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# ISOENZYMES OF LACTIC DEHYDROGENASE AND ESTERASES IN REGENERATING BONE

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The distribution of some enzymes in regenerating bone has been studied histochemically. These studies have shown, among other things, an increased activity of alkaline phosphatases (Pritchard & Ruzicka 1950), oxidative enzymes (Balogh & Hajek 1965) and esterases (Raekallio & Mäkinen 1968). The lactic dehydrogenase isoenzymes in regenerating rabbit bone have been investigated by Bruce & Strachan (1967). Isoenzymes are varieties of an enzyme possessing the same substrate specificity, but differing in electrical charge and/or molecular size.

In a preliminary study on esterases in experimental fracture callus we found the isoenzyme pattern to differ in certain respects from that of normal bone (Semb & Gudmundson 1969). This paper concerns a continuation of that study of esterases including an oxidative enzyme, lactic dehydrogenase.

## MATERIAL AND METHODS

Young adult rabbits weighing 2.5–3.0 kg were used. The animals were anaesthetized with sodium pentobarbital intravenously. Two centimeters proximal to the radiocarpal joint a defect was made in both radial diaphyses by removing a one centimeter long bone cylinder with a dental saw. The bone tissue regenerating in the defect was studied after various intervals. Seven groups of four animals were sacrificed at three, six, nine, fourteen, twenty-one, thirty and sixty days postoperatively. The forearms were X-rayed (Figure 1) and the tissue between the "fracture" ends was dissected and kept on ice during further preparation. The

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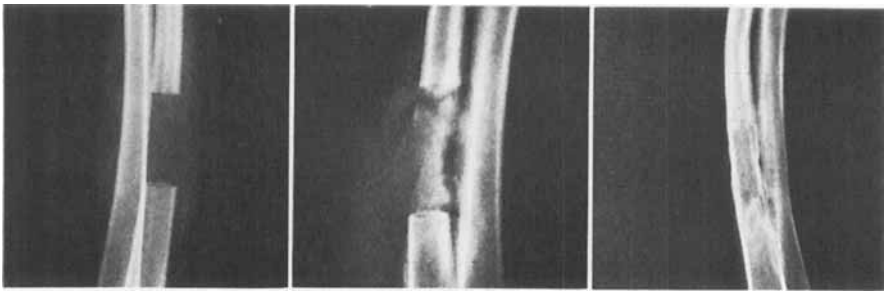

*Figure 1. Experimental fracture of rabbit radius with a healing time of 0 day (left), 14 days (middle) and 60 days (right).*

dissected material from each animal was prepared and analysed separately. The radial bone cylinders removed were treated in the same way as the regenerating tissue and were used as control samples of normal bone.

All blood was removed from the samples by rinsing them in cold isotonic saline. The specimens were frozen in liquid nitrogen, crushed in a cold steel mortar, weighed and extracted overnight in isotonic saline at +4°. The extracts were centrifuged at + 4° and 10,000 g for five minutes, and the supernatants were used for the analyses (Semb 1971).

Determination of lactic dehydrogenase (LDH) activity and separation and identification of LDH isoenzymes were performed as described earlier (Semb 1971). The relative activity of the various LDH isoenzymes was measured in a Vitatron photometer with a scanning device and automatic recorder.

Esterase isoenzymes were separated electrophoretically on starch gel according to Smithies (1955). A plexiglass electrophoresis tank was used. Starch was heated in Ashton buffer, pH 8.6, after which the gel was evacuated and casted on a glass plate, that was later cooled by running tap water during electrophoresis. A constant current of 150 mA was used. After the electrophoretic separation the various fractions were identified by incubation overnight at 37° in a substrate mixture of the following composition: 1 ml one per cent $\alpha$-naphtyl acetate, 1.5 mg Fast Red TR salt, 50 ml of phosphate buffer, pH 7.0, and 50 ml of distilled water. Fixing was performed in acetic acid, methanol, and distilled water in the volumetric proportions 1:5:5.

Specific inhibitors (eserine and diethyl-p-nitrophenylphosphate) were used to characterize the esterases. After electrophoresis the gel slides were divided horizontally. One of the slides was incubated in a $10^{-5}$ M solution of one of the inhibitors at room temperature for fifteen minutes before the two halves were incubated in the substrate mixture above described. Enzyme inhibition was evaluated by comparing the paired slides.

## RESULTS

The total activity of LDH, given as units/gram fresh tissue, was very high in callus with a maximum value about a hundred times normal after nine days. The LDH activity was still much increased after sixty

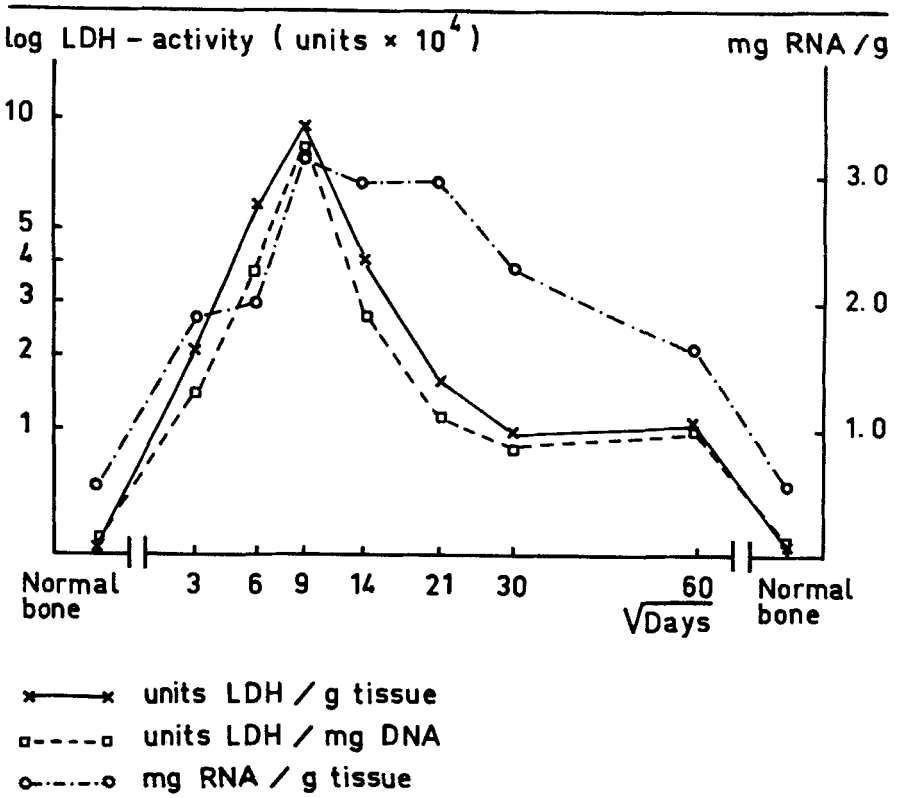


*Figure 2. Total activity of LDH expressed as units/g fresh bone and as units/mg DNA, respectively, in regenerating bone of various age. The concentration (mg/g fresh bone) of RNA in regenerating bone. The concentration of DNA and of RNA (mg/g wet tissue) were taken from Deutsch & Gudmundson (In press).*

days (Figure 2). There was a parallel but somewhat lower activity increase when the activity was expressed as units/mg deoxyribonucleic acid (DNA). There was a similar variation with time of the LDH activity and the RNA concentration (Figure 2). The values for the concentration of DNA as well as those of RNA in regenerating bone were taken from an investigation on regenerating tissue from the corresponding region in rabbits of the same weight as those used in the present investigation (Deutsch & Gudmundson 1971).

Figure 3 demonstrates the relative activity of the LDH isoenzymes of regenerating bone in various healing stages, compared with that of normal bone from the diaphysis of the radius. The percentage activity of LDH 1 was lower in regenerating bone, especially after six to thirty

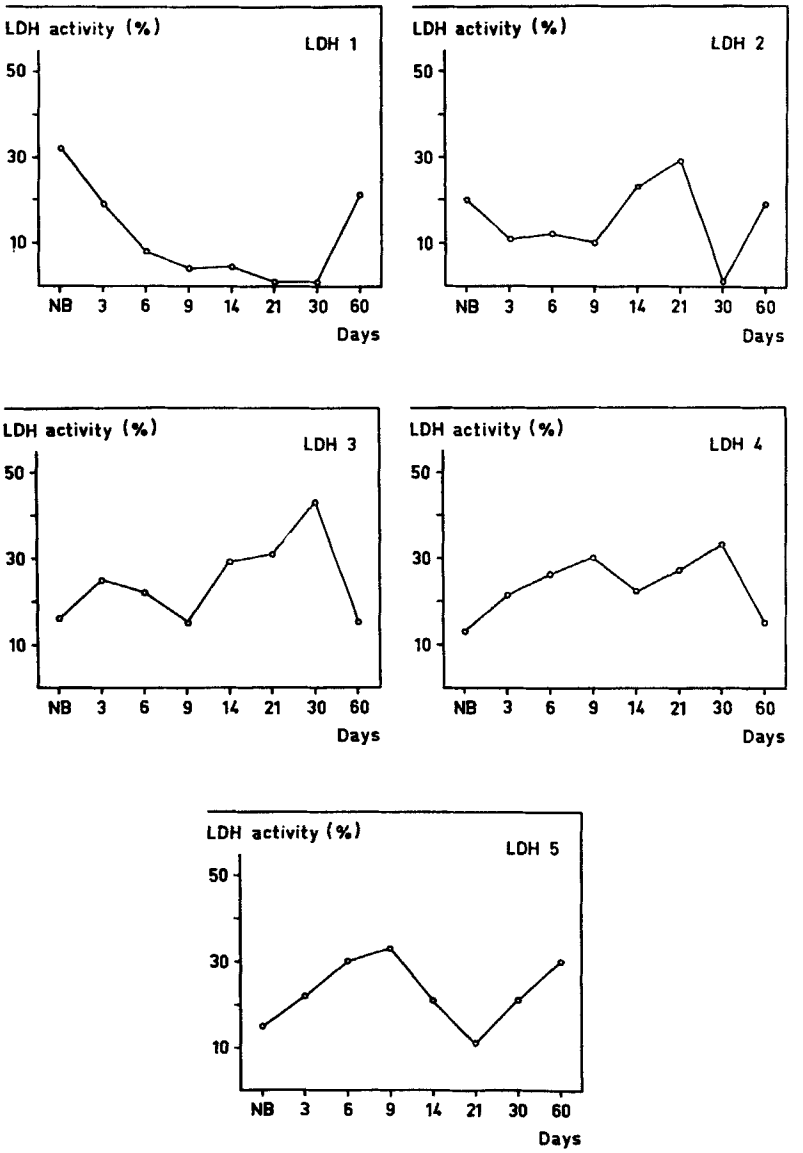


*Figure 3. Percentage activity of the LDH isoenzymes in regenerating bone of various ages and in normal diaphyseal bone (NB) from the rabbit radius.*

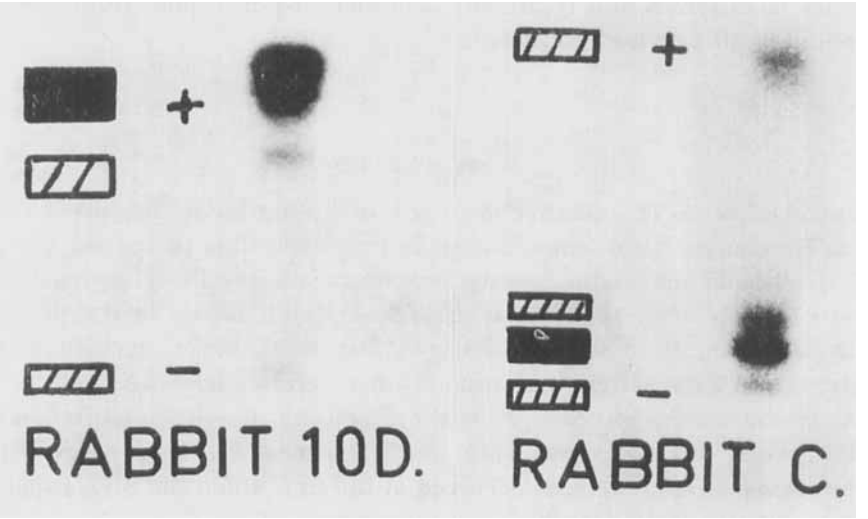


*Figure 4. Esterase isoenzymes in ten day old callus (left) and in normal diaphyseal bone (right) from the rabbit radius.*

days. There was also a drop in the percentage activity of LDH 2 up to nine days. The relative activity of LDH 3 was increased from fourteen to thirty days. The activity of LDH 4 and LDH 5 rose to peak values at nine days. Still after sixty days the relative activity of LDH 5 was much elevated. At that time, however, the remaining enzyme fractions tended to become normal (Figure 3). By X-ray was demonstrated that mineralization was weak at fourteen days but seemed to be completed after sixty days (Figure 1). Thus the activity of slow-moving isoenzymes (LDH 4 and LDH 5) increased and that of fast migrating (LDH 1 and LDH 2) decreased in young insufficiently mineralized callus.

Ocular estimation of the esterase isoenzyme activity showed increased activity in all callus preparations. In normal rabbit radial bone the esterase isoenzyme pattern showed a predominance of two slow migrating fractions and a smaller, fast moving anodic fraction. However, in regenerating bone the fast esterase isoenzyme was much more active than the slow fractions (Figure 4). With increasing maturity of the callus the fast zone was relatively weaker, resulting in a tendency towards normalization of the isoenzyme pattern.

Incubation in eserine of the gel plates after electrophoresis of extracts of healing and control bone did not result in inhibition of any

of the isoenzymes. But treatment with diethyl-p-nitrophenyl-phosphate inhibited all enzyme fractions.

## DISCUSSION

The occurrence of oxidative enzymes, including lactic dehydrogenase, has previously been demonstrated in fracture callus in the rat fibula. Considerable enzymatic activity was found mainly in osteoprogenitor cells already after three days (Balogh & Hajek 1965). In the present investigation, as in that by Bruce & Strachan (1967), a pronounced increase of LDH activity was found in regenerating bone. In the present study maximum increase of enzyme activity was found after nine days when the callus was very poorly mineralized. It is noteworthy that this maximal increase occurred at the time when the RNA concentration is maximal (Deutsch & Gudmundson 1971; Figure 2), and in later healing stages when the regenerating tissue was almost fully mineralized (Figure 1), the activity of LDH and the concentration of RNA curves declines almost simultaneously (Figure 2; Deutsch & Gudmundson 1971). The increased activity of LDH seems thus mainly to reflect the high rate of synthesis of organic matrix in healing fractures.

In the present study increased activity of LDH in healing bone was demonstrated both in relation to tissue content (wet weight) and to the DNA content (Figure 2). Considering the constant relationship between DNA concentration and the number of cells (Enesco & Leblond 1962), this may indicate that the enzyme activity in regenerating bone was increased more than what could be explained by the increased number of cells in this tissue.

Increased activity of esterases has been demonstrated histochemically very early in the granulation tissue of a fracture in rats (Raekallio & Mäkinen 1968). Also in the present investigation strong esterase activity was found early in bone healing. It is thus evident that esterases are involved in the metabolism of regenerating bone before the mineralization process reaches its maximum. There are two main groups of esterases, non-specific, and choline. As eserine, known to inhibit choline esterases completely, failed to inhibit any esterase fraction, the identified fractions were probably non-specific esterases. The esterase inhibition by diethyl-p-nitrophenylphosphate and the failure of inhibition by eserine implies that the demonstrated esterases

in healing and control bone are of the non-specific, organophosphate sensitive type.

The esterase isoenzyme pattern in regenerating bone showed a predominant anodic fast fraction. This fraction is weak in normal diaphyseal radial bone in the rabbit. This change in the isoenzyme pattern, which tended to become normal with time, might reflect the cell differentiation occurring during maturation of the callus.

LDH isoenzymes are tetramers of two polypeptide subunits, called H and M, which are randomly combined to form the five LDH isoenzymes: LDH 1 = HHHH, LDH 2 = HHHM etc. The polypeptide H is preponderant in tissues with mainly aerobic metabolism, such as heart muscle, while there is a preponderance of M subunit in tissues with anaerobic metabolism (Goodfriend & Kaplan 1963). In regenerating bone there was a decrease of LDH 1 or H subunits and an increase of LDH 4 and 5, M subunits, both of which tended to become normal in the later healing stages. This may imply a predominantly anaerobic metabolism in callus at a time when LDH activity and RNA concentration are at their highest and when there is a rich blood supply. Anaerobic metabolism with a rich blood supply seems conflicting. However, relative ischaemia might occur despite increased blood flow when the metabolic rate is very high, as in fracture repair (Gudmundson & Semb 1971). Thus, a clinical conclusion stressing the importance of normal oxygen transporting capacity of the blood in patients with healing fractures may be drawn from the isoenzyme pattern demonstrated. Against the other possible suggestion, that the cells in young fracture callus for some reason should prefer anaerobic metabolism, stands the well-known clinical experience that impaired blood supply retards the rate of fracture union.

The LDH isoenzyme pattern of callus in this investigation is incompatible with the results reported by Bruce & Strachan (1967). They studied the LDH isoenzymes in healing bone obtained from the rabbit mandible six to fifteen days after the defect had been made in the bone. In control mandibular bone, as in healing bone, LDH 1 predominated. This preponderance of LDH 1, however, was less in healing bone where the relative activity of the other isoenzymes was much increased. On the contrary, in our investigation the slow-moving isoenzymes (LDH 4 and 5) predominated in healing bone. In their investigation the isoenzymes were separated on acrylamid gel, but this cannot explain the conflicting results. The difference in region from which the tissue was obtained, mandible and radius respectively, might

explain the difference in isoenzyme pattern though a pilot study by one of us (H.S.) revealed no significant difference of the LDH isoenzyme pattern in bone tissue from various tubular bones in the same individual.

## SUMMARY

Isoenzymes of lactic dehydrogenase (LDH) and esterases were studied in bone regenerating in standardized osteotomies of the rabbit radius.

LDH isoenzymes were electrophoretically separated on agar gel and esterase isoenzymes on starch gel. The LDH enzyme activity (units/g wet weight) was substantially increased in fracture callus. The LDH activity per cell expressed as units/mg DNA was also increased in the same way. A parallel increase of LDH activity and RNA concentration was found.

LDH 4 and 5 were increased and LDH 1 was decreased in regenerating bone indicating a metabolism with relative ischaemia in healing fractures.

The esterase isoenzyme pattern of callus contained a predominant fast anodic fraction; that of normal bone, a predominant slow fraction.

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