970; Ross, 1971; Peacock, 1973*a*, *b*; Heughan and Hunt, 1975) refer to any specific study of eosinophils in this process. Those who describe the cells involved in wound healing have confined their attention chiefly to the infiltrating neutrophils, macrophages and lymphocytes of the early inflammatory phase and to the behaviour of fibroblasts. In the very few original papers (Ordman and Gillman, 1966; Levinson *et al.*, 1965) in which the presence of eosinophils has been mentioned, these cells have received only passing comments, since the authors' interests lay with other aspects of the healing process.

This paper, which amplifies the preliminary account published by Baker, Bassett and de Souza (1976), comprises a systematic quantitative and morphological study of eosinophils in healing rat skin wounds at different stages between 6 and 30 days after making the lesions. Using the same material and that from wound healing experiments of shorter duration (Days 1–10), we have also studied in detail the collagenous fibres both within the newly formed tissue of the scar and in the pre-existing dermis of the areas around wounds.

MATERIAL AND METHODS

Eosinophils

Animals.—Because an initial exploratory investigation showed that the number of eosinophils found in skin of wounded rats during the first 6 days after the lesion was not appreciably different from that in unwounded skin of controls, eosinophil counts were made only on samples from animals having wounds aged 6 days and over. Because the exploratory investigation revealed also that eosinophils in both

normal and wounded skin varied greatly in number between rats of heterogenous parentage in our random-bred colony, an experiment was designed to use sibling rats from within one litter. The experiment was simultaneously replicated on a second litter as a precaution against loss in the first litter during the experiment. Since all animals from both litters survived, the results for two separate experiments were available for statistical analysis.

The two litters consisted of 9 (Series I) and 8 (Series II) rapidly growing males, aged 8–10 weeks and thus just reaching sexual maturity, with a weight range of 200–230 g. They were numbered individually, housed in groups of 4–5 in wire cages, and fed standard pelleted diet and water *ad lib.* The room temperature was maintained at 20°.

From each of the two series, 4 rats were randomly selected (using a table of random numbers) to be unwounded controls. The remaining animals were randomly prenominated for wounding, so that the 5 treated rats in Series I would have 6-, 12-, 18-, 24- and 30-day wounds, and the 4 treated rats in Series II would have 7-, 14-, 21- and 28-day wounds. All the rats, both treated and control, were killed on the same day.

Wounding procedure.—The rats were anaesthetized with ether. An area approximately 4 cm wide along the whole length of the back of each animal was then prepared by closely clipping the hair with an electric clipper and swabbing with 70% alcohol.

The wound consisted of a 10-cm-long midline incision through the whole thickness of the skin, measured cranially from the tail base. The cut edges were closely apposed with interrupted 5·0 gauge Dermalon sutures which were removed after 7 days.

Removal and fixation of tissue.—The rats were killed by decapitation, under light ether anaesthesia. From each treated animal, a strip of skin which included the whole length of the wound with approximately 1 cm of skin on either side was removed, pinned flat on sheet cork and fixed for 7 days in 10% buffered formol saline (phosphate buffer) at pH 7·0. From each of the control rats a strip of skin with equivalent dimensions was similarly treated.

Sampling procedure.—After fixation, each of the strips was marked with calipers at points 1 cm apart, beginning from the cranial end of the strip. Ten slices (*i.e.* 10 different regions), each 3–4 mm wide, were then cut across the strip at each of these defined points at rightangles to the median plane.

Histological methods.—The slices were dehydrated in several changes of 2-ethoxy ethanol and double-embedded through 2% low-viscosity nitrocellulose in 2-ethoxy ethanol into Tissueprep wax, M.P. 56·5° (Fisher Scientific Co., Fair Lawn, New Jersey, U.S.A.).

Sections were cut at 4 μm in a plane at rightangles to the surface of the skin. In order to obtain satisfactory thin sections of such tough tissue, it was necessary first to trim away the wax covering the tissue surface and soak the block in a mixture of 1 part of aniline to 9 parts of glycerine (Lendrum, 1944) at 4° for 24 h, to rechill the block at intervals during cutting and to to use a cold knife. Sections could be completely flattened throughout their length only by floating on water on slides on a hotplate and heating at approximately 75° for a few seconds so that the wax was completely melted. The sections were stained with toluidine blue–phloxinate (Lillie, 1941), to show especially esoinophils and fibroblasts.

Eosinophil counts.—Sections in which the eosinophils were to be counted were selected in the following way:

To allow investigation of any "rat × region" interaction (*i.e.* a different distribution of eosinophils throughout the 10 regions from one rat to another), 2 sections were taken from each block (*i.e.* each of the 10 regions) from the controls of Series I, at distances of one-quarter and three-quarters through each block. They were then mounted and stained. Subsequent analysis of the eosinophil numbers in these sections showed that there was no significant interaction and that there was a significant difference between these control rats (see Results). Therefore, for the controls of Series II and for all the wounded rats (from both Series I and II) only one section was mounted and similarly stained, at a distance half-way through each block. In each section the eosinophils were counted in 60 fields of diameter 150 μm, under oil immersion at a magnification of × 1000.

The standard procedure by which these counts were made in normal (control) and in wounded skin is illustrated diagrammatically in Diagrams 1 and 2 respectively. Counts were made in 60 fields of diameter 150 μm distributed in 4 adjacent rows of 15 consecutive fields, all approximately parallel to the surface, in the region between the panniculus carnosus muscle and the epidermis. In sections from the control animals, the deepest row was selected at a site in the relatively loose connective tissue of the dermis immediately adjacent to the panniculus carnosus muscle. The eosinophils in a row of 15 consecutive fields (2·25 mm) approximately parallel to the muscle were counted and the results recorded separately. Eosinophils were then counted in each of 15 consecutive fields in 3 further rows parallel and adjacent to each other, progressing through the denser part of the dermis above the original row next to the muscle towards the skin surface. By this procedure counts were made through a considerable distance in the denser part of the dermis so that in some sections practically all of the distance

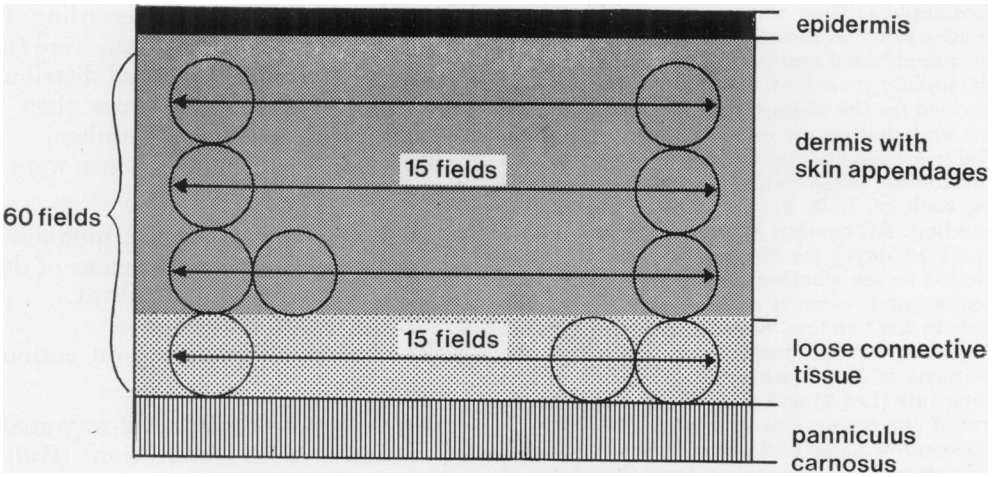


DIAGRAM 1.—The general structure of sections of skin from unwounded rats and the procedure for counting eosinophils.

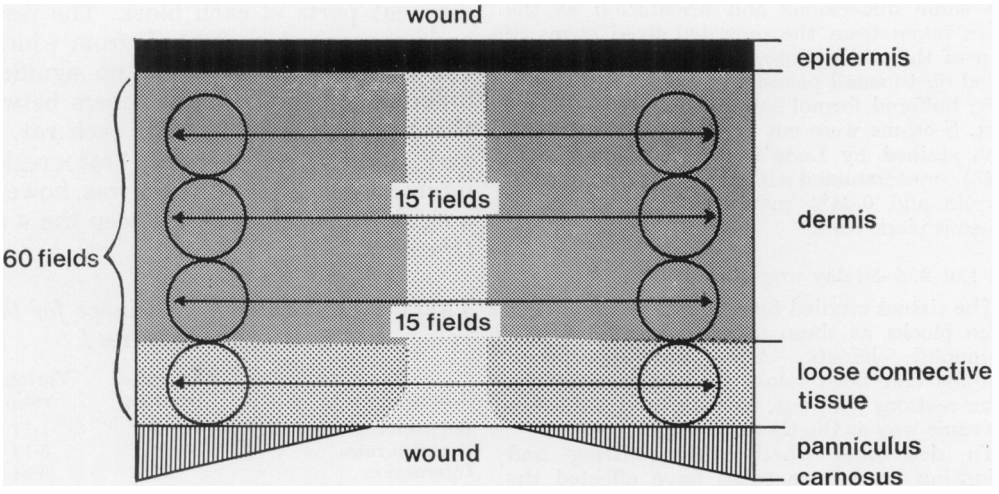


DIAGRAM 2.—The structure of sections of skin from wounded rats and the procedure for counting eosinophils.

between the muscle and the epidermis was included.

In sections from the wounded animals, the counts were made in a similar way, except that the deepest row of 15 fields was specifically sited so that the centre of the row lay at about the centre of the wound, between the severed ends of the panniculus carnosus muscle. Since the wound was never more than 0·3 mm across most of the counts were made in the areas on each side of the wound.

Observations of morphology.—After the eosinophil counts had been made, sections were systematically examined for morphological features in cells at the different stages of wound healing.

Collagenous and reticular (argyrophilic) fibres

These were studied in two different lots of rats: one lot with wounds between 1 and 10 days old, and one lot (the same Series I and II sibling rats used for the eosinophil studies) with wounds between 6 and 30 days old.

(i) Lot 1. 1–10 day wound stages.

Animals.—The rats were 28 males of heterogeneous parentage from the same source as the rats used in the initial exploratory investigation

of eosinophils. Their weights were within about the same range as those of the sibling litters, and they were housed and fed in the same way.

Wounding procedure.—This was the same as described for the sibling rats except that all rats were wounded on the one day. They were then killed in groups of 4, so that there were 4 controls (unwounded) taken on the day of wounding, and 4 at each of 1, 2, 3, 4, 6 and 10 days after wounding. An overlap of post-wounding stages (6 and 10 days) for the two lots of rats was included to see whether the appearances of the areas around wounds differed at comparable stages in the two lots. Since in fact we found no differences we felt justified in including the specimens of Lot 1 with the specimens of the sibling rats (Lot 2) and regarded the two lots as parts of one continuous sequence.

Removal of tissue and histological methods.—A single sample only was taken from the centre of the mid-dorsal region of each rat since, for eosinophil numbers, no significant "rat × region" interaction had been detected. All samples had the same dimensions and orientation as the slices taken from the mounted fixed strips of skin of the sibling rats. Fresh slices were flattened on to small pieces of paper, then fixed in 10% buffered formol saline and processed into wax. Sections were cut at 4 μm, mounted and then stained by Long's silver method (Long, 1948) counterstained with Weigert's iron haematoxylin and 0·04% methyl blue in saturated aqueous picric acid.

(ii) Lot 2. 6–30 day wound stages.

The tissues studied for this lot were from the same blocks as those used to investigate the eosinophil changes. After the appropriate sections had been taken to stain eosinophils, other sections were cut, mounted and stained in the same way as the tissues in Lot 1.

To determine whether the cutting and mounting procedures could have affected the microscopical appearance of the fibres in the areas around wounds additional sections (though more difficult to cut and less flat) were prepared from the same blocks with and without the preliminary soaking and were treated at the minimal heat (approximately 45°) required to stretch the floating sections.

RESULTS

Eosinophils: statistical analysis of eosinophil counts

(Using the sibling rats in Series I and II)

The results of the intial exploratory investigation showed that eosinophils were distributed throughout normal unwounded rat skin approximately according to a Poisson distribution. The data were therefore transformed to a normal distribution and statistical analyses were then performed on the eosinophil numbers.

Two-way analyses of variance were performed to test the following:

1. Differences in eosinophil numbers between the 10 different regions of dorsal skin from each individual rat.
2. "Rat × region" interaction.
3. The effect of wounding on eosinophil numbers.

Series I: control rats.—A 2-way analysis of variance with replication (Huitson, 1966) was carried out on the eosinophil numbers for the 4 control rats, using the counts made on the 2 sections taken from different parts of each block. The results are summarized in Table I, from which it can be seen that there was no significant difference in eosinophil numbers between the 10 different regions for each rat, nor was there a significant "rat × region" interaction ($P > 0{\cdot}05$). There was, however, a significant difference between the 4 rats ($P < 0{\cdot}01$).

TABLE I.—*Analysis of Variance for the 4 Control Rats in Series I*

Source of variation	Degrees of freedom	Variance ratio
Between regions	9	1·11
Between rats	3	5·14
Interaction	27	0·81
Residual variation	40	
Total	79	

Series I: wounded rats.—The analysis proceeded by performing a 2-way analysis of variance on the eosinophil numbers from the 5 wounded rats. The resultant variation between rats was compared with that of the controls, using an F-test, and was found to be significantly higher than that from the controls ($P < 0{\cdot}05$).

Series II: control rats.—A similar analysis was then carried out on these controls, but in the light of results from the controls from Series I, a 2-way analysis of variance without replication was performed. The

TABLE II.—*Analysis of Variance for the 4 Control Rats in Series II*

Source of variation	Degrees of freedom	Variance ratio
Between regions	9	1·77
Between rats	3	1·78
Residual variation	27	
Total	39	

results are presented in Table II, from which it can be seen that, again, there was no significant difference in eosinophil numbers between regions, but for these controls there was no significant difference between rats ($P>0·05$).

Series II: wounded rats.—The eosinophil numbers for the 4 wounded rats were analysed by the same methods as were those in Series I and again it was found that the variation between the wounded rats was significantly higher than the variation between the controls ($P<0·05$).

Interpretation of increased variation in eosinophil numbers as a result of wounding.—In order to interpret this increased variation, an attempt was made to relate the eosinophil numbers to the age of the wound in days. To this end, regression analyses (Draper and Smith, 1966) were performed on the figures for the wounded rats for each series.

In both series there was a significant quadratic regression ($P<0·01$ in Series I; $P<0·05$ in Series II). Graphs in which eosinophil density (*i.e.* eosinophils in 600 fields) is plotted against age of wound in days (Diagrams 3 and 4) confirm by eye that eosinophil density does not change

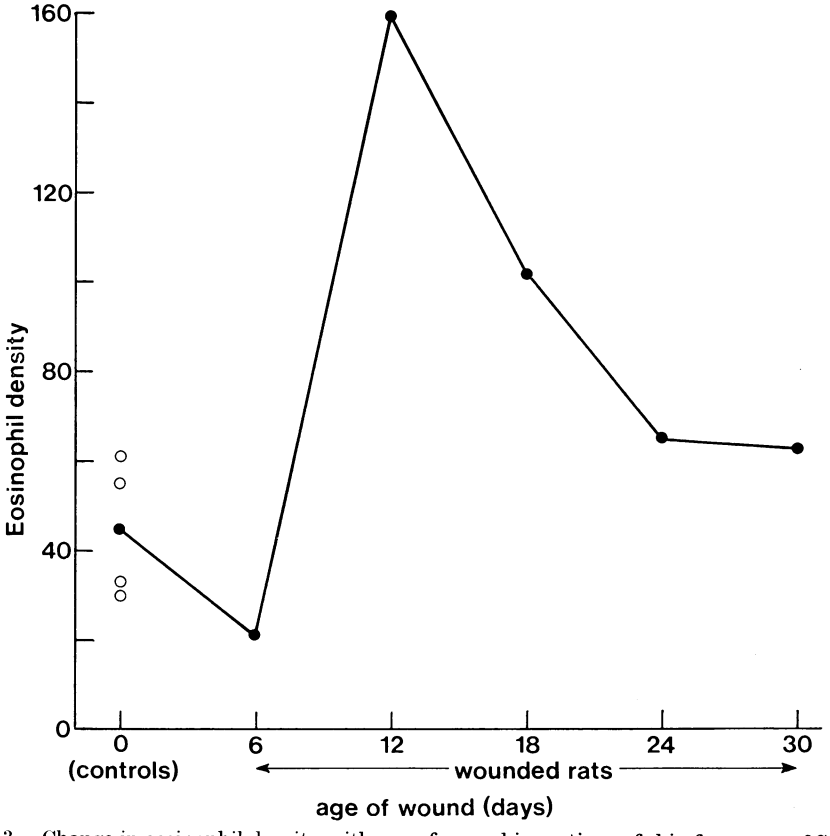


DIAGRAM 3.—Change in eosinophil density with age of wound in sections of skin from rats of Series I. Points indicate the total number of eosinophils in 600 fields for each rat (eosinophil density).

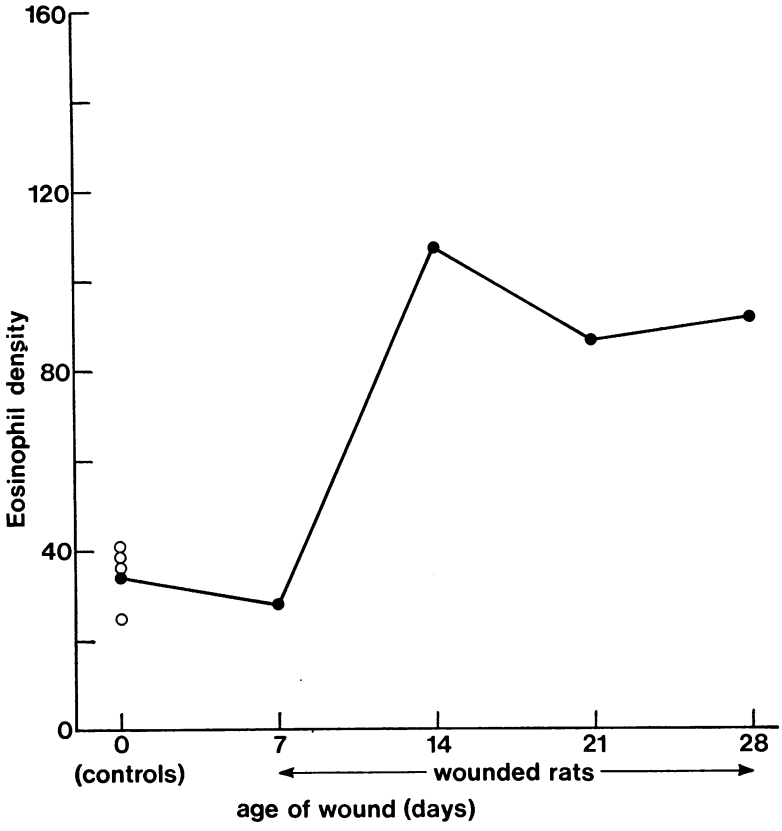


DIAGRAM 4.—Change in eosinophil density with age of wound in sections of skin from rats of Series II. Points indicate the total number of eosinophils in 600 fields for each rat.

linearly with increasing age of the wound. It can be seen that the density begins to rise only after the 6–7th day and reaches a peak at the 12–14th day. It then falls, but even at 30 days does not entirely reach the same level as that of the control animals.

Further regressions of eosinophil numbers on $\log_{10}$ (age in days) gave a superior fit and indicated that eosinophil number is more clearly related to the $\log_{10}$ (age of wound) than to the age of the wound itself. This suggests that the rate of change in the numbers of eosinophils slows as the wound ages.

The quadratic regressions involving $\log_{10}$ (age) calculated for the wounded rats in each of the two series were not significantly different ($P>0{\cdot}05$). This supports the view that the changes in eosinophil numbers after wounding followed the same pattern in each series.

Comparison of eosinophil numbers in different parts of the dermis.—A χ^2 dispersion test was performed on the cell counts of the rats in Series II to see if there was a difference in eosinophil numbers in the area comprising the row of 15 fields adjacent to the panniculus carnosus muscle compared with those in the remaining 45 fields in the more superficial area (Diagram 1).

In each of the 4 control rats there was no significant difference in eosinophil numbers between these two areas ($P>0{\cdot}05$). In the 4 wounded rats, however, the eosinophil numbers were always higher in the row adjacent to the panniculus carnosus muscle. This difference, which was highly significant ($P<0{\cdot}001$) in 3 of the rats (but

not significant in the other, $P>0{\cdot}05$), suggests that the major changes occur in the area next to the panniculus carnosus muscle, but does not exclude the possibility of some changes in the more superficial areas also. When an analysis of the eosinophils in the 45 more superficial fields was performed, there was, as before, a significantly greater variation between wounded rats than between controls ($P<0{\cdot}05$) and thus it can be concluded that the changes in eosinophil numbers which occur during wound healing are not restricted to the area immediately overlying the panniculus carnosus muscle.

Observations of morphology

Several variations in the morphology of eosinophils within the tissues were observed, not only in sections sampled at 12 and 14 days after wounding when these cells were most numerous, but also at later stages. Details of these variations, which could be appreciated fully only by examining the cells at a magnification of $\times 1000$ under oil immersion, can be seen in figs. 17–21.

Many of these cells resembled eosinophils of rat blood (Schermer, 1967), being more or less round, well-granulated and with annular nuclei characteristic of this species. Such cells appeared to be most plentiful in the region adjacent to the panniculus carnosus muscle, both within the scar among the basophilic fibroblasts and in the areas around wounds, and surrounding small blood vessels within the newly formed tissue of the scar (Fig. 17), although some were seen also within the more superficial areas around wounds (Fig. 21). In contrast to these apparently intact cells, degranulated eosinophils (Figs 18, 20 and 21) were often found, chiefly within the areas around wounds, but also occasionally within the scar. In some instances, the shed granules could be seen scattered into the surrounding intercellular tissue. Many of these eosinophils were so degranulated that they could be identified (and thus counted) only by their annular nuclei and by constant focussing to distinguish their few orange-pink granules. Such cells sometimes appeared to be "lined up" along the junction of the scar tissue with the area around the wound (Fig. 18).

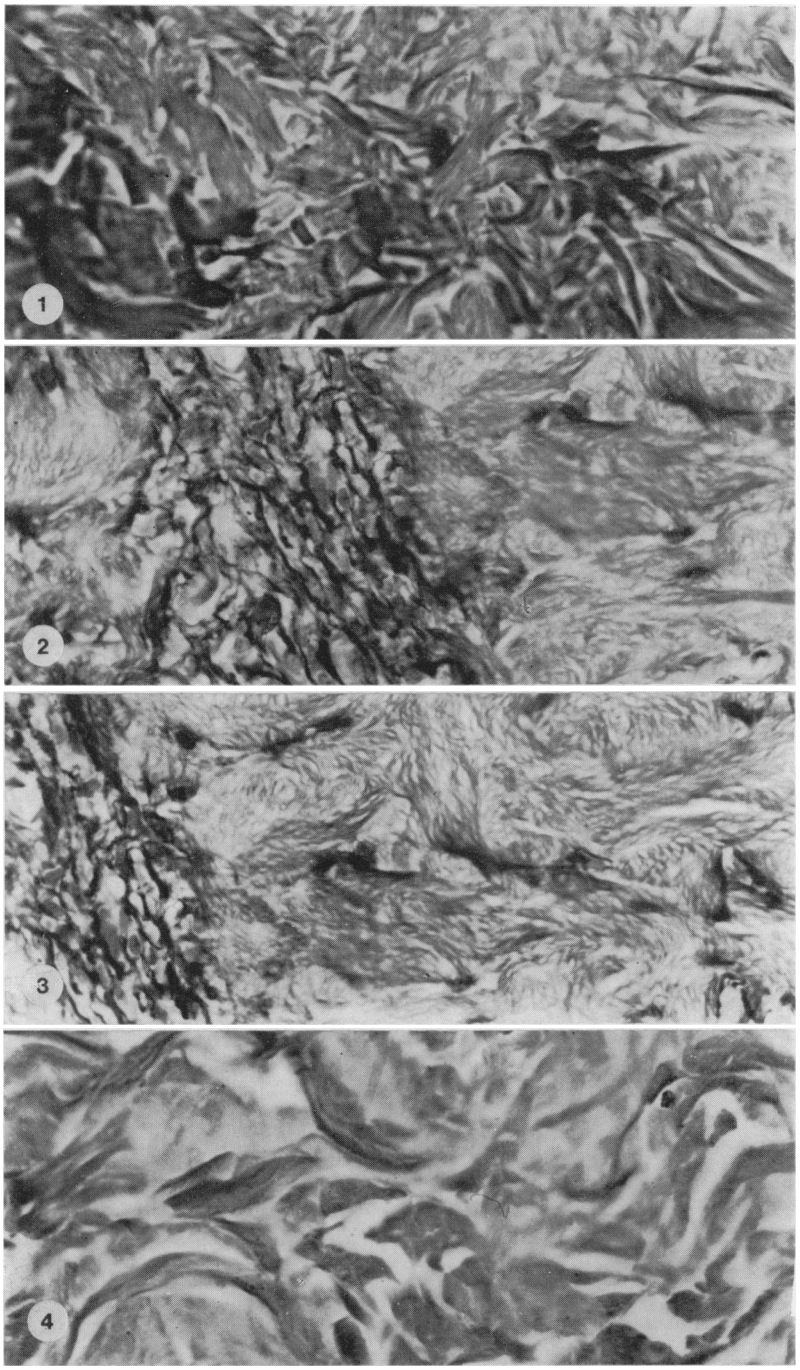
Yet another notable feature, especially within the more superficial areas around wounds, was the close association of eosinophils with enlarged highly basophilic cells, presumably fibroblasts (Fig. 21). These eosinophils did not always appear to be degranulated and were often in direct contact with the fibroblasts (Fig. 19).

Collagenous and reticular (argyrophilic) fibres

The appearances of fibres in the newly formed tissue of the scar, in the contiguous areas around wounds and in areas about 1 cm from the wounds are described at various stages between Days 1 and 30.

Control (unwounded) rats.—Sections from both lots of control rats appeared similar. Thick bundles of the golden-brown-stained collagenous fibres, which had shrunk away from each other during histological progressing, were orientated in all directions (Fig. 1, 22). Although it was sometimes difficult at lower magnifications to identify the individual fibres, especially within the thickest bundles, they could usually be distinguished from each other at high magnification.

Wounded rats: general comments.—(a) Width of wound—In the early stages, from Days 1 to 4, the width of the incision was about 20 μm, with slight variations between different places along the length of individual wounds and between different samples at the same stages. From the 6th to the 12th days the scar gradually widened and again showed within-scar and between-sample variations. From Days 12 to 30 it was difficult to detect any real increase in width because of marked variations (150–300 μm) between stages. At no stage, however, was the wound more than about 300 μm wide (*i.e.* the combined diameters of two fields at a magnification of 1000).



FIGS. 1–16.—Collagenous and Reticular (Argyrophilic) Fibres 6–30-day wounds (rats from Lot 2)

Sections of rat dermis, cut at 4 μm at right angles to line of wound, and stained with Long's (1948) silver impregnation method, counterstained with Weigert's iron haematoxylin and methyl blue picric acid. All $\times$ 548.

(b) Staining characteristics of the fibres —Within the scar itself, the staining characteristics of the fibres of the newly formed tissue changed as the wound aged, being argyrophilic in the early stages and losing this property through the later stages. On the other hand, within the immediate areas around wounds (whether or not the fibres were altered) and the areas more remote from the wound, the staining characteristics remained the same during all stages of aging of the scar. These fibres always stained a warm golden-brown, like those in sections of dermis from the control animals.

Detailed comments on fibres in the different post-wounding stages. Day 1.—At each edge of the incision (Fig. 24), in sections from all 4 animals, the bundles of collagenous fibres were dense and compact and seemed no different from those in the areas about 1 cm lateral to the wound (Fig. 23), where they resembled fibres in the dermis of the controls (Fig. 22). The cut ends of the bundles bordering the incision were blunt (unfrayed) and occasional fine argyrophilic fibres could be discerned protruding from the edges of these bundles into the fibrin clot between the wound margins.

Day 2.—The bundles of fibres in the areas around wounds, and their cut ends (Fig. 25) seemed to be the same as on Day 1 (Fig. 24). There appeared, however, to be more argyrophilic fibres protruding from the cut surfaces of the bundles.

Day 3.—(Fig. 26). At this stage, the areas on each side of the incision were distinctly different from those observed at Days 1 and 2. The fibres of the collagenous bundles were now somewhat dispersed, especially at their cut ends, where the fine argyrophilic fibres protruding from their surfaces were noticeably more numerous than at Day 2. Also, at this stage, fine argyrophilic fibres were now scattered sparsely within the substance of the clot.

Day 4.—(Fig. 27). The fibres of the areas around wounds were more dispersed than at Day 3. Numerous argyrophilic fibres protruded from the golden-brown-stained fibres of these areas at the margins of the incision and were distributed randomly through the substance of the clot.

Days 6 and 7.—(Figs 1–4 & 22–29). There was no apparent difference between the Day 6 samples from the two different lots of rats nor between Days 6 and 7 samples from rats in Lot 2.

A clear distinction could be made between fine black argryophilic and grey-stained fibres in the newly formed scar, where they were orientated approximately at rightangles to the epidermis, and the brown-stained pre-existing dermal collagen. (Fig. 2, 28). On either side of the scar was an area approximately 0·30–0·75 mm wide in which the collagenous bundles were dispersed so that the fibres were very clearly distinguishable from one another and no shrinkage effect was apparent (Figs 2, 3, 28). The fibres were most obviously dispersed in the immediate areas around wounds and gradually formed more compact bundles further towards the edge

FIG. 1.—Normal dermis.

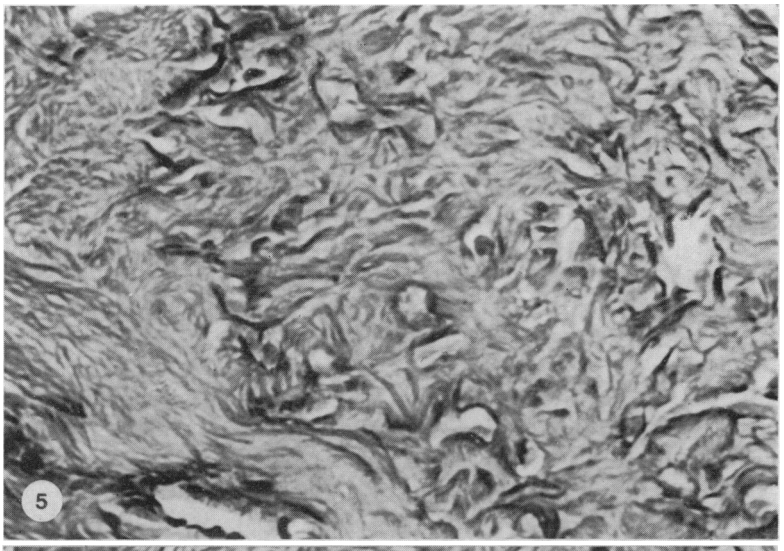
FIGS 2–4.—Scar and surrounding dermis in the same section through a 5-day wound. (All in the same section).

FIG. 1.—Dermis of control unwounded rat. Dense bundles of collagenous fibres orientated in all directions. Individual fibres not discernible in all bundles.

FIG. 2.—In the centre the scar, consisting of newly formed fine black argyrophilic fibres intermingled with fine pale grey fibres all orientated at rightangles to the epidermis, is clearly demarcated from the dispersed fibres of the pre-existing collagen of the areas bordering the wound on either side.

FIG. 3.—A field further into one of the wound border areas, where the individual fibres of the dispersed collagenous bundles can be determined even more clearly than in Fig. 2. The small dense patches scattered among the dispersed fibres, appearing black in the photograph, are concentrations of collagenous fibres at these points.

FIG. 4.—The appearance of the dermis near one edge of the section about 1 cm from the scar. The dense bundles of collagenous fibres resemble those in the dermis of unwounded rats, as shown in Fig. 1.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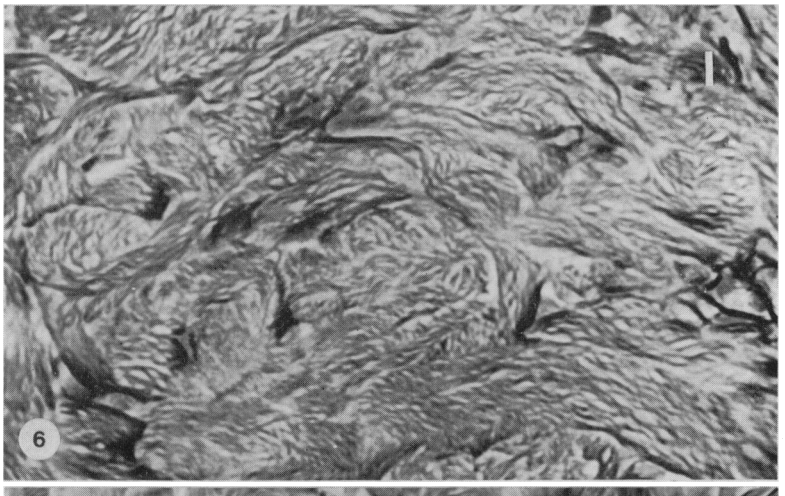
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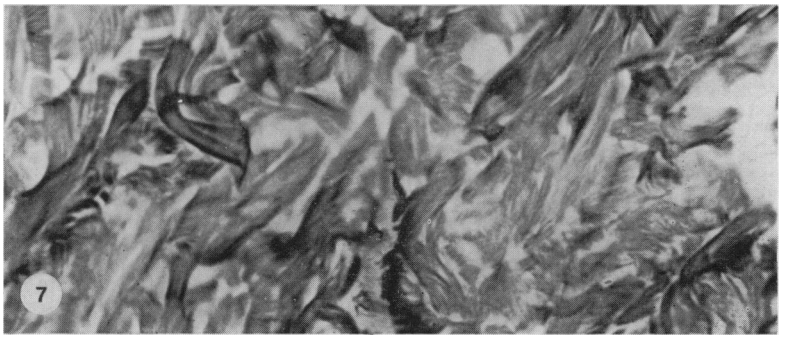
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of the section (*i.e.* approximately 1 cm from the scar), where the arrangement of the bundles and the shrinkage effect (Fig. 4) resembled that of normal dermis (Fig. 1, 22).

Day 10.—(Fig. 29). At this stage, the argyrophilic fibres in the scar, which were fewer than at Day 6, were intermingled with fine brown-stained fibres and orientated across the scar, approximately parallel with the epidermis. The fibres of the scar appeared to merge with the ends of the now very diffuse bundles of collagenous fibres in the periwound areas.

Days 12 and 14.—(Figs 5–7). There was no apparent difference between the sections of skin taken at these two stages. In the scar, fine black argyrophilic fibres and fine brown-stained fibres were intermingled, evenly distributed and not aggregated into bundles (Fig. 5). The collagenous fibres in the areas adjoining the scar on both sides had the same dispersed, individually distinguishable appearance as they did at 6 and 7 days (Fig. 6). Laterally, these areas merged with parts of the section more remote from the wound which had an appearance characteristic of normal dermis (Fig. 7).

Day 18.—(Figs 8–10). Most of the fibres in the scar were fine and brown-stained, with only a few argyrophilic ones interspersed. They were more closely apposed than in earlier stages and sometimes formed small bundles (Fig. 8). The modified wound areas (Fig. 9) were again clearly distinguishable from the scar and also from the edge (Fig. 10), although the individual fibres appeared less dispersed than at the earlier stages.

Days 21–24.—(Figs 11–13). No differences could be found between these two stages. In the scar, no argyrophilic fibres were apparent and the fine brown-stained fibres, orientated across the scar, were more closely apposed and more obviously formed into bundles than at 18 days (Fig.11). In the modified wound area (Fig. 12) the bundles of collagenous fibres appeared somewhat less dispersed than at 18 days, but there was still a clear distinction between these areas, the scar and the more remote dermis (Fig. 13).

Days 28–30.—(Figs 14–16). All the fibres of the scar were brown-stained and were even more obviously aggregated into bundles than they were at 21–24 days (Fig. 14), but they were not nearly as thick as the collagenous bundles in the dermis remote from the wound (Fig. 16). Again, the arrangement of the collagenous fibres of the wound areas (Fig. 15) was different from that of the more remote part of the dermis, but the distinction between these two regions was not so evident as in any of the earlier stages.

Results of trials of the effect on collagenous fibres of different methods of cutting and mounting of sections

Sections from blocks of tissue subjected to different modifications of presectioning treatment and of mounting showed that, at all post-wounding stages, the scar tissue appeared the same after all treatments. In areas around wounds, the greatest dispersion of fibres of the collagenous bundles was seen when the blocks had been soaked in Lendrum's solution and flattened at high temperature. This dispersion of fibres appeared slightly less after the other treatments but the difference in the arrangement of collagenous fibres in the wound borders and that at the edge of the

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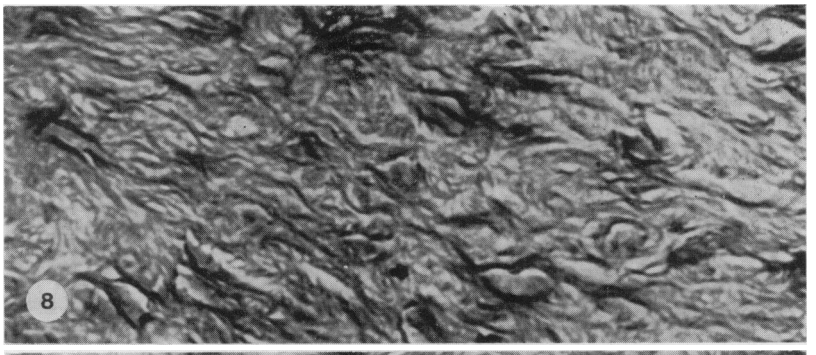
FIGS 5–7.—Scar and surrounding dermis in the same section through a 12-day wound.

FIG. 5.—Intermingled fine argyrophilic and collagenous fibres of the scar, now orientated across the wound, comprise about three-quarters of the picture, at the right (the apparent spaces, with argyrophilic outlines, contain enlarged fibroblasts seen clearly under the microscope, but not brought out in the photograph). A small area of dispersed collagenous fibres of the wound border, clearly distinguishable from the scar, is at left.

FIG. 6.—Further into the same wound border area as shown in Fig. 5. As at 6 days, the individual fibres of the dispersed collagenous bundles are clearly discernible.

FIG. 7.—Dense bundles of collagenous fibres near one edge of the section.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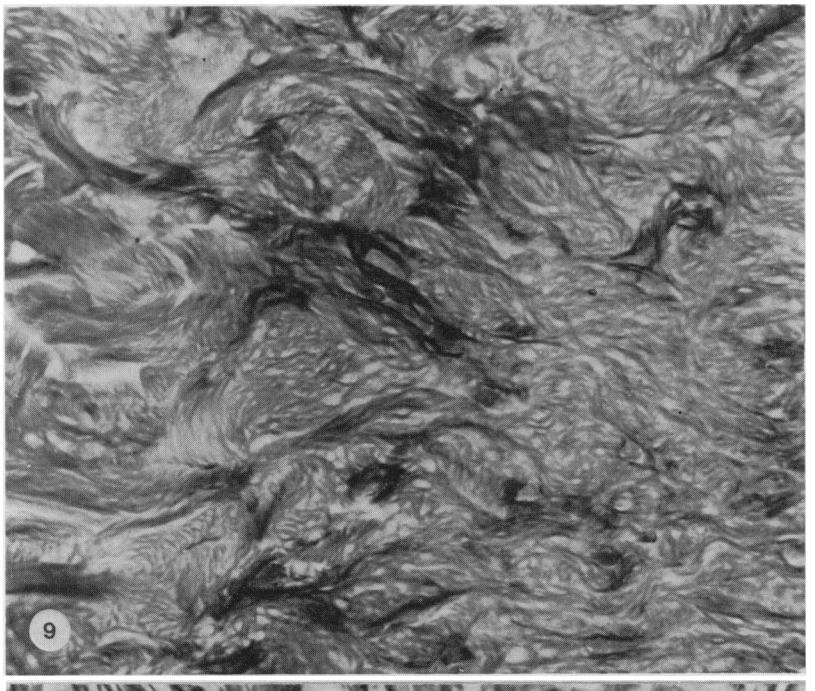
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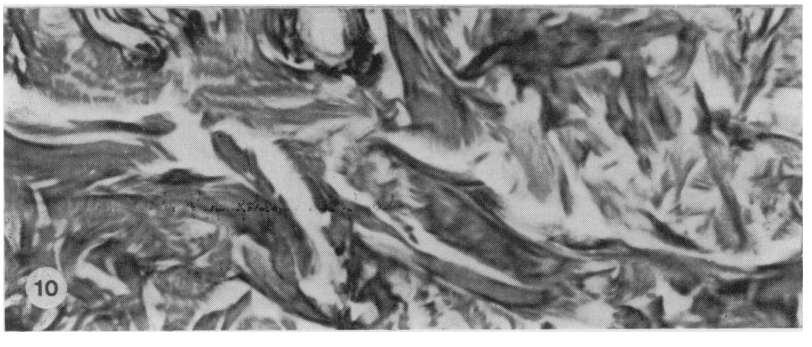
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sections at about 1 cm from the scar was still very obvious.

DISCUSSION

This investigation of the effects of experimental wounding of rat skin has brought to light two different aspects which do not appear to have been reported before. The first of these concerns numerical and morphological changes in eosinophil leucocytes, and the second concerns structural modifications of the pre-existing dermal collagen in the areas adjacent to the scar.

Wounding had a significant effect on the number of eosinophils within the newly formed tissue of the scar and especially within the adjacent pre-existing dermis. The numbers rose to a peak in wounds between 7 and 12–14 days old—the period when collagen remodelling becomes intensified (Madden and Peacock, 1971) after the inflammatory phase (Ross, 1968) —and were still elevated at 30 days when the experiment ended. We have not been able to find any account of a systematic investigation on the behaviour of eosinophils in connective tissue following the mechanical injury inflicted by an incision through the full thickness of the skin. Our findings are comparable, however, with those of Maximow (1902), who systematically studied connective tissue which had been mechanically injured in other ways: by inserting foreign bodies in the form of glass, agar and celloidin chambers into the muscles of rabbits, dogs and pigeons. Maximow noted the behaviour of eosinophils in the newly proliferated connective tissue at various stages after the insertions. He found that eosinophils became more numerous from the 9th day onwards, "just when the fibroblasts were starting to form "firm" tissue", and that their number was still elevated at the 65th day.

In contrast to the little that is known about the behaviour of eosinophils in healing wounds, there is much in the literature about the behaviour of neutrophils, which appear during the inflammatory phase within the first 6 h after wounding, reach a maximum number during the first day, decrease after the second or third day Ross (1968), and have completely disappeared by the 12th day, at the time when the peak of eosinophil numbers occurs. Thus, two waves of emigration of granulocytes, relatively different numerically, are associated with successive phases of the wound healing process—large numbers of neutrophils during the inflammatory phase and fewer eosinophils during the remodelling phase.

Many of the eosinophils in the sections of wounded skin appeared morphologically similar to those in normal rat blood, but some had become degranulated into the extracellular tissue. Another notable phenomenon was the close contact between eosinophils (apparently not degranulated) and enlarged basophilic cells resembling active fibroblasts. Both of these features have been recorded previously in other contexts.

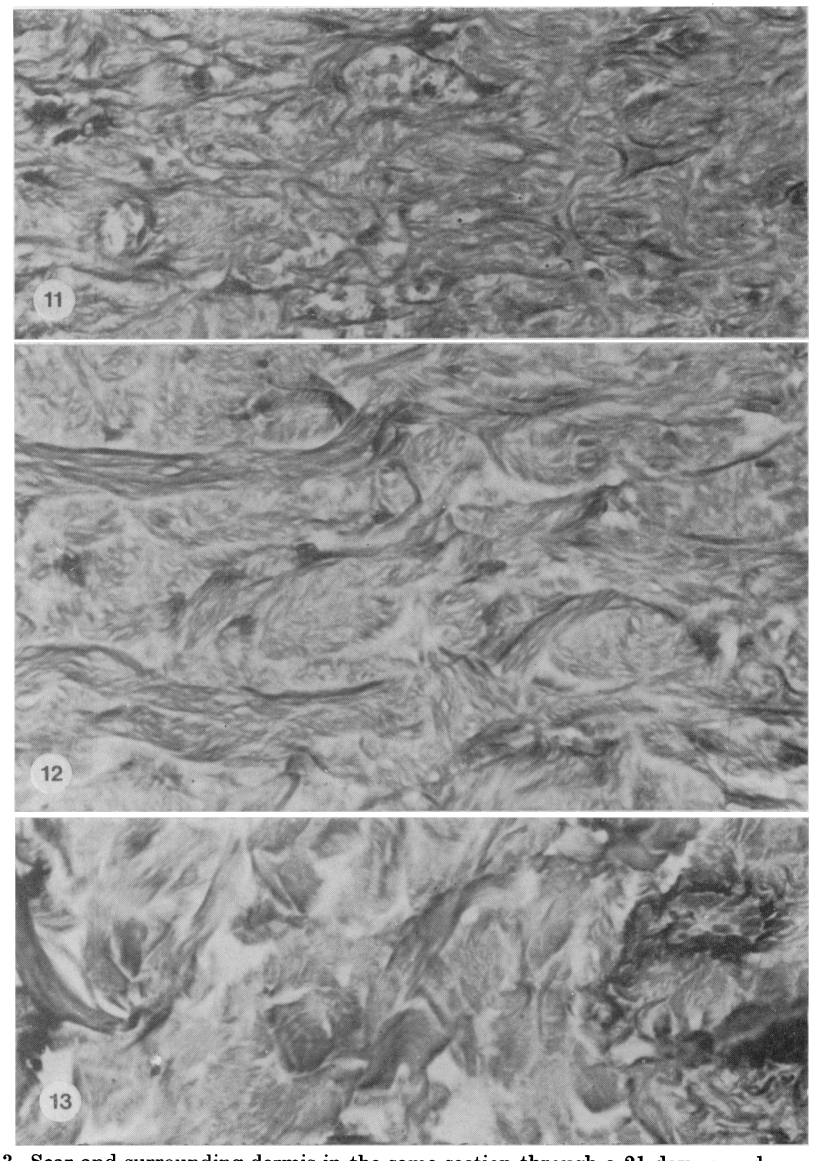
Degranulation of eosinophils has been observed in passive cutaneous anaphylaxis (Parish, 1965), in experimentally induced acute inflammation (Riddle and Barnhart, 1965), in the rat uterus during the oestrous cycle (Ross and Klebanoff, 1966; Tchernitchin, 1973), pathological bone marrow (Skinnider and Ghadially, 1974), in the vulval skin of the oestrous

FIGS 8–10.—Scar and surrounding dermis in the same section through an 18-day wound.

FIG. 8.—Fine collagenous fibres of the scar, a few of them argyrophilic but most of them stained brown (shown here as pale grey). The fibres appear evenly distributed, with no suggestion of bundle formation.

FIG. 9.—The fine collagenous fibres of the scar, to the right, merge with the pre-existing fibres of the wound border area, to the left. The fibres of the wound border area appear somewhat less dispersed and less easily distinguishable from each other than they did at 6 and 7, 12 and 14 days, but yet are markedly different in arrangement from those near the edge of the section.

FIG. 10.—Dense bundles of collagenous fibres near one edge of the section.



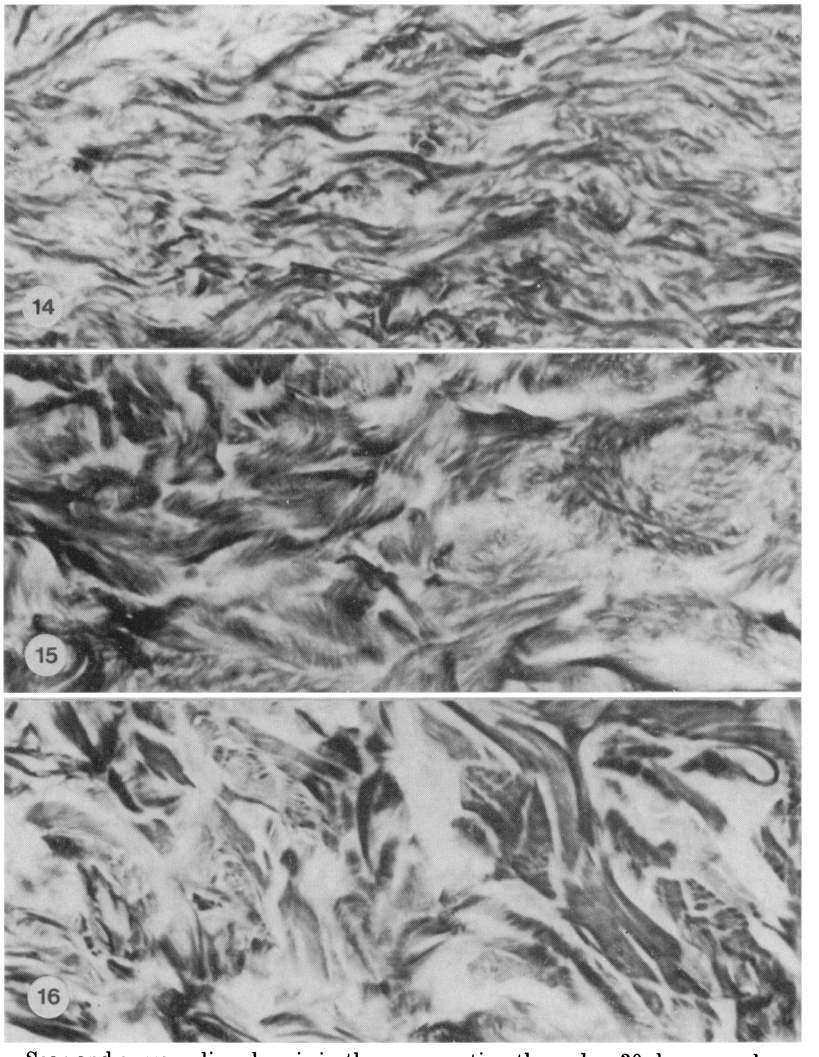
FIGS 11–13.–Scar and surrounding dermis in the same section through a 21-day wound.

FIG. 11.—Fine collagenous fibres of the scar, which appear more closely apposed and more definitely formed into bundles than at 18 days.

FIG. 12.—One of the wound border areas. The bundles of collagenous fibres are less dispersed than at any of the earlier stages.

FIG. 13.—Dense bundles of collagenous fibres near one edge of the section.

ferret and in the uterus of the spayed rat after oestrogen treatment (Bassett, unpublished). Much earlier, Schwarz (1914), in his review of eosinophils in inflammatory processes, had stated that disintegration is characteristic of tissue eosinophils in some conditions. Since we have seen intact as well as degranulated eosinophils within the same section, and because degranulation has been observed by both light and



FIGS 14–16.—Scar and surrounding dermis in the same section through a 30-day wound.

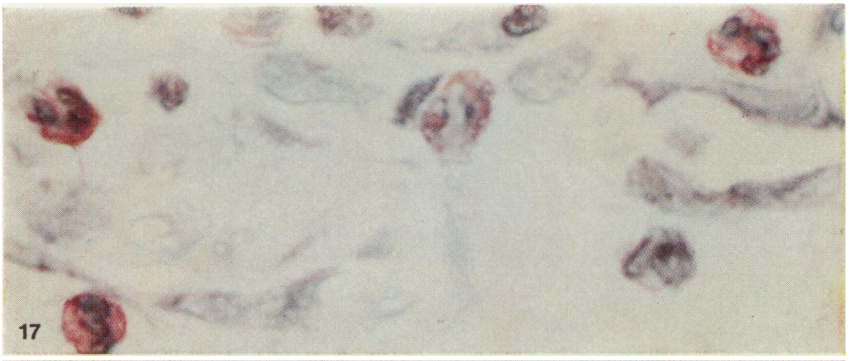
FIG. 14.—The fibres of the scar are now less evenly distributed than in the earlier stages illustrated, and show a tendency to be aggregated into bundles.

FIG. 15.—One of the wound border areas. The fibres appear even less dispersed than at 21 days, and the individual fibres are less obviously distinguishable from each other than at earlier stages.

FIG. 16.—The appearance of the collagenous bundles towards one edge of the section.

electron microscopy in such a variety of other conditions, it seems unlikely to be an artefact created during tissue processing. These observations clearly do not support the comments which Cohen (1974) has made in his review that "the eosinophil is a hardy cell completing its life cycle intact and morphologically unchanged."

Close contact between eosinophils and connective tissue cells (fibroblasts and macrophages) has been described in several different tissues, including various sex hormone-sensitive sites modified during pregnancy and oestrus in a number of different species, especially in the vulval skin of the oestrous ferret (Bassett, 1962), the rat uterus during the oestrous cycle (Ross and Klebanoff, 1966; Tchernitchin,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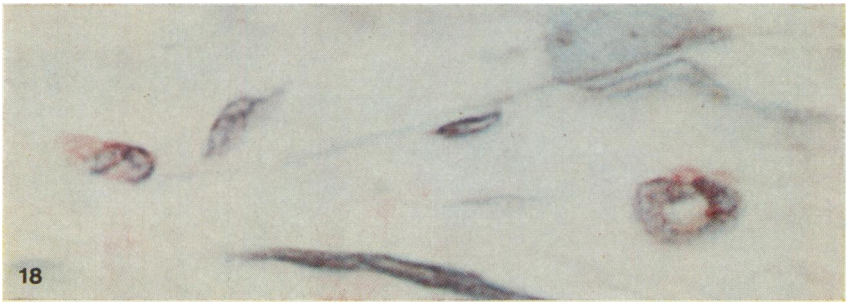
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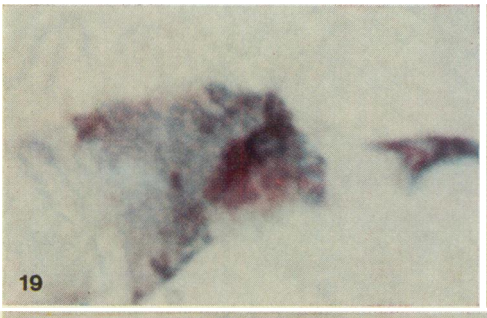
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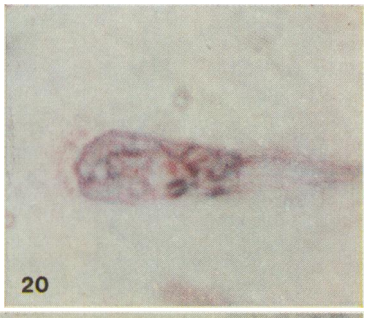
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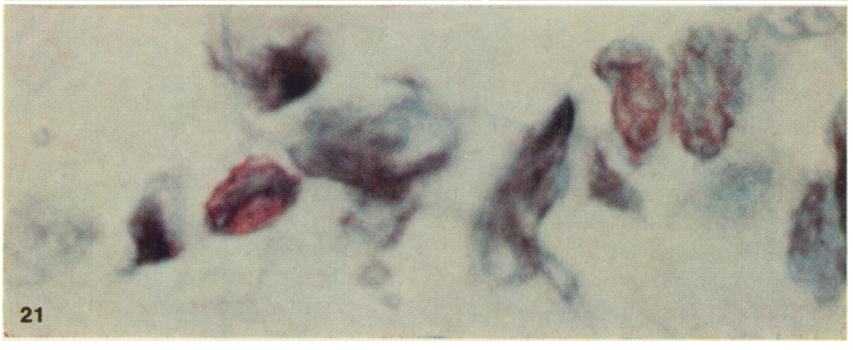
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1973) and the monkey skin in passive anaphylaxis (Parish, 1965).

Our observations that the staining characteristics and orientation of the newly formed collagenous fibres undergo changes in the wound itself confirm other descriptions of developing scar tissue in incised dermal wounds (*e.g.* Ordman and Gillman, 1966), although we detected fine argyrophilic fibres at the margins of the incision on the first day after wounding—a day earlier than others have. This finding of new collagen synthesis in wounds as early as the first day is in agreement with results from biochemical studies on collagen formation in rat skin wounds (Moore, Diegelmann and Cohen, 1975). We have, however, found no previous descriptions of the distinct changes which we have observed in the bundles of collagenous fibres in the areas adjacent to the incision. For the first two days after wounding, these bundles were dense and compact as in normal dermis of unwounded rats, and their cut ends were blunt. From the third day onwards, up to the 14th day, the collagenous fibres in the bundles in these areas progressively became very dispersed. Between 14 and 30 days this effect gradually diminished and the fibres became increasingly aggregated into bundles, until at 30 days they were almost as dense as those in normal dermis. This dispersion of collagenous fibres was very marked in the areas approximately 0·5 mm on either side of the scar and diminished laterally until, at about 1 cm from the scar, the appearance was similar to that in normal dermis. The first signs of collagenous fibre dispersion in the areas around wounds coincided with an apparent fraying of the cut ends of the collagenous bundles which merged with fine argyrophilic fibres protruding from their surfaces into the fibrin clot. These changes in the region of the wound became more and more obvious during the first 7 days—at the same time as the argyrophilic fibres increased in numbers within the scar—thus suggesting that the two processes could have been initiated by the same stimulus.

The appearances of this modification of the wound areas were not materially affected by variations in the processing techniques used in our laboratory. Furthermore, similar changes in the arrangement of collagenous fibres in these areas have also been observed in another wound-healing experiment in a different laboratory, on a different strain of rats and using Michelle clips instead of sutures to unite the wound (Bassett, Harkness and Harkness; unpublished). It is possible that these strikingly obvious changes in collagenous fibres have not been detected before because others apparently have not made use of a silver impregnation staining method (Long, 1948) which one of us (E.G.B.) has found repeatedly to be particularly good for demonstrating collagenous as well as reticular fibres. Had this stain not been used in our experiments, the phenomenon could easily have passed unnoticed.

FIGS 17–21.—Cells in wound and periwound. Sections of dermis from 12-day wound. Cut at 4 μm and stained with toluidine blue–phloxinate (Lillie, 1941). Eosinophil granules shown pink. Basophilic fibroblasts and nuclei shown dark blue.

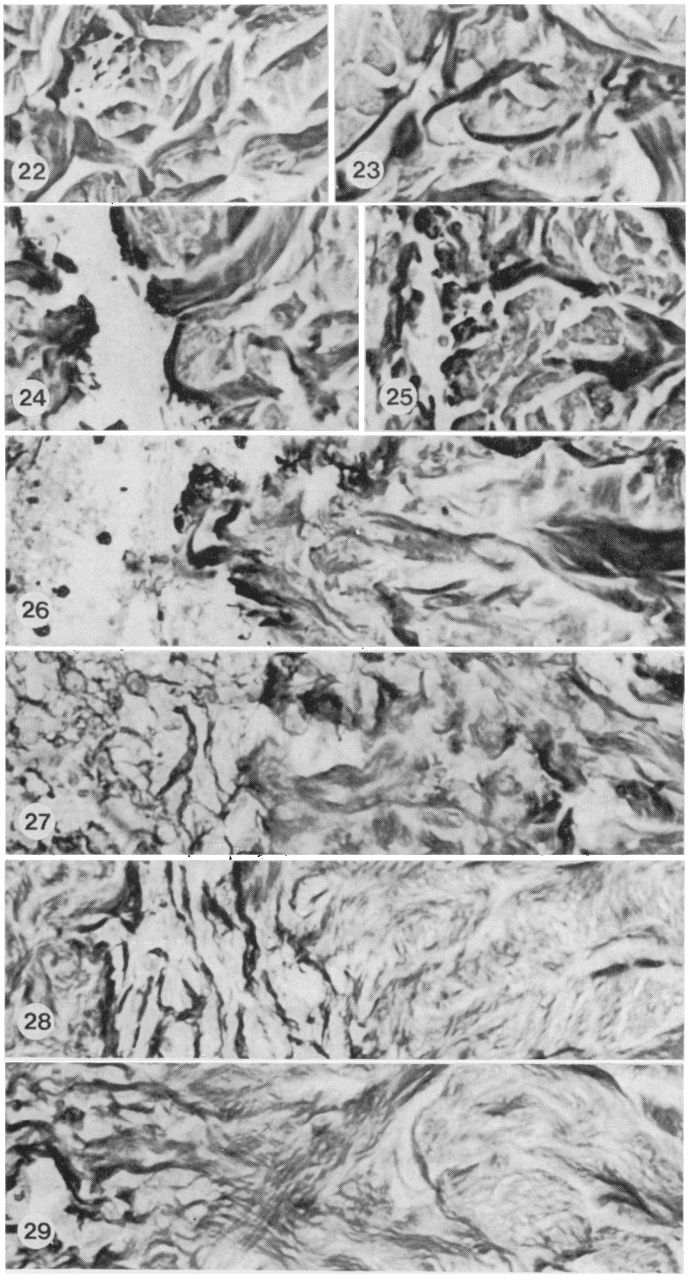
FIG. 17.—Four well-granulated eosinophils, with an appearance characteristic of those in normal rat blood, in the vicinity of a blood vessel within the newly formed tissue of the scar. Several enlarged basophilic fibroblasts are present also. × 1000.

FIG. 18.—Two very degranulated eosinophils within the scar, "lined up" near the margin of the wound border area. Note the annular nuclei characteristic of rat eosinophils, shown flat in the cell at the right and twisted into a figure-of-eight in the cell at the left. × 1000.

FIG. 19.—Close contact of a granulated eosinophil with a large basophilic cell, apparently a fibroblast, within the wound border area. × 1200.

FIG. 20.—Eosinophil with an annular nucleus and only a few granules, within the wound border area. × 1200.

FIG. 21.—One well-granulated eosinophil at the left and two somewhat degranulated eosinophils at the top, within the wound border area. Several enlarged basophilic cells, apparently fibroblasts, are within the same field. × 1200.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That there should be morphological changes in the collagen of the areas around incised wounds in the mid-line dorsal skin of rats, as well as in the wound itself, is not surprising in light of biochemical changes reported in skin of similarly treated rats. For instance, Peacock (1963) who studied such skin over a period of 21 days from the time of wounding, recorded a significant increase in saline-extractable collagen as far distant as the lateral thorax (*i.e.* approximately 2 cm from the wound). In the same context, Carlsen, Helin and Helin (1973) found an increase in sulphated glycosaminoglycans (ground substance) during approximately the same post-wounding period of time, but within a more limited distance (5 mm) from the wound edge. In another but somewhat different investigation, on the biochemical changes at distances from midline ventral incisions in the musculofascial abdominal wall of the guinea pig, Adamsons, Musco and Enquist (1966) described increases in both hydroxyproline (total collagen) and hexosamine (ground substance) at distances up to 7·5 mm on each side of the incision, over approximately the same period of time.

In our investigation on eosinophil numbers, the experiment was designed simply to find out whether or not wounding had a significant effect. The length of each row of fields in which the counts were made, and which included both the scar and the adjacent areas around wounds, was arbitrarily chosen. The effect of wounding was assessed as change in total numbers, with no distinction between the numbers in the scar and in the adjacent areas, or, within these areas, between different distances from the scar. Now that it has been established that wounding does have a significant effect on total eosinophil numbers within this arbitrarily chosen region, we consider that the relative

FIGS 22–29.—Collagenous and reticular fibres, 1–10-day wounds (rats from Lot 1). Sectioned and stained as described for Figs 1–16. All ×490.

The heavily argyrophilic margins of the wounds shown in Figs 24 and 25 are staining artefacts and not of any significance in the wound-healing process (see Ordman and Gillman, 1966, pp. 866–867), since a similar effect was observed on the freshly cut margins of the same sections, about 1 cm from the wound. On the other hand, the fine argyrophilic fibres protruding from the wound margins are unlikely to be artefacts, since none could be found at the surfaces of freshly cut margins distant from the wound.

FIG. 22.—Normal dermis of control rat, with dense irregularly arranged bundles of collagenous fibres. See also Fig. 1.

FIG. 23.—Dermis about 1 cm from the 1-day wound shown in Fig. 24. Compare with normal dermis in Fig. 22, and see also similar areas from the several wound-healing stages illustrated in Figs 1–16.

FIG. 24.—The two sides of the incised dermis, one day after wounding. The collagenous bundles in the pre-existing dermis of the area immediately bordering the wound have the same appearance as those about 1 cm from the wound and as in normal dermis, and their cut ends are blunt. Occasional fine argyrophilic fibres protrude from the surface.

FIG. 25.—The two sides of the incised dermis 2 days after wounding. There is no apparent difference in the collagenous bundles between this and the previous stage. Occasional fine argyrophilic fibres protrude from the surface.

FIG. 26.—One side of the pre-existing dermis in a 3-day wound to the right. The fibres of the wound border area are more diffuse than at 1 and 2 days and their ends are no longer blunt. Many fine argyrophilic fibres protrude from the surface and occasional ones are scattered within the fibrin clot (*e.g.* top left corner).

FIG. 27.—The appearance in a 4-day wound. The collagenous bundles in the wound border area are more diffuse again than at the earlier stages. There are numerous fine argyrophilic fibres both at the surface of the pre-existing dermis, to the right, and within the fibrin clot to the left.

FIG. 28.—The areas bordering the wounds, and scar 6 days after wounding. The very diffuse collagenous fibres, at the right and extreme left, and the argyrophilic fibres within the scar, mid-left, present a picture almost identical with that observed in 6 and 7-day wounds of rats from Lot 1 (Fig. 2).

FIG. 29.—The appearance at 10 days. At this stage, the collagenous fibres of the wound border areas, at the right, are the same as at 6 days in sections from both lots of rats, (Figs. 10 and 28) and as at 12 days in sections from rats of Lot 2 (Fig. 6). In the scar, (extreme left), however, the proportion of the relatively thicker argyrophilic fibres to fine collagenous fibres is much less marked than at 6–7 days, and the fibres are now orientated across the scar, parallel with the epidermis.

numbers in the scar and in tissue at different distances laterally from it need to be investigated. Such experiments should be designed to include areas more remote from the wound than those included in the present investigation, in order to define the limits of the region about the wound in which the changes in eosinophils take place.

This finding concerning the behaviour of eosinophils in regions of morphologically altered collagenous fibres, both in the scar and in the adjacent areas, and the discovery of collagenase activity in rat peritoneal eosinophils (Bassett *et al.*, 1976) suggests that eosinophils could be involved in connective tissue catabolism in such conditions, especially since eosinophil granules are known to be shed into the surrounding tissue. The close contact between eosinophils and connective tissue cells resembling active fibroblasts, which are concerned with collagen synthesis (Ross, 1968), may also support our theory of eosinophil involvement in connective tissue metabolism, since these cell contacts could represent some type of functional intercellular junction (Staehelin, 1974).

Since eosinophils accumulate within both the scar and the adjacent areas, where the types of connective tissue are morphologically dissimilar, it would be desirable for any theory of their role in connective tissue metabolism to account for this. Eosinophils increase in numbers at approximately the same time as the newly formed tissue of the scar undergoes remodelling. Thus it is possible that eosinophil collagenase could mediate the breakdown of collagen which occurs concomitantly with biosynthesis during this process (Woessner, 1968).

In comparison with the scar, much less is known about connective tissue metabolism in the areas around wounds. In our experiments, alteration in the arrangement of collagenous fibres was seen very early in the healing process, well before the rise in eosinophil numbers, so that it is improbable that eosinophils could be involved in fibre dispersion. On the other hand, the fact that a reversal of this process (*i.e.* re-aggregation of fibres) apparently began shortly after the peak and during the maintained elevation of eosinophil numbers, suggests that at these stages eosinophils may be involved in a remodelling process to reorganize the fibres from their dispersed state. A possible second role of the eosinophils in the areas around wounds, which could be related to the first, is suggested by the observations noted in the review by Jackson and Bentley (1960) that saline-extractable collagen (which Peacock (1963) showed to increase in areas distant from the wound), contains the collagen molecules most recently synthesized. Thus in this situation, as in the scar, the eosinophils could be involved in remodelling of new collagen. Adamsons *et al.* (1966), in discussing their finding of increased (total) collagen adjacent to the wound, suggested that such newly formed collagen is probably reorganized, but they did not postulate any cellular mechanism which might be involved.

The theory that eosinophils may be involved in collagen catabolism during the remodelling process through action of their collagenase accords with what is known of the roles played by neutrophils as well as eosinophils in healing wounds (see Bassett *et al.*, 1976, for full references and dicussion). Because neutrophils, which also contain a collagenase, are very numerous in the early inflammatory phase, the early drastic degradation of collagen is thought to be mediated by this collagenase. On the other hand, eosinophils, which appear in relatively small numbers during the remodelling phase, may perform some more subtle regulatory function in collagen metabolism.

A collagenolytic enzyme has been detected in cultured samples from granulation tissue in skin wounds (Grillo and Gross, 1967—rats; Riley and Peacock, 1967—humans). In these experiments the enzyme activity was associated particularly with the epidermis and less so with the dermis alone. Such findings support the concept of metabolic changes associated with collagen

taking place within the scar, but do not contribute materially to our hypothesis.

The dispersion of the collagenous fibres in the areas adjacent to the wound is unlikely to be mediated by eosinophils, since it takes place before eosinophils have increased significantly in numbers. It seems likely that this effect could be related to the increase in sulphated glycosaminoglycans which occurs within distances from the wound edge approximating those in which the changes in fibre arrangement were observed. There is considerable evidence (Jackson and Bentley, 1968) that glycosaminoglycans, especially chondroitin sulpate, are necessary for directing the organization of collagen fibrils. It is also possible that the fibres could be dispersed initially by the action of neutrophils, which are still present in appreciable numbers at the third day (Ross, 1968). But this second hypothesis seems less probable than the first, since at the 14th day, long after the neutrophils would have disappeared, the fibres were even more clearly dispersed than they were at the 3rd day.

Our findings, which suggest that eosinophils play a significant role in connective tissue metabolism, appear to be at variance with current views that these cells have a role in immune reactions, with which their presence is often associated (Litt, 1961; Bessis, 1973; Cline, 1975). We will therefore briefly discuss the considerable volume of collateral evidence which supports our view, and then turn to a possible way in which the two opinions might be reconciled.

Schwarz (1914) reviewed numerous situations in which the numbers of tissue eosinophils paralleled the extent of connective tissue changes, occurring experimentally or in pathological conditions. These included aseptic and septic inflammation, chronic inflammation of the eye, aleuronate inflammation of the liver, aleuronate pleuritis, open tuberculosis and granulomas. There are also many later references to eosinophils in association with modified connective tissue in granulomas at various specified sites, including the lung (Auld, 1957; Anderson and Foraker, 1959), vulva (McKay *et al.*, 1953), stomach (Vaněk, 1949; Vazquez and Ayestaran, 1975), all divisions of the gastrointestinal tract (Ureles *et al.*, 1961) and bone (Rodrigues and Lewis, 1971). Examples of other sites of pathological conditions in which an association between eosinophils and altered connective tissue has been recorded are the gallbladder (Fox and Mainwaring, 1972), honeycomb lung (Heppleston, 1956), gastric fibroma (Salm, 1965), retroperitoneal fibrosis (Mitchison, 1970; Skeel *et al.*, 1975), disseminated eosinophilic collagen disease (Cryer and Kissane, 1974) and myocardial infarction (Mallory, White and Salcedo-Salgar, 1939). In many of these places or conditions, plasma cells and lymphocytes were present also, and where appropriate stains were used, reticular fibres were revealed as well as fibres of mature collagen.

In the above conditions the eosinophils have usually appeared after the initial acute inflammatory phase and during the later phases of connective tissue proliferation and remodelling, just as in our wound-healing experiment. Sometimes, however, eosinophils may appear much earlier, soon after the initial reaction. Thus, Chase and Hunziger (1974), in discussing contact sensitization, state that eosinophils and new collagen are present in the contact site after 72 h.

All of the above reports, as well as the present investigation, describe eosinophils associated with connective tissue which has been modified either experimentally or in pathological conditions. A similar association does, in fact, occur in some places, in entirely normal physiological conditions, such as in sex-hormone-sensitive sites at certain phases of the female reproductive cycle, and also in the same tissues in spayed animals in which the normal condition is simulated by treatment with sex hormones. In the rat and mouse, the uterus contains numerous eosinophils, especially during oestrus; they are absent in spayed animals, but

reappear after treatment with oestrogen (Saito, 1928; Gander, 1930). Bassett (1965) who confirmed these observations, noted in addition (unpublished) that after oestrogen treatment the fibroblasts in the uteri of spayed animals appeared very active (*i.e.* they contained vesicular nuclei, prominent nucleoli and basophilic cytoplasm), and that sometimes eosinophils and fibroblasts were in close contact with each other. Furthermore, the coarse densely arranged bundles of collagenous fibres seen in the uteri of spayed animals became fine, loosely arranged and argyrophilic at the same time as the above cellular changes which took place under the influence of oestrogen.

Eosinophils have been seen at the same time as active fibroblasts in modified connective tissue in several other pelvic sites as well as the uterus, in a number of different species during the oestrous cycle, at the end of gestation and in the early stages of involution following cessation of the stimulus for proliferation (oestrus or pregnancy; Bassett, 1962). In the same tissues, these altered fibroblasts were accompanied by changes in the collagenous fibres, which were more loosely arranged and more argyrophilic than they were in anoestrous or non-pregnant animals, when the fibroblasts appeared inactive (Bassett, 1959). As in many of the pathological conditions noted above, lymphocytes and plasma cells were sometimes seen in the early stages of involution or in the later stages after the eosinophils had disappeared.

A phenomenon similar to that seen in spayed female animals has been reported in males too. Voss (1935) induced emigration of numerous eosinophils into the seminal vesicles of castrated mice by treatment with androgen, and noted that at the same time there was a loosening of the connective tissue.

Although this parallelism between eosinophil infiltration and modification of connective tissue has been described repeatedly in these various different conditions, it has rarely been suggested that there might be any connection between these two events. Schwarz (1914), who gave careful consideration to this possibility, felt that no significant association could exist since, at the time of his writing, such an idea lay entirely outside his experience of the function of leucocytes. Voss (1935) did, however, suggest that eosinophils might be involved in catabolism of connective tissue and a similar idea was put forward by Bassett (1962).

A theory that fibroblasts may be responsible for the cellular basis of connective tissue remodelling has been put forward in a review by Ten Cate and Deporter (1975). Ten Cate and Freeman (1974) had described collagen-containing vesicles within the fibroblasts of tissues at the margins of wounds, and this subsequent review collates similar observations by others in various sites of remodelling connective tissue. Ten Cate and Deporter's theory and the one proposed here, involving eosinophils in connective tissue remodelling, are not incompatible, however, since it could be postulated that eosinophil collagenase first acts specifically on the collagen fibrils, which are then phagocytosed by adjacent fibroblasts.

Eosinphils become more numerous within tissues under such a wide variety of situations that it is difficult to suggest any common basic mechanism which could be involved. Both Litt (1961) and Cohen (1974), who have discussed the problem of tissue eosinophilia at some length, felt that the common denominator of induction could have an immunological basis, but they were interested only in the eosinophilia and did not mention any associated connective tissue changes. In fact, most of the authors who have recorded observations on tissue eosinophils in immune reactions have concentrated on these and other cells in the site of the reaction, and have virtually ignored the effect, if any, on the surrounding connective tissue. Chase and Hunziger (1974) commented that contact reactions in man and animals,

such as the occurrence of eosinophils and new collagen, are often overlooked because the usual staining procedures do not reveal them: this may also apply to connective changes in the sites of other immune reactions. Likewise, those who describe connective tissue changes but make no reference to eosinophils may have not recognized them—especially if they were degranulated—simply because a suitable staining procedure, such as the toluidine blue–phloxinate method of Lillie (1941), was not used and because the sections were not examined at a sufficiently high magnification. In light of our own experience, in which many eosinophils, especially degranulated ones, were discernible only at a magnification of $\times 1000$ under oil immersion after staining by the toluidine blue–phloxinate method, we are convinced that eosinophils could be present but undetected in many more sites of modified connective tissue than the ones in which they have been recorded.

Should the esosinophilia and associated connective tissue changes in all of the experimental and pathological conditions described prove to have an immunological basis, it would seem reasonable to extend the immunological concept to normal situations such as certain pelvic tissues in oestrus and pregnancy as well. One possibility which needs to be investigated is that of a temporary alteration in the state of tolerance to the protein which is synthesized during oestrus and pregnancy, and which, after termination of the stimulus (*i.e.* after oestrus or parturition), becomes redundant to the needs of the organism.

CONCLUSION

When our observations are considered together with the relevant findings of others, it is evident that knowledge of behaviour of the different components of the surrounding areas during the healing of a wound is far outweighed by information concerning that of the newly formed tissue of the scar. Gillman (1968), in his review, emphasized that "attention has not yet been given to the process of remodelling of the intact tissue bordering a wound, and especially to the way in which the new collagen fibres, which form within the wound, become united to, or interdigitated with, the pre-existing connective tissue fibres." The information in this text shows that consideration should be given, not only to the tissue at the immediate junction of the wound with the pre-existing tissue, but also to that at distances considerably further from it, since it is apparent that a relatively much greater area of tissue than that of this junction is involved in the final integration of the scar with its surroundings.

We thank Mr K. W. Turner, Department of Anatomy, for skilled technical assistance.

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