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ENZYME STUDIES OF FRACTURES WITH NORMAL AND DELAYED UNION

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Fracture healing has been extensively studied by morphological methods (e.g. Enneking 1948, Pritchard & Ruzicka 1950). Cell proliferation in healing bones has been studied by autoradiographic technique (Tonna & Cronkite 1961). The mineral accretion and turnover (Bauer 1954) as well as collagen synthesis and turnover (Stacher & Firschein 1967) have also been studied in experimental fractures. The glycosaminoglycans have been identified in fracture callus by Solheim (1965).

The mechanism underlying the development of delayed fracture healing or non-union is, however, still obscure. The uptake of phosphorus is increased irrespective of the rate of healing (Bohr 1955). Wendeberg (1961) found the uptake of strontium in healing tibial shaft fractures in man to be increased to roughly the same extent whether healing was normal or delayed. A slight disturbance of the distribution of glycosaminoglycans has been demonstrated on one occasion in pseudarthrosis of the human femur (Solheim 1966).

Some publications are available on the role played by enzymes in bone repair. Thus, alkaline phosphatase (Pritchard & Ruzicka 1950), oxidative enzymes (Balogh & Hajek 1965) and esterases (Raekallio & Mäkinen 1968) have been demonstrated histochemically in fracture callus. Lactic dehydrogenase activity and isoenzymes have been studied in regenerating rabbit bone (Bruce & Strachan 1967, Gudmundson & Semb 1970).

The purpose of this study was to elucidate the activity and subfractions (isoenzymes) of some enzymes in callus tissue from fractures with normal and delayed union. Rat femur fractures were used because they frequently heal very slowly (Bohr 1955, Pritchard & Ruzicka 1950).

MATERIAL AND METHODS

Forty mature female Sprague-Dawley rats, eighteen months of age weighing 242 ± 18 (S.D.) grams at the beginning of the experiment, were used. Their left femur was manually broken in the middle of the diaphysis under ether anaesthesia. No attempt was made to immobilize the fractures. Eight weeks later the right femur was broken with the same technique. The animals were kept in three large cages and were fed a standard laboratory diet with water *ad libitum*. Immediately after fracture the animals walked on three legs, but within a few days, on all four.

The animals were killed fifteen weeks after the first fracture. The fracture of the right femur was then seven weeks old. Both femurs were dissected and X-rayed. Femurs with stable and unstable fractures were grouped separately according to fracture age. The fracture area was cleaned of soft tissues and the callus was removed for enzyme analyses. Specimens of callus tissue from healed and unhealed fracture, respectively, of each fracture age were randomly divided into four groups.

Specimens from diaphyses of femurs obtained from adult female rats, weighing about 250 grams, were used as controls. Furthermore, six pooled samples of fracture callus from twenty-one-week-old rat femur fractures were used for comparison of the isoenzyme pattern of esterases in later healing stages. All these fractures were stably healed.

The specimens were kept on ice, and their blood was removed by rinsing them in isotonic saline at + 4°. The specimens were then deep frozen in liquid nitrogen and crushed to powder in an ice-cold steel mortar (Semb 1970).

Extraction was performed overnight at + 4° in distilled water in the proportions 2:1 (v/w). The extracts were centrifuged at + 4° and 10,000 *g* for five minutes, and the supernatants were used for the analyses after dilution with distilled water. The total activity of lactic dehydrogenase (LDH) was measured spectrophotometrically as described earlier (Semb 1970). After addition of sodium pyruvate in phosphate buffer 0.1 M pH 7.4 and NAD.H dissolved in 0.001 N NaOH, the decrease in light absorption was measured at 340 mμ. This is a measure of the oxidation of NAD.H, which is a function of LDH activity. The activity was expressed as units, one unit being equivalent to an extinction decrease of 0.001 per minute per millilitre of the sample.

The tartrate inhibited acid phosphatase (Acpase) activity was determined spectrophotometrically according to Jacobsson (1960). Samples of the tissue extracts were added to p-nitrophenyl-phosphate, di-sodium salt, 0.4 per cent, in citrate buffer, pH 5.5. After incubation at 37° the amount of p-nitrophenol liberated was measured photometrically at 405 mμ in a Beckman B spectrophotometer. The total activity of alkaline phosphatase (Alkpase) was measured according to the same principle using p-nitro-phenyl-phosphate as substrate with the exception that a buffer with pH 11.0—11.3 (2-amino-2-metyl-1-propanol + $MgSO_4$) was used.

All values of enzyme activity were related to the wet weight of the specimens and also to the DNA concentration. The DNA content was estimated using the diphenylamine reaction (Dische 1930).

LDH isoenzyme were separated by electrophoresis on agar gel and were identified by incubation at 37° in a substrate mixture containing lithium lactate, NAD, and nitroblue tetrazolium as coupler as described by Semb (1970). The density of the

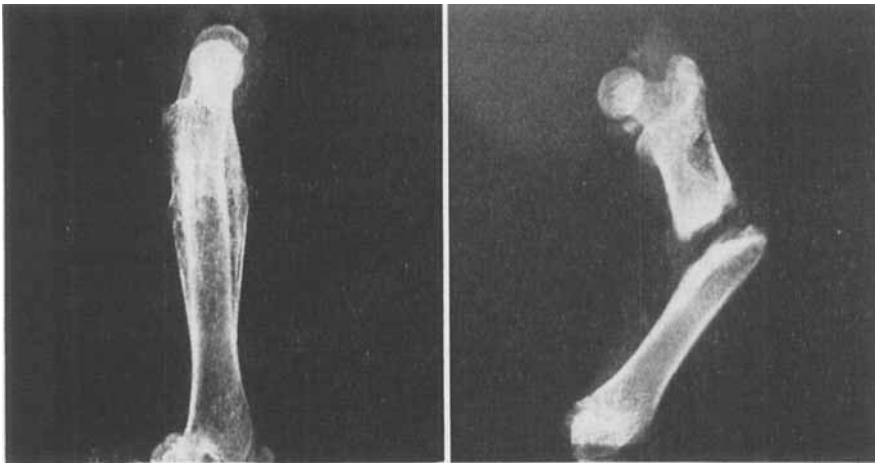

Figure 1. Fifteen-week-old fractures of the rat femur with normal (left) and delayed (right) union.

LDH isoenzymes was measured in a Vitatron photometer with a scanning device and an automatic recorder.

Esterases and Alkpase and Acpase were examined by starch gel electrophoresis according to Smithies (1955). The starch gel was cast on glass plates, which were placed on top of the plexiglass electrophoresis tank and were continuously cooled with tap water. Esterases and Acpase were separated in Ashton buffer, pH 8.6 and Alkpase in borate buffer pH 8.5.

For identification esterases were incubated at 37° overnight in α-naphtyl acetate, phosphate buffer pH 7.0 and Fast Red TR salt as coupler. Alkpase was incubated in α-naphtylacid-phosphate-sodium-salt, Tris buffer pH 8.6 and Fast Blue RR salt. After preincubation at 37° in acetate buffer pH 4.0 for 40 minutes, Acpase was identified by incubation in α-naphtyl-acid-phosphate-sodium-salt, acetate buffer pH 5.2 and Fast Blue RR salt.

RESULTS

The type of fracture healing was assessed by testing the stability of the femur diaphyses by hand. Of the seven-week-old fractures, 17 were not healed (43 per cent), and 12 fractures (30 per cent) were not healed after fifteen weeks. The X-ray films (Figure 1) invariably confirmed the type of healing recorded.

During the studied period of 15 weeks the weight gain of the animals amounted to 39 ± 7 (S.D.) grams. There was no difference in weight gain or carcass weight between rats with stably healed fractures and rats with one or two fractures with delayed union ($p > 0.2$).

Table 1. Enzyme activities (units ± S.D.) in callus from fractures with normal and delayed union.

	Stable fract. 7 weeks	Unstable fract. 7 weeks	Stable fract. 15 weeks	Unstable fract. 15 weeks	Normal bone
Alkpase u/g	424 ±40	160 ±11	250 ±47	152 ± 6	169 ±39
Alkpase u/mg DNA	235 ±23	77 ± 5	191 ±36	111 ± 4	252 ±56
Acpase u/g	83 ±19	149 ±20	88 ±15	104 ±11	45 ± 5
Acpase u/mg DNA	48 ±11	71 ±10	67 ±11	76 ± 8	64 ± 7
LDH u/g × 10^3	61.3±13.2	72.4± 3.9	71.8± 8.1	63.5±12.8	9.2± 2.4
LDH u/mg DNA × 10^3	35.2± 7.6	34.6± 1.9	54.7± 6.2	46.3± 9.3	10.3± 3.4

The total activity of LDH was increased in healed as well as in unhealed fractures of both seven and fifteen weeks of age (Table 1). This increase was always highly significant ($p < 0.001^{***}$), whether relating the activity to wet weight or DNA concentration.

Alkpase activity was in the same range in normal bone as in fractures with normal union as compared to the DNA content. However, the Alkpase activity was significantly higher in stable fractures than in unstable ones after seven ($p < 0.001^{***}$) as well as after fifteen ($0.01 > p > 0.001^{**}$) weeks irrespective of whether the activity was correlated with wet weight or DNA content (Table 1, Figure 2).

Acpase activity was most increased in unhealed fractures after both periods (Table 1, Figure 2). The difference in Acpase activity was, however, only statistically significant after a healing period of seven weeks ($0.01 > p > 0.001^{**}$) and insignificant after fifteen weeks ($p > 5\ \%^{-1}$). When correlated with the DNA concentration there was neither any significant Acpase activity difference between control bone and healing bone with delayed union nor between control bone and fifteen-week-old stably healed bone.

After electrophoreses of Alkpase and Acpase in starch gel, only one fraction of each enzyme could be seen. This fraction had the same electrophoretic mobility whether extracted from normal bone or from callus.

Electrophoretic separation of esterases revealed some differences

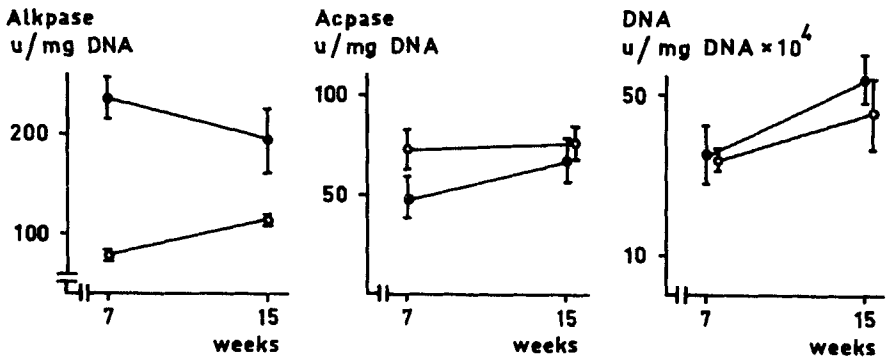

Figure 2. Total activity of alkaline and acid phosphatases and of lactic dehydrogenase (units/mg DNA ± S.D.) in seven and fifteen week old fractures with normal (●——●) and delayed (○——○) union.

between normal bone and fracture callus. Both tissues showed two main fractions, a slow one and a fast one. But the fast fraction was more active in callus tissue than in normal diaphyseal bone. There was no difference between healed and unhealed fractures in this respect. However, in callus from twenty-one-week-old fractures the slow fraction was more active than the fast one (Figure 3). Thus the esterase zymograms of twenty-one-week-old fractures resembled those of normal diaphyseal bone.

Five distinct zones of LDH were seen after electrophoretic separation on agar gel (Figure 4). In normal diaphyseal rat bone LDH 5 is predominant (42 per cent). In stable as well as in unstable fractures the activity of LDH 1 was significantly ($p < 5\ \%^*$) lower and that of LDH 5 significantly higher, than the corresponding isoenzyme activities

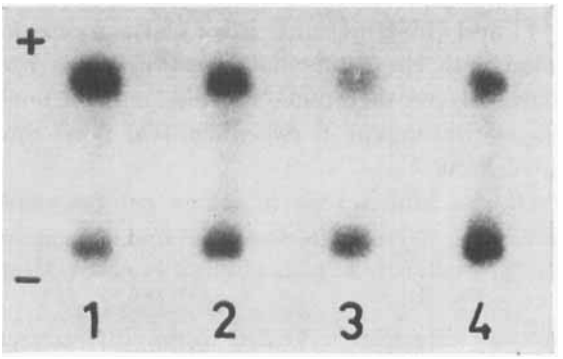

Figure 3. Isoenzymes of esterases in fifteen-week-old stable fractures (1–2) and in twenty-one-week-old stable fractures (3–4).

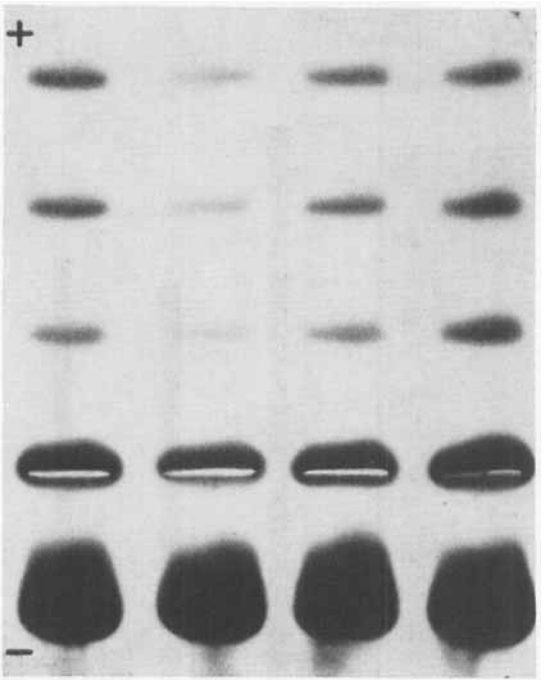

Figure 4. Isoenzymes of lactic dehydrogenase in fifteen-week-old stable fractures.

in normal bone. Stable fractures of seven weeks of age displayed the most pronounced increase in LDH 5 activity and, correspondingly, the most pronounced decrease in LDH 1 activity. These differences were significant ($p < 5\ \%^*$) as compared with the activity in the callus from the other fractures as well as with that in the normal bone. Comparison between stable and unstable fifteen-week-old fractures did not reveal any significant difference in the LDH isoenzyme pattern (Figure 5).

DISCUSSION

Some factors are known to be able to delay or prevent union of fractures, e.g. regional impairment of blood flow, insufficient immobilization, and infection. But sometimes delayed union cannot be ascribed to any of these known factors. Previous investigations have demonstrated a normal uptake of phosphorus in rat femoral fractures with delayed union (Bohr 1955) and of strontium in pseudarthrosis of the tibia in man (Wendeberg 1961). However, no investigations have been published of enzymes in fractures with delayed union or non-union.

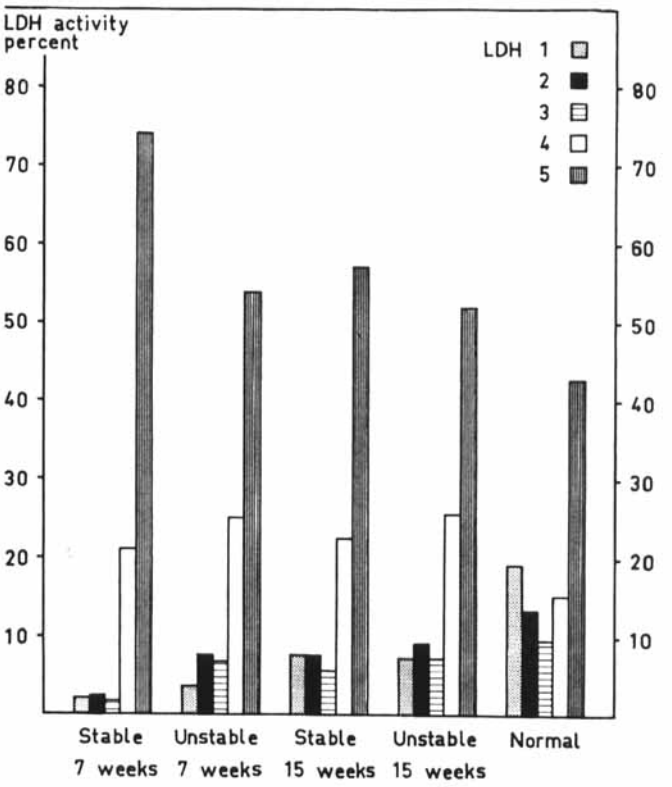

Figure 5. Percentage activity of lactic dehydrogenase isoenzymes in callus from seven and fifteen-week-old fractures with normal and delayed union and in normal rat femoral diaphyses.

In this study fractures of the rat femur were chosen because such fractures often heal slowly or result in delayed union (Bohr 1955). The frequency of delayed union after seven and fifteen weeks was very high (about 35 per cent). However, most fractures of the rat femur seem to heal spontaneously after some time. Thus, all fractures observed at twenty-one weeks were healed. It must be emphasized, however, that twenty-one weeks is a long part of the rat's life-span.

The function of Alkpase is still obscure, but it is in some way related to the mineralization process. A normal mineral uptake has been found in fractures with delayed union (Bohr 1955) and in pseudarthroses of the tibia (Wendeberg 1961). However, in our investigation, Alkpase activity was significantly lower in fractures with delayed union as compared to normally healing fractures. It has been

suggested that Alkpase limits the accumulation of calcification inhibitors, e.g. inorganic pyrophosphate (Jibril 1967). Moreover, serum Alkpase activity increases significantly long before the mineralization process of the fracture callus begins (Semb et al. 1970). Thus, the enzyme is probably of considerable importance in the premineralization phase of fracture healing, and the decreased enzyme activity of our fractures with delayed union may be an important limiting factor for a normal fracture healing.

No difference in electrophoretic mobility of Alkpase was found. Only one fraction was seen in all preparations, which is in accordance with some earlier investigations of isoenzymes of Alkpase in serum, bone, and other tissues (Hodson et al. 1962, Hill & Sammons 1967).

It has been found that Acpase belongs to lysosome-like particles in bone cells (Vaes 1965). Increased activity of this enzyme has been demonstrated in bone with an increased resorption rate induced by parathyroid hormone (Vaes 1967). In the present investigation Acpase activity was higher in fractures with delayed union than in normally healing fractures, particularly after seven weeks.

During bone formation the highest activity of Acpase has been found in the osteoclasts (Buring & Semb 1970), which implies that this enzyme is of importance in the bone remodelling. The increased Acpase activity in fractures with delayed union may result in a high remodelling rate, which is in accordance with reported data on mineral uptake in fractures with delayed union or pseudarthroses (Bohr 1955, Wendeberg 1961).

A high activity of LDH has been demonstrated histochemically in osteoblasts as well as in osteoclasts in healing fractures (Balogh & Hajek 1965). In the present investigation the activity of LDH, an oxidative enzyme, was increased to about the same level independent of normal or delayed fracture healing. These results support the theory about a high remodelling rate also in fractures with delayed union.

The LDH isoenzymes are tetramers of two polypeptide subunits, called H and M; LDH 1 = HHHH, LDH 2 = HHHM etc. *In vitro* a predominance of H in tissues with aerobic metabolism has been found. On the other hand, there is a preponderance of M in tissues with anaerobic metabolism (Hellung-Larsen 1968). In the present investigation the activity of LDH 5 was higher and that of LDH 1 was lower in stable seven week-old fractures than in callus from the other fractures. Moreover, on comparison between all kinds of callus tissue and normal diaphyseal bone, the relative activity of LDH 5 was higher, and that

of LDH 1 lower, in callus tissue (Figure 5). This implies a more anaerobic metabolism in callus than in normal bone and a predominance of that kind of metabolism in stable seven-week-old fractures. Thus, in healing rat femur fractures and especially in those with the fastest healing rate, there might be an increased oxygen demand or relative ischaemia in spite of the profuse blood supply.

SUMMARY

Enzyme studies were performed on seven and fifteen-week-old rat femoral fractures with normal or delayed union. The frequency of delayed union was about thirty-five per cent.

Alkaline phosphatase activity was higher in stable fractures than in unstable ones, particularly at seven weeks. On the other hand, acid phosphatase activity was somewhat higher in fractures with delayed union. No change was seen in the isoenzyme patterns of alkaline or acid phosphatases.

The total activity of lactic dehydrogenase (LDH) was higher in fractures than in normal bone irrespective of the stage or duration of healing of the fracture. There was a preponderance of LDH 5 isoenzyme in normal bone as well as in callus, but this isoenzyme was more active in callus than in bone.

The enzyme studies suggest a more anaerobic metabolism in callus than in normal diaphyseal bone, and may indicate a high remodelling rate also in fractures with delayed union.

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