

Quantification of Damage to Striated Muscle after Normothermic or Hypothermic Ischemia

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Muscular ischemia of different duration was induced by use of a tourniquet applied to the rear left leg of rabbits under normothermic or hypothermic conditions, and variations in serum creatine kinase, lactate dehydrogenase, K^+ , Ca^{2+} , and lactate were determined at various intervals after blood flow was restored. A monocompartmental model analysis applied to the activities of the two enzymes in the serum demonstrated that: (a) the total enzyme activity leaked into the blood is proportional to the duration of normothermic but not of hypothermic ischemia; (b) this enzyme leakage persists until at least the third day after blood flow is restored; (c) a substantial part of the ischemic damage seems to occur as a consequence of re-perfusion, not of the ischemia itself; and (d) hypothermia greatly minimizes the ischemic damage. The data we obtained for K^+ and for lactate confirm the protective effect of hypothermia.

Additional Keyphrases: electrolytes · creatine kinase · lactate dehydrogenase · lactate · rabbits

In surgical restoration and revascularization of a severed or severely injured limb, quantification of ischemic damage is of the utmost importance for evaluating the viability of the tissue that has undergone ischemia and the efficacy of the therapeutic approaches.

Tissue ischemia results in increased permeability of the cellular membrane, with consequent leakage of cytoplasmic enzymes into the extracellular compartment (1-4); in the case of experimental myocardial infarction the total amount of enzyme leaked has been shown to be directly proportional to the extent of the ischemic area (5) and inversely related to the amount of oxygen and of ATP available at the site (6). Thus it seemed possible to evaluate the severity of the ischemic damage suffered by a replanted or a revascularized limb by measuring the total activity of certain enzymes in the blood immediately after surgery and at various intervals thereafter.

We assessed the total amount of creatine kinase (CK, EC 2.7.3.2) and of lactate dehydrogenase (LDH, EC 1.1.1.27), part of which was released from the ischemic limb, in serum of rabbits submitted to different intervals of tourniquet ischemia, by the use of a mathematical model originally worked out to determine the extent and evolution of acute myocardial infarction in man (7).

Because ischemic damage as evaluated by morphological, plethysmographic, and cineradiographic techniques has been shown to be decreased by hypothermia (8), we also measured the release of CK and of LDH in this condition. Further to define the influence of hypothermia, we also measured variations in potassemia, calcemia, and lacticemia.

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Received Sept. 23, 1980; accepted Nov. 12, 1980.

Materials and Methods

Animals. We used male rabbits of the "Fulvo di Borgogna" strain, weighing about 2.5 kg. They were housed at constant temperature (20 °C) throughout the experimental period and were maintained on a nutritionally complete diet of laboratory chow (Piccioni, Brescia, Italy) and water, *ad lib*.

Experimental design. Ischemia was induced in rabbits that were under pentobarbital anesthesia, by use of a circular rubber sleeve, inflated so as to give a pressure of 300 mmHg (40 kPa) (9), applied at the proximal end of the rear left leg. The ischemic limb was kept at room temperature (about 20 °C) in the "normothermic ischemia" experiments, or wrapped in a plastic bag filled with melting ice immediately after the onset of ischemia in the "hypothermic ischemia" experiments. Ischemia lasted for 2, 4, or 6 h. It was ended by removing the rubber band. At the same time the ice bag was removed.

Blood was sampled for determination of the activity of CK and LDH and of the concentration in serum of K^+ , Ca^{2+} , and lactate, via a heparinized cannula inserted into the jugular vein of the anesthetized animal.

For each interval of ischemia the rabbits were randomly distributed into five groups (four or five in each group): Two groups were sampled every 4 h in the time intervals 0-12 and 12-24 h after re-perfusion; the other three groups were sampled three, seven, and 15 days after re-perfusion.

Hematochemical determinations. Kits were used to determine the activity of CK and of LDH in serum [Merckotests, UV test, CK-Nac (10), and LDH (11); Merck, Darmstadt, F.R.G.]. Lactate was measured by the Test-Combination Lactate UV-method (12) (Boehringer GmbH, Diagnostica; Mannheim, F.R.G.). Absorbance changes were measured with a Gilford Model 2500 recording spectrophotometer. Total Ca^{2+} was determined with a kit [Calcium, (13); F. Hoffmann-La Roche and Co. A.G., Diagnostica, Basel, Switzerland]. K^+ was determined with a Unicam SP 90A atomic absorption spectrophotometer.

Mathematical analysis. We used the monocompartmental model analysis developed by Shell et al. (7) to calculate the total activity of CK and of LDH released into blood from the ischemic limb at a given time after re-perfusion. With this model the total amount of enzyme released into blood in the time interval Δt is expressed by the equation:

$$E_R = E + K_d \cdot \int_{t_0}^t E dt$$

where E_R = total enzyme activity (in U/L) released into the blood in the time interval $t - t_0$, E = activity (U/L) of the enzyme in the serum at time t , and K_d = fractional disappearance rate of the enzyme from serum (h^{-1}).

The values of K_d (0.156 and 0.181 h^{-1} for CK and LDH, respectively) were calculated from values for half-life (second phase) of the two enzymic activities in rabbit serum (14).

Results

Our preliminary experiments (not shown) demonstrated that during ischemia the values for of all the analytes measured in this work were not different from those obtained for

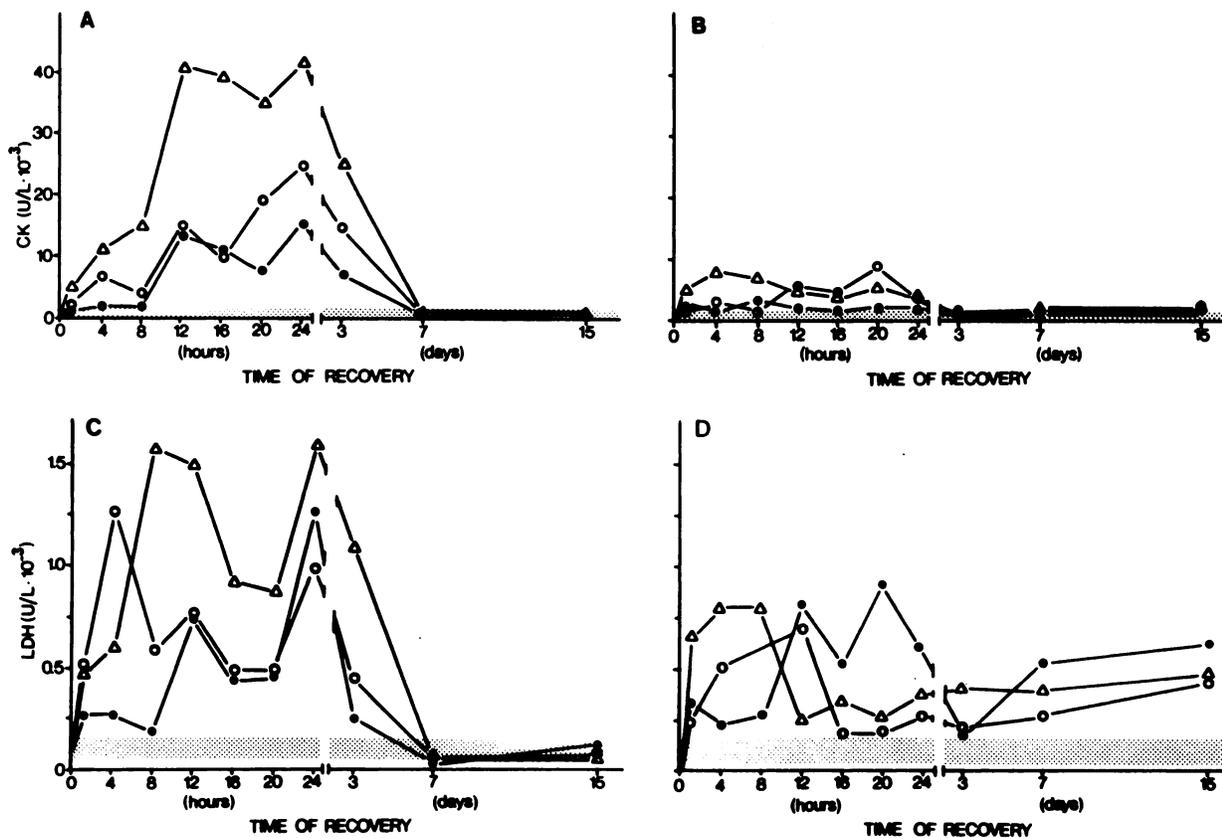


Fig. 1. Activity of CK and LDH in serum during recovery from 2 (●—●), 4 (○—○), and 6 (Δ—Δ) h of normothermic (A, C) and hypothermic (B, D) ischemia

The points are the mean of 4–5 animals; the SEM (not represented) ranged between 20–25% of the mean. The shaded area represents the mean value of the control \pm SEM

normal animals. This indicates that under our experimental conditions both blood flow and lymphatic flow in the leg were completely abolished during ischemia.

The possibility that part of the effects seen in the “hypothermic ischemia” experiments could be ascribed to hypothermia per se was also ruled out by control experiments.

Creatine Kinase and Lactate Dehydrogenase

Re-perfusion of the limb after normothermic ischemia is followed by a concomitant increase in the activity of CK and of LDH in serum (Figure 1, A and C). For both enzymes, activities in serum returned to normal by the seventh day. Hypothermia (Figure 1, B and D) greatly decreases the amount of CK and of LDH released, but the behavior of the two enzymes differed: CK became normal by the third day, while LDH was higher than the control all during the period of observation.

Using the monocompartmental model analysis of the data presented in Figure 1, we obtained the curves shown in Figure 2. These curves, which represent the total amount of enzyme

released (E_R) into the blood as a function of the time elapsed from the end of ischemia, demonstrate that for both normothermic and hypothermic ischemia the release of CK and of LDH continues until at least the third day after re-perfusion. Observations were limited to 72 h, because thereafter the time intervals between the experimental points are too long for us to give a consistent description of the phenomenon.

Except for LDH, after hypothermic ischemia, this prolonged release was not predictable from the curves shown in Figure 1 by simply relating the activity of the enzyme in serum to time.

The curves shown in Figure 2 demonstrate also that: (a) hypothermia greatly diminishes the total activity of enzyme leaving the ischemized muscle and (b) hypothermia has a greater influence on the release of CK than of LDH. The ratio between the enzyme activity released in normothermic and in hypothermic ischemia varies between 1.7–3.7 and 6.9–11.2 for LDH and CK, respectively.

The regression equations in Table 1 relating the total enzyme activity released by the 72nd hour from the restoration

Table 1. Correlation between Duration of Ischemia (h_i) and Total Activity (U/L) of CK (CK_R) and LDH (LDH_R) in Blood during the First 72 h after Normothermic and Hypothermic Ischemia^a

| Type of ischemia | Regression equation | r | p |
|------------------|---|------|------|
| Normothermic | $CK_R = -(8.5 \pm 40.0) + (59.8 \pm 9.1) \cdot h_i$ | 0.99 | 0.02 |
| Normothermic | $LDH_R = (0.7 \pm 7.0) + (2.5 \pm 0.7) \cdot h_i$ | 0.97 | 0.07 |
| Hypothermic | $CK_R = (6.1 \pm 11.8) + (3.0 \pm 3.2) \cdot h_i$ | 0.69 | 0.44 |
| Hypothermic | $LDH_R = (1.1 \pm 2.2) + (0.5 \pm 0.6) \cdot h_i$ | 0.70 | 0.45 |

^a Regression equations $y = (a \pm SD) + (b \pm SD) \cdot x$ have been calculated from the data shown in Figure 2. r, correlation coefficient; p, significance of the linear regression.

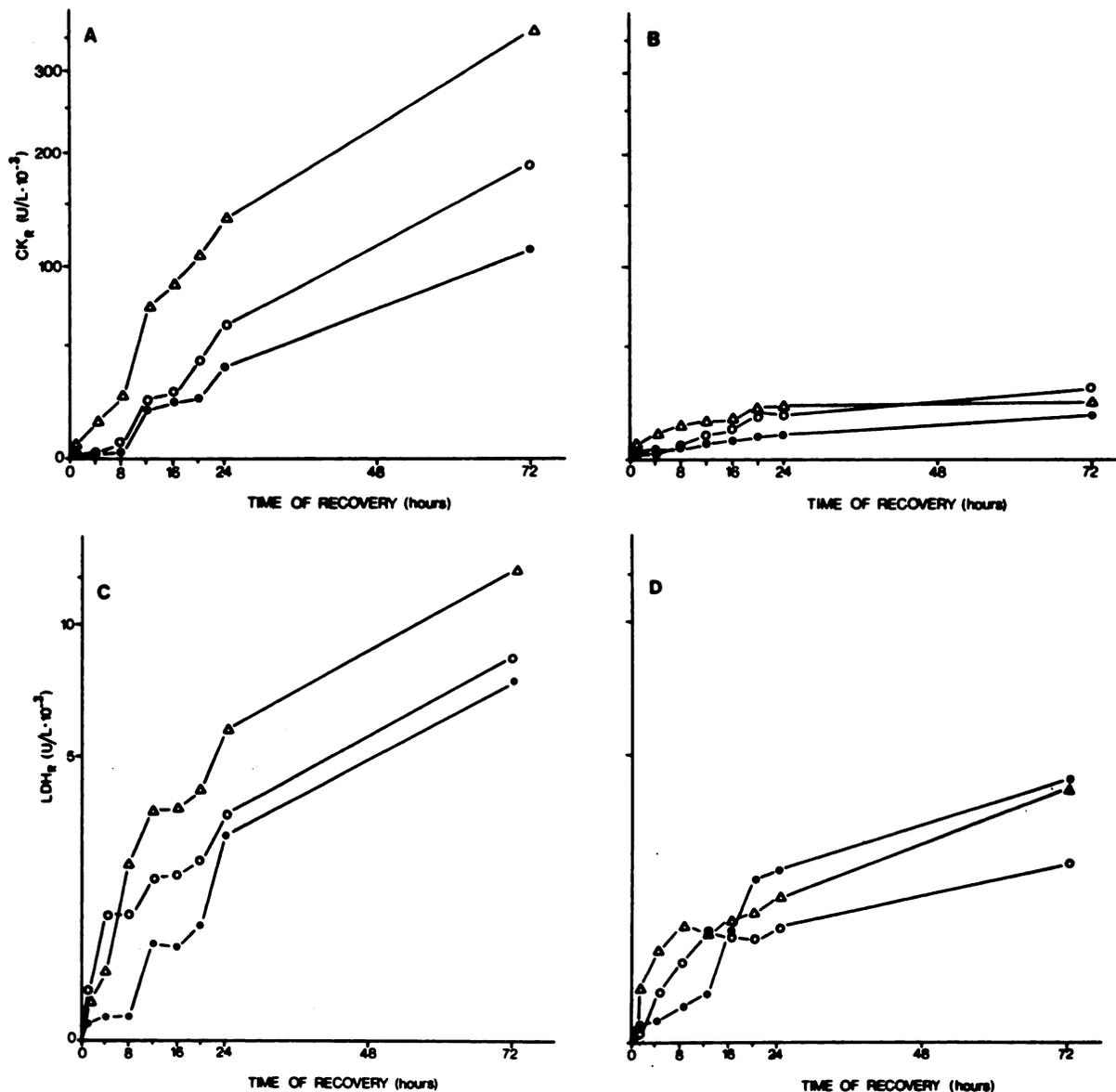


Fig. 2. Total CK and LDH activity released into the blood by the ischemic limb during the recovery from 2 (●—●), 4 (○—○), and 6 (Δ—Δ) h of normothermic (A, C) and hypothermic (B, D) ischemia

The curves were obtained with the monocompartmental model analysis described in *Materials and Methods* and applied to the data shown in Figure 1

of blood flow to the time of ischemia demonstrate the strict correlation between these two variables in the case of normothermic but not in the case of hypothermic ischemia.

Electrolytes

Re-perfusion of an experimentally ischemic tissue is followed by a decrease in intracellular K^+ and an increase of intracellular Ca^{2+} (15–17). Because these modifications are obviously accompanied by an opposite situation in the blood, we studied the influence of normothermic and hypothermic ischemia on potassemia and calcemia (Figures 3 and 4).

Potassium: Normothermic ischemia of 4 and 6 h (but not of 2 h) causes a rapid and significant increase in serum K^+ , which peaks at the eighth hour. The K^+ concentration then decreases, reaching the value of the control at the 12th h. At all intervals studied, hypothermic ischemia is followed instead by a significant decrease in serum K^+ , which persists at least until the 24th hour.

Calcium: Independently of the type of ischemia applied—normothermic or hypothermic—re-perfusion is ac-

companied by a decrease in serum Ca^{2+} that lasts until the third day.

Lactate

Lactate production in aerobiosis is a marker of an altered mitochondrial function. Thus determination of serum lactate activity may give valid information on the energy-producing capacity of a muscle that is recovering from ischemia.

Re-perfusion after normothermic ischemia entails a significant increase in the concentration of lactate in blood (Figure 5A), which persists until the 15th day.

In a first phase, which lasts about 20 h, hyperlactacidemia correlates well with duration of ischemia; subsequently the correlation is lost.

In hypothermic ischemia (Figure 5B), immediately after re-perfusion, serum lactate increases only in the case of ischemia; subsequently, serum lactate increases greatly, but there is no correlation between the duration of ischemia and the concentration of lactate in serum.

The concentration of lactate in serum at the seventh and

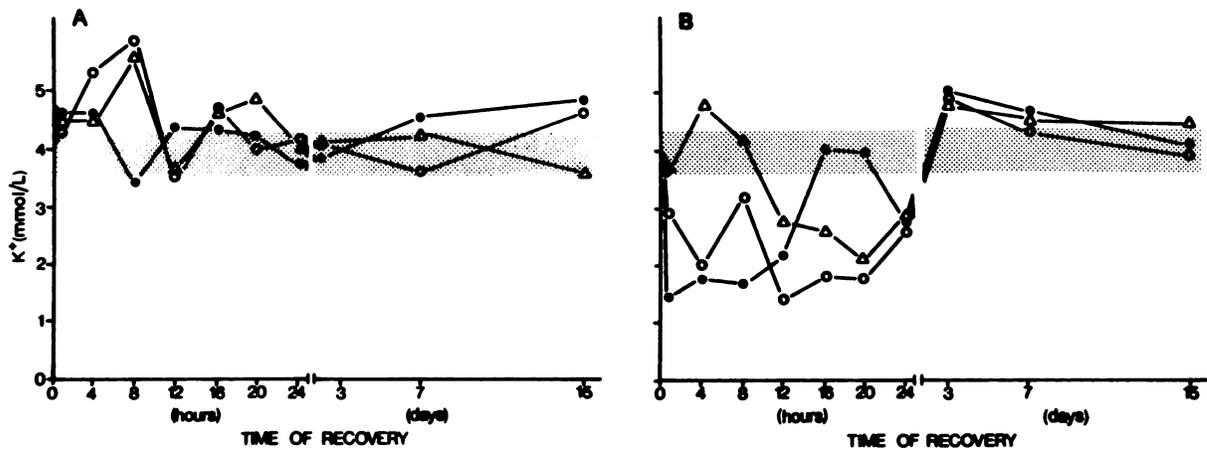


Fig. 3. Potassium in serum during the recovery from 2 (●—●), 4 (○—○), and 6 (△—△) h of normothermic (A) and hypothermic (B) ischemia

The points are the mean for four or five animals; the SEM (not represented) ranges between 20 and 25% of the mean. The shaded area represents the mean value of the control \pm SEM

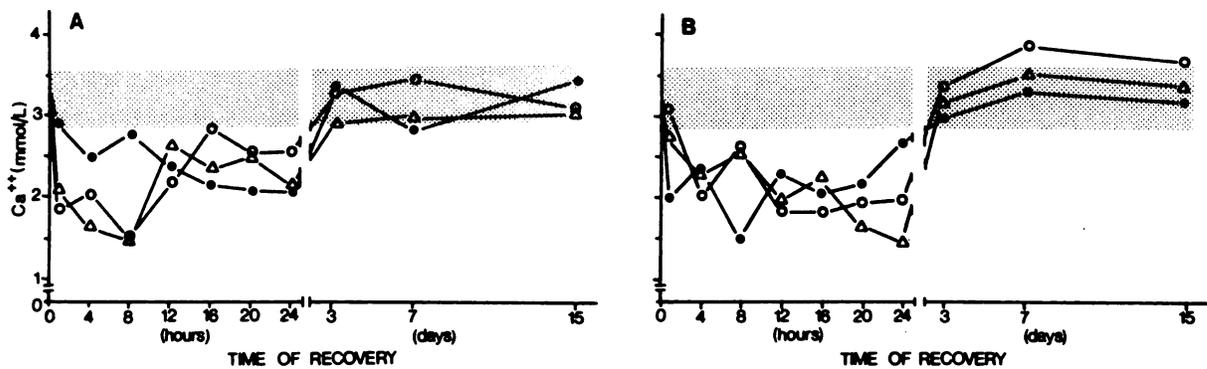


Fig. 4. Calcium in serum during the recovery from 2 (●—●), 4 (○—○), and 6 (△—△) h of normothermic (A) and hypothermic (B) ischemia

The points are the mean for four or five animals; the SEM (not represented) ranges between 20 and 25% of the mean. The shaded area represents the mean value of the control \pm SEM

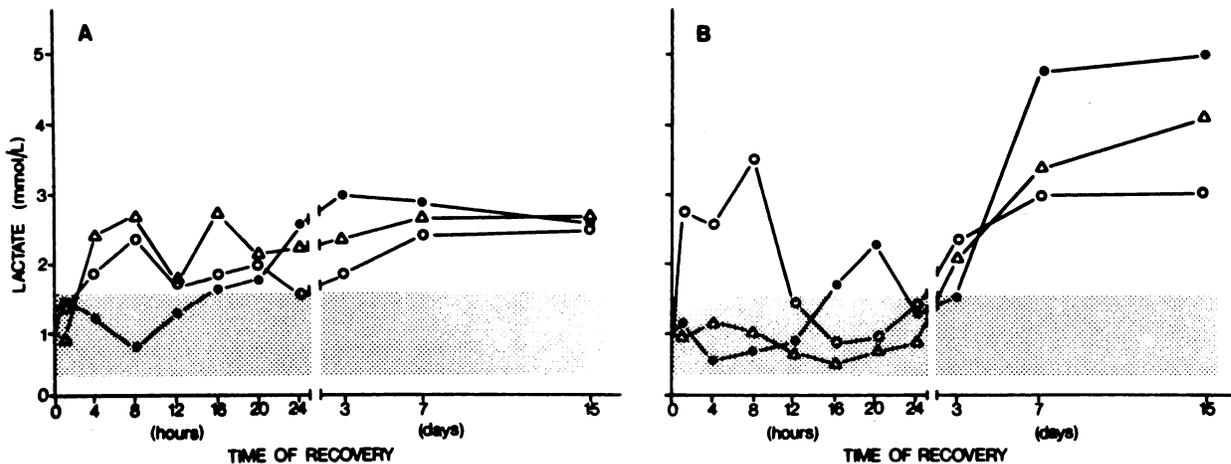


Fig. 5. Lactate in serum during recovery from 2 (●—●), 4 (○—○), and 6 (△—△) h of normothermic (A) and hypothermic (B) ischemia

The points are the mean for four or five animals; the SEM (not represented) ranges between 20 and 25% of the mean. The shaded area represents the mean value of the control \pm SEM

15th day after re-perfusion is much greater in the case of hypothermic than in the case of normothermic ischemia.

Discussion

Two points deserve discussion: (a) assessment of the ischemic damage to a striated muscle by measuring the total

enzyme activity released into the blood from the ischemic tissue and (b) the influence of hypothermia on the severity of the ischemic injury.

(a) Demonstrably, the total CK activity in the blood after acute myocardial infarction correlates well with the size of the infarct as evaluated by morphological techniques (5). We

therefore investigated the correlation between the ischemic damage suffered by striated muscle and the total circulating enzyme activity (CK and LDH) during the period of recovery after ischemia of various durations.

The significant correlation between the total CK and LDH activity in serum during the three days of recovery and the duration of ischemia applied (Table 1) demonstrates that measuring the total enzyme activity gives an index to the extensiveness of the ischemic damage.

Use of a shorter sampling interval was also considered. As shown in Figure 2, 24 h of recovery is the shortest period that could be used to detect a correlation between duration of ischemia and enzyme activity released into the blood; thus a 24-h period of sampling may adequately substitute for a 72-h one if prompt quantification of the damage is required. Nevertheless, longer followup of the release of the enzymes is useful for evaluating the course of damage.

Our data show also that in the first hours after re-perfusion, little CK or LDH is released into the blood. Because blood flow resumes immediately after re-perfusion, as shown by the rapid normalization of muscle temperature (15 to 30 min, A. Veicsteinas, personal communication), the appearance of more of the two enzymes in blood late after re-perfusion indicates that most of the cellular damage occurs during re-perfusion rather than during ischemia. This is agreement with the increase in enzyme depletion from an isolated heart reperfused at high pO_2 (18) and with the increase in Ca^{2+} deposition in the mitochondrial matrix after re-perfusion of the experimentally ischemic myocardium (17). Morphological data support this view (19, 20).

From Figure 2 (A and C) it is also evident that enzyme depletion, and hence cellular damage, persists at least until the third day of recovery, even though a decrease in the K_d , owing to the saturation of the mechanisms of enzyme removal, may contribute to this phenomenon.

(b) Ischemia entails an immediate anaerobiosis and a shortage of nutrients, with a consequent profound decrease in the energy available (21, 22). In this respect hypothermia, by decreasing cellular metabolism and hence the energy need, would be expected to lessen the cellular damage.

The smaller amount of CK and of LDH activity present in the blood after hypothermic ischemia (Figure 2) confirms this hypothesis. Moreover, the lack of correlation between the total amount of enzyme released after three days of recovery and the time of ischemia (Table 1) indicates that tissue damage is within reasonable limits, independent of the duration of hypothermic ischemia. Evidently, in hypothermic ischemia, independent of the duration of ischemia, the same number of cells are damaged, a number that seems also to coincide with the number of cells damaged during the first hours of re-perfusion after normothermic ischemia, when no definite correlation seems to exist between enzyme leakage and time of ischemia (Figure 2). This suggests that muscle cells are heterogeneous with respect of their susceptibility to ischemic damage. A first group of cells, probably the "red" fibres (23, 24), are always and immediately damaged, independent of the severity of the injury. With increasing severity of the injury, new groups of cells, probably the "white" fibres (23, 24), also undergo demonstrable damage.

We excluded the possibility that the decrease in the amount of CK and LDH activity released into the blood after hypothermic ischemia was due to intracellular inactivation. After incubating fragments of the soleum muscle for 6 h at 4 and 37 °C, we saw no differences in the specific activities of the two enzymes (result not shown). It seems therefore possible to conclude that hypothermia significantly decreases ischemic damage to the skeletal muscle. The results obtained for K^+ and lactate support this view. The increase in serum K^+ after normothermic ischemia, consequent to an alteration of the

Na^+/K^+ pump (16), is in fact completely prevented by hypothermia; instead hypopotassemia is observed, probably owing to an alteration in the K^+ reabsorbing capacity of the kidney. The sustained lactate concentrations after both normothermic and hypothermic ischemia, at the late stages of recovery, are most likely the consequence of an irreversible impairment of the mitochondrial function consequent to the precipitation of Ca^{2+} as insoluble hydroxyapatite in the mitochondrial matrix (17, 19). The trend of variations in serum Ca^{2+} (Figure 4) is in agreement with this view. However, the higher concentrations of blood lactate after hypothermic than after normothermic ischemia in the late stages of recovery indicate that hypothermia preserves the glycolytic capacity of the muscle cell, with more energy being then available. Thus hypothermia appears to be an useful therapeutic approach in revascularization and replantation surgery. Our results on enzyme release demonstrate that much or most of the damage occurs after restoration of blood flow. Therefore we suggest that hypothermia should be maintained also in the early phases after re-perfusion and that the temperature and the blood flow of the ischemized part be restored to normal very gradually.

We thank Dr. G. B. Gervasi, of Zambelletti S.p.A., Milan, for K^+ determination, and Mr. A. Minischetti and Miss F. Rizzieri for skilled technical assistance. Part of this work was presented at the "XVII Congresso Nazionale della Società Italiana di Chirurgia della Mano," September 1979, Gardone Riviera, Italy.

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