CHEMICAL AND HISTOCHEMICAL STUDIES
ON THE ORGANIC CONSTITUENTS IN FRACTURE REPAIR IN RATS

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The process of fracture healing has been studied in many different ways. John Hunter, Paget, Syme and Macewen studied the gross anatomy (Keith 1918); Galli and Robertson (1919) and Urist and McLean (1941) made histological studies. More recently, biochemical, biophysical and histochemical methods have been used. Most of these studies have concerned the inorganic constituents of bone (Bauer 1954, Neuman and Neuman 1958, Nilsonne 1959, Engström 1960, Wendeberg 1961). Fewer studies have been made of the organic constituents of bone such as collagen and mucopolysaccharides. The importance of these constituents in the healing of fractures was, however, clearly indicated by Nilsonne (1961) who stated that "non-union is not the result of primary disturbance in the mineral process, but rather a change in the organic matrix which diminishes or prevents its calcifiability." In the present study we have used chemical methods to study the collagen and mucopolysaccharides of bone during the various stages of fracture healing. We also compared the histochemical findings with the histological appearances in order to obtain a clear picture of the behaviour of the organic constituents of bone after fracture.

MATERIAL AND METHODS

Sixty growing albino rats weighing about 100 grammes were selected. Under inhalation anaesthesia with ether the right humerus of each animal was broken by hand. Up to the time of killing the animals were given the usual food and unlimited water. At the end of each week up to the sixth after fracture, ten rats were killed and the fractured bones and the intact ones of the opposite sides were removed.

Histochemical and histological investigations—The bones were fixed in 10 per cent formal saline for twenty-four hours and were then decalcified with formic acid. When the bones were fully decalcified they were dehydrated in alcohol, embedded in paraffin and cut in slices 5 μ thick. For histological studies the usual haematoxylin and eosin stains were used and for histochemical studies of mucopolysaccharides and collagen, Hale's colloidal iron stain as modified by Rinehart and Abul-Haj (1951) was employed. Although toluidine blue and periodic acid-Schiff stains were also used in the beginning to identify mucopolysaccharides, they were given up in favour of the colloidal iron stain because in the latter method the mucopolysaccharides took a blue colour and collagen fibres a red.

Biochemical investigation—Dickerson's (1962) method was followed in general. The soft tissues were carefully removed from the bones which were weighed immediately after removal and were then dried in an oven at 115 degrees Centigrade until their weight became constant. The difference between the two weights was a measure of the water content. The bones were then powdered in a mortar. The powdered bone was sealed in an ampoule after the addition of 1 millilitre of 6 N hydrochloric acid to each 50 milligrams of bone and was then hydrolysed in a bath at 100 degrees Centigrade for forty-eight hours. The contents were then filtered and the filtrate was neutralised with 2 N sodium hydroxide solution and made up to 10 millilitres. One millilitre of this solution was taken out and studied for hydroxyproline content (Neuman and Logan 1950) and another millilitre was studied for hexosamine content (Rondle and Morgan 1955). Rat collagen contains 14·2 per cent of hydroxyproline, so that the collagen content could be derived from the value obtained for hydroxyproline. Although by estimating only the total hexosamine one would not be able to assess accurately the total mucopolysaccharide content, yet the hexosamine value would give a rough indication of the changes occurring in that component.
RESULTS OF HISTOLOGICAL AND HISTOCHEMICAL STUDIES

First week: fibroblastic phase—The greatest cellular activity takes place during the first week. Immediately after injury, cells of mesenchymal origin rapidly proliferate from all sides to fill the gap. The fibroblastic proliferation becomes intense in the region of the fracture within twenty-four hours and by forty-eight hours these cellular activities can be demonstrated clearly (Fig. 1). These fibroblasts mostly arise from the periosteum of the broken ends (Fig. 2); but they come also from the other surrounding structures such as fibrous tissues around injured muscles. In the most peripheral region of the fractured bone one can see the proliferation of osteoblasts just under the fibrous layer of the periosteum. In between these two zones of proliferating fibroblasts and osteoblasts another zone of varying size consisting of active chondroblasts adjacent to the fractured bone can be identified (Fig. 3). The rapidity of cellular proliferation seems to depend on various factors. If the distance between the broken fragments is small the fibroblastic proliferation may not be so extensive, but should the fragments not be in perfect apposition and should there be an extensive gap, the fibroblastic activity becomes intense and is derived from all surrounding structures.

Once these cells start proliferating they begin to secrete their intercellular materials. The proliferating fibroblasts produce mucopolysaccharides which ultimately help to form collagen fibres. These are seen around the fibroblasts in the area of fracture within forty-eight hours. By selective histochemical stains their quantity can roughly be assessed. The quantity of mucopolysaccharides usually decreases after about a week as they are replaced by collagen fibres. When the collagen fibres make their appearance after the first week the cellular activity gradually becomes less until the gap is completely bridged by mature collagen fibres.
The chondroblasts also secrete their own intercellular materials, the main constituents of which are collagen fibres and mucopolysaccharides rich in chondroitin sulphuric acid. These materials can also be identified well by histochemical methods.

The proliferating osteoblasts start secreting mucopolysaccharides which are quickly converted into collagen fibres. These calcify to form new bone. Thus, in the most peripheral region of the fracture the proliferation of osteoblastic cells starts almost immediately after injury. New bone formation can be seen in the peripheral area within forty-eight hours of fracture. At the end of first week one can clearly see the zones of new bone formation (Fig. 3).

Second week: collagen phase—All the changes seen in the first week manifest themselves more clearly in the second. The clotted blood which at first occupied the gap between the fragments is now replaced by collagen fibres. The fibroblastic activity gradually becomes less as the collagen fibres are laid down to bridge the gap. As the collagen fibres become mature by joining one another and by forming dense bundles of fibres, not only is the cellular activity retarded but also the blood supply to the region becomes scanty because of the compression of the capillaries by the rapidly shrinking collagen fibres. Thus, by the end of the second week, if the distance between the broken fragments is not very wide, the mature collagen fibres bridging the gap can easily be made out. If the space between the broken ends is great, one may still encounter certain patchy areas of fibroblastic activity, but even in such cases most of the region becomes filled with collagen fibres during subsequent days.

Third and fourth weeks: osteogenic phase—Just adjacent to the region of the collagen fibres and very close to the broken bones one can distinctly observe the activity of cartilage cells. It is in this area that this phase of healing mostly takes place. The chondroblasts would be gradually replaced by bone in the same way as in endochondral ossification in the embryo or in growing bones near the epiphysial cartilage (Fig. 4). The chondroblasts proliferate very rapidly and then become hypertrophied. They begin to secrete alkaline phosphatase which quickly brings about calcification of the intercellular substance. As soon as calcification takes place the cartilage cells die and they and some of the intercellular materials disintegrate. The empty space so produced is gradually filled by proliferating osteoblasts which again produce the intercellular material which goes to form new bone. This is the sequence of ossification of the cartilaginous area (Ham and Leeson 1961). In this respect there seems to be very little difference between the osteogenic activity of growing bones and the healing of fractures.
One feature not seen in growing bone is the filling of the gap by new bone. Our histochemical studies provided new evidence about this process. The central area filled by collagen fibres is surrounded on all sides by the proliferating cartilage cells. As these cells grow and proliferate into the gap they gradually penetrate into the area occupied by the collagen fibres by pushing these fibres aside. During this period the growing cartilage cells can clearly be seen to be surrounded by mature collagen fibres (Fig. 5). As the cartilage cells become hypertrophied and mature they start secreting alkaline phosphatase which precipitates calcification around them. They die and their area is taken over by the proliferating osteoblasts which then form new bone. Therefore, for bridging the gap, the central zone which was occupied by fibroblast and mucopolysaccharide in the first week and by collagen...
fibres in the second week becomes successively the seat of the chondroblastic and osteoblastic activity in the third and fourth weeks (Fig. 6). These osteoblastic cells not only come from the periosteal side but also from the endosteal side to bridge the gap.

Occasionally, if the blood supply is good and the osteoblastic cells have great regenerating power, the gap may be filled simply by osteoblastic cells which penetrate directly into the collagenous area, just as occurs in intramembranous ossification in the embryo. In this process the osteoblastic cells penetrate the framework of collagen fibres and secrete specific intercellular material and alkaline phosphatase so that calcification occurs and later bone is formed.

Fifth and sixth weeks: remodelling phase—Initially the new bone is not compact but has many trabeculae containing capillaries. As the new bone grows the trabeculae become smaller and smaller until the tissue gradually attains the structure of compact bone. When the whole area becomes fully occupied and most of the collagenous and cartilaginous structures are replaced by new bone the remodelling phase starts (Fig. 7). The fusiform mass of cancellous bone around the broken fragments is transformed into compact bone and any excess is resorbed.

![Graphs showing changes in mucopolysaccharide and collagen content](image)

**Figure 8**—The mucopolysaccharide content of bone as calculated from measurements of hexosamine content at various phases of healing. After an initial rise the mucopolysaccharide falls in the subsequent weeks until it reaches the normal level in the sixth week. Between the second and third weeks there is little change in mucopolysaccharide because of excess of chondroblastic proliferation during this period. **Figure 9**—The changes in the collagen content of bone after fracture. After the initial fall in the first week there is a rise in the second. This is followed by a fall caused by deposition of excess of minerals. The collagen content returns to normal level during the remodelling phase.

**RESULTS OF CHEMICAL STUDIES**

First and second weeks—In the first week, as soon as sufficient fibroblastic proliferation occurs, there is also accumulation of mucopolysaccharide. There is evidence that fibroblastic proliferation has a close relationship with the production of these substances. It is uncertain whether the entire substances or only the materials essential for their production are secreted by the fibroblasts. Nor is it clear what part the mast cells, which are very intimately connected with the elaboration of these substances elsewhere, play in their production around a fracture. The more intense the fibroblastic proliferation the greater is the production of mucopolysaccharides.

There are about 291 milligrams of hexosamine in 100 grammes of healthy rat bone. In the first week after fracture the hexosamine content rises to about 600 milligrams per 100 grammes of bone (Fig. 8). There are about 14.2 grammes of collagen in 100 grammes of intact bone.
The collagen content falls to 9 grammes in the first week after fracture (Fig. 9). This is probably caused by decalcification and consequent removal of collagen fibres from the area of the fracture. In the second week the mucopolysaccharide content gradually becomes less, probably because collagen is laid down by the fibroblasts. By the end of the second week the hexosamine content falls to 450 milligrams per 100 grammes of bone, whereas the collagen content increases to 12·4 grammes. This early accumulation of mucopolysaccharides followed by their disappearance as collagen is formed occurs also in the healing of soft-tissue wounds.

TABLE I

THE CHANGES IN THE WEIGHT AND IN THE MUCOPOLYSACCHARIDE AND COLLAGEN CONTENT OF BONE DURING HEALING

<table>
<thead>
<tr>
<th>Weeks after fracture</th>
<th>Percentage gain in weight of bone</th>
<th>Milligrams of mucopolysaccharides per 100 grammes of dry bone</th>
<th>Grammes of collagen per 100 grammes of dry bone</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9·5</td>
<td>597</td>
<td>8·9</td>
</tr>
<tr>
<td>2</td>
<td>20·8</td>
<td>450</td>
<td>12·4</td>
</tr>
<tr>
<td>3</td>
<td>26</td>
<td>424</td>
<td>11·2</td>
</tr>
<tr>
<td>4</td>
<td>37·6</td>
<td>351</td>
<td>11·4</td>
</tr>
<tr>
<td>5</td>
<td>24·7</td>
<td>244</td>
<td>12·8</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>319</td>
<td>13·7</td>
</tr>
</tbody>
</table>

Third and fourth weeks—In the third week the chondroblastic and osteoblastic proliferations keep the hexosamine content—440 milligrams per 100 grammes of bone—only slightly lower than that in the second week. At the end of the fourth week, when the process of new bone formation usually comes to an end, the hexosamine content falls to 300 milligrams per 100 grammes of bone. The collagen content falls from 12·4 grammes in the second week to 11·2 grammes in the third week and remains at 11·5 grammes in the fourth week (Table I). The fall in the collagen content is only apparent because in the third and fourth weeks there is disproportionate increase in the mineral deposition in the callus and consequent increase in the bone weight (Fig. 10). Once the collagen fibres have been laid down in the second week there is not much addition to them in the subsequent two weeks. It may be that formation of collagen fibres in the osteoblastic region and removal of collagen fibres from the necrosed portion of the broken bone are so balanced that there is not much change in the total collagen content of the regenerating bone in the third and the fourth weeks.

Fifth and sixth weeks—In the fifth and sixth weeks remodelling starts, and the composition of the bone tends to revert to normal (Figs. 8 and 9). The mucopolysaccharides come down to normal and even below normal values in the fifth week and rise to normal levels in the sixth. The collagen content rises during the fifth and sixth weeks, to reach almost its original level at the end of the sixth. This happens because of the fall in total weight of the bone
(Fig. 11). It does not indicate so much the actual production of collagen fibres during the period, but shows rather that during remodelling the excess of calcium phosphate and other minerals is removed by the specific action of osteoblasts and other agencies. At the end of sixth week the normal ratio between the collagen and the mineral contents is restored.

![Graph](https://via.placeholder.com/150)

**Fig. 11**
The relationship between the collagen and mucopolysaccharide content of the bone and the bone weight during various healing. The cause of the secondary fall in collagen content is clearly shown.

**DISCUSSION**

In the middle of the last century it was thought that the healing of fractures was mediated by the cells of the surrounding tissues. The school of thought led by Ollier held that the periosteal cells were responsible for healing; the other school, led by Macewen, considered that the periosteum had nothing to do with the healing and that repair was mainly done by the newer blood vessels and mesenchymal cells of the surrounding region (Keith 1918). We think that there is some truth in the opinions of both schools. Tonna and Cronkite (1961) investigated the sources of cellular response in fracture repair. By injecting thymidine labelled with tritium, they localised the sources of actively proliferating and dividing cells. They demonstrated cellular proliferation around the fracture as early as sixteen hours after injury. The cells were derived partly from the preosteoblastic cells of the periosteum and partly from the surrounding mesenchymal cells. The greatest proliferation of these fibroblasts around the region of fracture was seen about thirty-two hours after injury. The peak of proliferative activity of these cells became localised to the region of fracture by five days. It remained high up to fourteen days, after which moderate activity continued until healing was complete.

Our histochemical results are in agreement with these findings. We observed high cellular activity within twenty-four hours of injury and estimated that the peak was reached at forty-eight hours. We confirmed that the injured bone was repaired both by the cells of periosteal origin and also by those of the surrounding mesenchymal tissue. Most of the periosteal cells changed into osteoblasts, and proliferated near the broken bone. Some of them also converted themselves into chondroblasts in close proximity to the bone ends and proliferated as such till they were replaced by osteoblasts (Ham and Harris 1956, Ham and Leeson 1961).

The gap between the fragments is at first filled mostly by the proliferating fibroblasts derived from the surrounding mesenchymal cells. Their cellular activity continues high till the entire gap is bridged by collagen fibres somewhere towards the end of the second week.
Then the region is invaded by chondroblasts which ultimately give place to osteoblasts for producing bony union. The cells of endosteal origin do not contribute much to the initial formation of callus, though in the later stages they may add their share to some extent. Similarly, the contribution to the cells originating from the osteocytes of the Haversian canals or trabeculæ is meagre and scanty.

Normal bone has comparatively little mucopolysaccharide, but the content of this increases after injury. Rogers (1949) found that human bone contained about 200 to 260 milligrams of hexosamine per 100 grammes of dry bone in youth and 130 to 190 milligrams in the adult. Kasavina and Zenkevich (1961) found 100 to 125 milligrams per 100 grammes of dry rabbit bone. Our studies indicated a hexosamine content of 291 milligrams per 100 grammes of dry rat bone. Immediately after injury this rises considerably. In rats it went up to 590 milligrams; in Kasavina and Zenkevich's rabbits it rose to 1,760 milligrams. There was a sudden fall in hexosamine content between the first and second weeks: in the subsequent weeks it gradually returned to normal. This sequence of events is explained by the proliferation of cartilage cells during the third and fourth weeks contributing to the mucopolysaccharide content. At the end of the sixth week the mucopolysaccharide content tends to become normal.

An increase in mucopolysaccharide content is present not only during healing but also during regeneration and growth of bone. The mucopolysaccharide content of bone remains very high during early foetal life and also in the period of growth soon after birth (Dickerson 1962). As the mucopolysaccharide content becomes less the growth rate of bone also gradually decreases. Thus, one can say that the rate of fall in the mucopolysaccharide content provides indirect evidence of the rate at which the bone is growing. Studies using radioactive sulphur as an indication of mucopolysaccharide content confirm these findings (Singh, Arnkar and Udupa 1961; Udupa, Arnkar and Singh 1961).

The collagen content of bone varies with species, age and type of bone. All these factors must be kept in mind in any quantitative study of collagen content (Eastoe 1956).

There is strong evidence that the collagen is laid down extracellularly out of the secretions made by one of the mesenchymal cells such as fibroblast, chondroblast or osteoblast (Jackson and Smith 1955). In the initial period of their proliferation all these cells secrete substances which will help the formation of mucopolysaccharides. In the latter period they try to secrete substances like precollagen or tropocollagen which ultimately join together to form collagen fibres (Schmitt, Gross and Higberger 1955). How much the mucopolysaccharide actually takes part in the formation of collagen is not certain, but the mucopolysaccharide content usually becomes less as soon as the collagen fibres are formed.

Although all the three mesenchymal components, osteoblast, chondroblast and fibroblast, secrete almost similar intercellular substances, their nature and character differ. Thus when osteoblasts proliferate and start secreting substances required for formation of mucopolysaccharides and collagen, they soon calcify, presumably because of the simultaneous secretion of alkaline phosphatase (Ham and Leeson 1961). When chondroblasts proliferate the constituents of the intercellular substances secreted differ in retaining a large quantity of mucopolysaccharide even after the formation of collagen fibres. When fibroblasts proliferate they secrete substances which are essentially required for the formation of collagen fibres only. As soon as these are formed the substances like mucopolysaccharides gradually disappear (Dunphy and Udupa 1955). Thus, the production of abundant collagen fibres seems to be a general reaction of the body for repairing the breach after injury. The only difference in the case of bone is that the fibres which act as a framework for the chondroblasts are followed by osteoblasts which become calcified.

The curve of collagen content after fracture exactly coincides with that of tensile strength produced by McKeown, Lindsay, Harvey and Howes (1932) and by Lindsay (1934). This perhaps confirms that the tensile strength of most tissues depends upon the amount and the quality of their collagen. The apparent fall of collagen content in the third and fourth
weeks exactly coincides with a fall of tensile strength of the bone. No satisfactory explanation for this fall was given by McKeeown and his colleagues (1932). From our histochemical observations we came to the conclusion that once the collagen has been formed it does not disappear from the area. The apparent fall in the collagen content in the third and fourth weeks is caused by the increase in the weight of the bone mainly brought about by the deposition of calcium phosphate (Fig. 11). As soon as the remodelling phase starts the excess of calcium and phosphate deposited in the earlier period are removed without much loss of collagen; hence the collagen content rises. It is difficult to say whether any collagen is actually added during the remodelling phase.

The fall in tensile strength as observed by Lindsay (1934) might be partly caused by decrease in the collagen per unit of bone and partly by invasion of the region of collagen fibres by chondroblasts followed by osteoblasts resulting in the temporary disorganisation of collagen fibres. Probably because of these changes in the region of the actual gap occupied by collagen fibres, the tensile strength falls. However, as soon as the remodelling phase starts and the excess of minerals is removed, the tensile strength reaches the normal value.

SUMMARY

1. The process of repair after fracture of the humerus of the growing rat has been studied by histological, histochemical and biochemical methods.
2. Both periosteal and surrounding mesenchymal cells take part in the process of repair.
3. The primary framework of collagen bridging the gap is mainly formed by the mesenchymal cells, while calcification and ossification of the framework is largely a function of the periosteum.
4. The mucopolysaccharide content rises rapidly in the first week after injury, and is followed by a rise in the collagen content during the second week. The deposition of calcium phosphate during the third and fourth weeks causes an apparent fall in the collagen content during that period. The collagen content tends to return to normal during the phase of remodelling in the fifth and sixth weeks.
5. The tensile strength of the healing bone bears a close relation to its collagen content.

REFERENCES

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