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The Mechanism of the Tetrazolium Reaction in Identifying Experimental Myocardial Infarction

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Summary. Tetrazolium salts (NBT) stain normal myocardium whereas infarcts are not stained. We tried to elucidate the staining mechanism which discriminates normal from infarcted canine myocardium. The left anterior descending coronary artery (LAD) was occluded in dogs for between 4 and 32 h. The activities of four different tissue dehydrogenases were measured after 4, 8, 16, and 32 h of ischaemia. Nicotinamide adenine dinucleotides (NAD, NADH, NADPH) were determined in needle biopsies taken from the ischaemic region $^1/_2$, 1, $1^1/_2$, 2 and 4 h after occlusion of the LAD. In another set of experiments the NBT stain was altered by the addition of NADH, NAD, NADPH, NADP, succinate, lactate and phenazine methosulfate respectively and the effect of the added substances on the previously nonstained infarcts was examined. We further compared histochemically determined infarct size to the ultrastructural extent of infarcts. Activities of the tissue dehydrogenases did not change after 4 h of ischaemia, although the NBT stain revealed a large infarction. At that time total NAD, the sum of NAD+NADH, had decreased from about 600 pmoles/mg tissue to about 200 pmoles/mg tissue and addition of the coenzymes or succinate could "repair" the biochemical lesion. After 24 h of ischaemia the activities of dehydrogenases and diaphorases were markedly decreased. Our data indicate that loss of the reduced coenzymes plays a key role in identifying myocardial infarction with tetrazolium salts. In older infarctions loss of coenzymes is joined by decreased activities of dehydrogenases and diaphorases. The principal mechanisms of staining is an enzymatic cycling.

Key words: Myocardial infarction – Tetrazolium salts – NAD – Oxidoreductases

Introduction

A comprehensive study on the usefulness of the redox indicator triphenyltetrazoliumchloride (TTC) in histology and histophysiology was reported by Doerr

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in 1950. Sandritter and Jestädt (1958) used this tetrazolium salt in identifying early myocardial infarction, i.e., after 4 h of coronary artery occlusion. Normal myocardium reduced TTC to a red formazan stain, whereas the infarcted tissue remained unstained. A better contrast between normal and necrotic myocardium was obtained by Nachlas and Shnitka (1963), who stained heart slices with nitroblue tetrazolium (NBT). The reduced form, a deep blue formazan, is bound firmly to the cut surfaces of normal heart slices causing a sharp border with the nonstained infarcted region. The NBT stain is not only important for the pathological diagnosis of myocardial infarction but also for measuring infarct size. Schaper et al. (1979) demonstrated that infarct size measured histochemically correlated very well with the size determined by histological examination. As these previously cited studies proved tetrazolium stains to be good qualitative and quantitative indicators of myocardial infarcts, we tried to elucidate the biochemical difference discriminating normal from infarcted myocardium. Sandritter and Jestädt (1958) believed that decrease of dehydrogenase activities plays the key role, whereas Nachlas and Shnitka (1963) held that the loss of coenzymes and substrates in the early stage and the added loss of respiratory enzymes in the later stage are together responsible for the difference in staining.

Materials and Methods

Apart from the bioluminescent test kits all enzymes and coenzymes were obtained from Boehringer Mannheim, W. Germany. The bioluminescent assays for NADH and NADPH measurements and the Lumacounter M 2080 were products of Lumac, Basel, Switzerland. Phenazine methosulfate (PMS) and nitrobluetetrazolium (NBT) were purchased from Serva, Heidelberg, W. Germany, thiazolylblue (MTT) from Merck, Darmstadt, W. Germany. All other reagents used were of analytical grade and are commercially available.

Experimental Design. Thirty-two mongrel dogs of either sex and with an average body weight of 28 kg were anesthetized with 2.5 mg/kg subcutaneous piritramide and with 5–10 mg/kg intravenous sodium pentobarbital. Anesthesia was maintained with 50:50 N_2O/O_2. The open chest model was used for infarctions up to 8 h, longer lasting infarctions were induced with a transfemoral technique in closed chest animals (Gottwik et al. 1979; Puschmann et al. 1980).

Four different sets of experiments were performed. In the first set of experiments the left anterior descending coronary artery (LAD) was occluded in 12 anaesthetized dogs for 4 h, 8 h, 16 h, and 32 h respectively. Collateral blood flow was determined with radioactive tracer microspheres (Flameng et al. 1977) and left ventricular oxygen consumption was calculated in the shorter lasting experiments (Bretschneider 1972). After sacrifying the animals hearts were cut into slices, parallel to the heart basis and stained with NBT solution (0.25 g/l 0.1 m phosphate buffer pH 7.1). Then tissue activities of four different dehydrogenases, lactate dehydrogenase, LDH (E.C. 1.1.1.27), malate dehydrogenase MDH (E.C. 1.1.1.37), NADP dependent isocitrate dehydrogenase ICDH (E.C. 1.1.1.42), and L-3-hydroxyacyl CoA dehydrogenase HADH (E.C. 1.1.1.35) were measured in normal and infarcted myocardium. HADH activity was determined as porposed by Brdiczka et al. (1968). The other enzyme activities were tested with kits obtained from Boehringer Mannheim (for more detailed information see Klein et al. 1981 a).

In the second set of experiments we tried to influence the NBT stain by adding different reagents to the incubation medium. Infarctions of 6 h and 24 h were produced. Heart slices were first stained with ordinary NBT solution, until the infarcted region became clearly visible (15–20 min). We then added one of the following reagents to the incubation medium of different slices, NADH, NAD, NADPH, NADP (all 0.5 mg/ml), PMS (3 mg/ml), sodium succinate (100 mg/ml), and sodium lactate (100 mg/ml). The influence of autolysis on the NBT stain was tested in one experiment of a 24 h infarction. The sacrificed animal was kept at room temperature for another 20 h, before the heart was removed and stained.

In the third set of experiments we determined the myocardial tissue concentrations of NAD and NADH in six dogs. The concentrations of NADPH were measured in eight dogs. Transmural needle biopsies were taken from the permanently ischaemic myocardium of these dogs $^1/_2$ h, 1 h, $1^1/_2$ h, 2 h, and 4 h after occlusion of the LAD. Biopsies from well perfused myocardium at the beginning and at the end of the experiment served as controls. The obtained tissue was immediately blotted, divided into subepicardial and subendocardial halves and deep frozen in liquid nitrogen.

Measurement of Coenzymes. An extensive description is given in Klein et al. (1981 b) NAD and NADH were extracted as proposed by Klingenberg (1974). Measurements were performed in a photometer at a wave length of 578 nm according to Bernofski and Swan (1973).

NADPH was extracted with ice cold 0.02 n NaOH containing 0.5 mm Cysteine (Burch et al. 1967). After heating at 60° C for 10 min an aliquot of the supernatant was added to 300 µl 0.1 m phsophate buffer pH 6.9 and the reduced coenzyme concentrations were determined with a luminescent reagent. In this assay NADPH is oxidized by a specific oxidoreductase system, which reduces a flavinmononucleotide (FMN). In the second step reduced FMN is oxidized by a luciferase enzyme, which produces light proportional to the coenzyme concentration. This emitted light was integrated in the lumacounter over 30 s after starting the reaction. Finally NADPH tissue concentrations were determined using three NADPH standards, which were prepared as suggested by Passonneau and Lowry (1974).

Comparison of Infarct Size Determined Histochemically and Ultrastructurally: The last described experiments were performed to examine the histochemical infarct size with electron microscopy. Two infarctions of 6 h and another two of 6 h duration followed by 90 min of reflow were produced. Before removing the hearts six transmural needle biopsies were taken from the lateral borders and the centre of the infarcts. They were further subdivided into subepicardial, intramural, and subendocardial specimens. These samples were then checked for the occurrence of irreversible cell damage by electron microscopy (Jennings et al. 1965). The tissue adjacent to the biopsy tracks was stained with NBT and the transmural extent of the infarcted area was determined and compared with the ultrastructural results.

Results

Activities of Dehydrogenases in Infarcted Myocardium

In one dog the LAD was occluded for 4 h at a low oxygen consumption of 6 ml O_2/min · 100 g. The NBT stain did not reveal an infarction nor did the measured enzyme activities decrease in this experiment. In the other four hour experiments the LAD was ligated at the high oxygen consumption of 12–13 ml O_2/min · 100 g. Although the NBT stain now identified a large infarction (Fig. 1), enzyme activities had not changed (Fig. 2). Decrease of the activities of the dehydrogenases started in the infarcted subendocardium between the 4th and 8th hour of occlusion. After 12 h of ischaemia they were reduced to about 50% and reached their lowest values after 16–32 h (Fig. 3).

Modification of the NBT Stain by Addition of Different Substrates

After a transient ischaemia of 6 h in two dogs the NBT stain identified large anterior wall infarcts. The addition of NAD, NADH, NADP, NADPH, and succinate respectively rendered the previously nonstained infarcted area stained, so that infarctions became indistinguishable from well perfused myocardium. We observed no change in staining behaviour when lactate alone was added to the incubation medium. PMS accelerated the speed of staining without altering

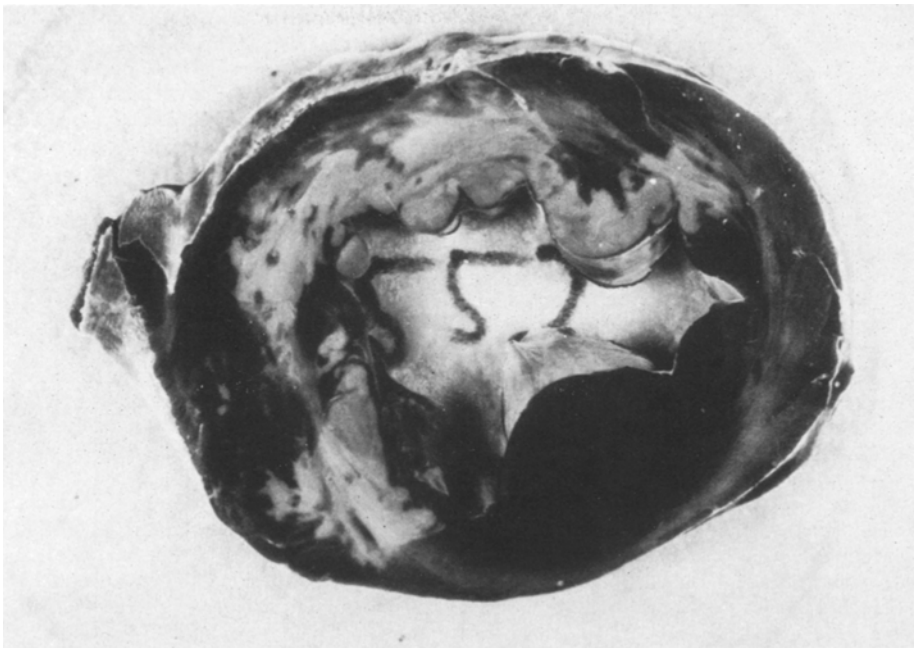

Fig. 1. NBT stain of a heart after 4 h ischaemia at high oxygen consumption. Infarcted tissue is unstained

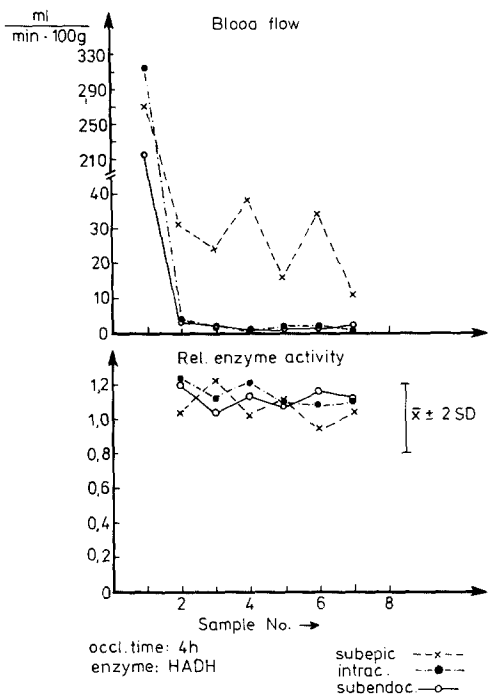

Fig. 2. HADH activity after 4 h ischaemia. Enzyme activity and regional myocardial blood flow were measured in the slice of Fig. 1. On the lower panel enzyme activities of subepicardial, intramural, and subendocardial samples are expressed as percentages of the control enzyme activity, which was obtained from nine samples of the well perfused posterior wall. $\bar{x} \pm 2$ S.D. was also calculated from these samples. On the upper panel the corresponding myocardial blood flow to each enzyme activity is plotted. The first samples belong to normal perfused myocardium, the following ones are taken from the ischaemic-infarcted tissue

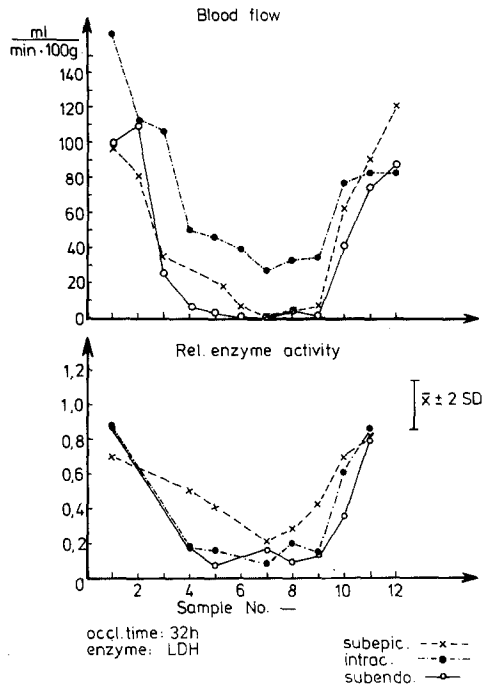

Fig. 3. LDH activity after 32 h ischaemia. For an explanation of the illustration see Fig. 2. After this long occlusion time LDH activity decreased markedly in the infarcted tissue

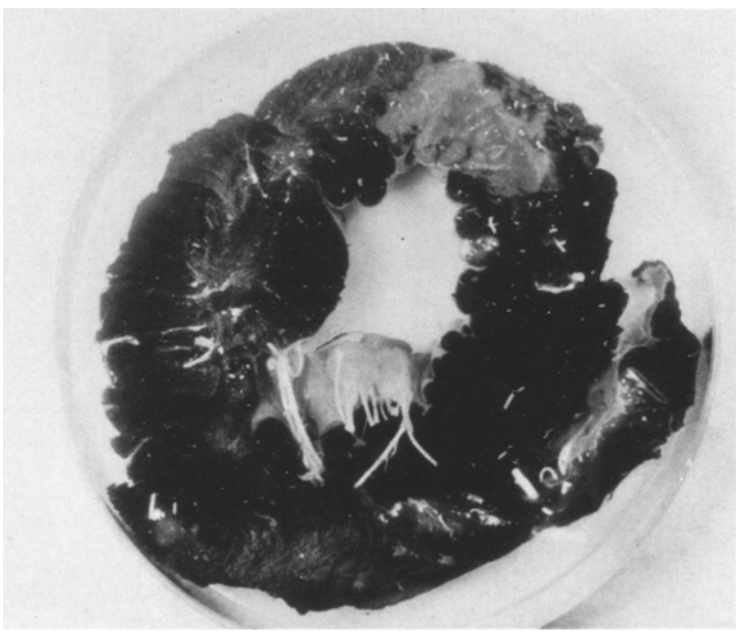

Fig. 4. NBT stain after 24 h occlusion. The white spots are due to a post mortem angiography

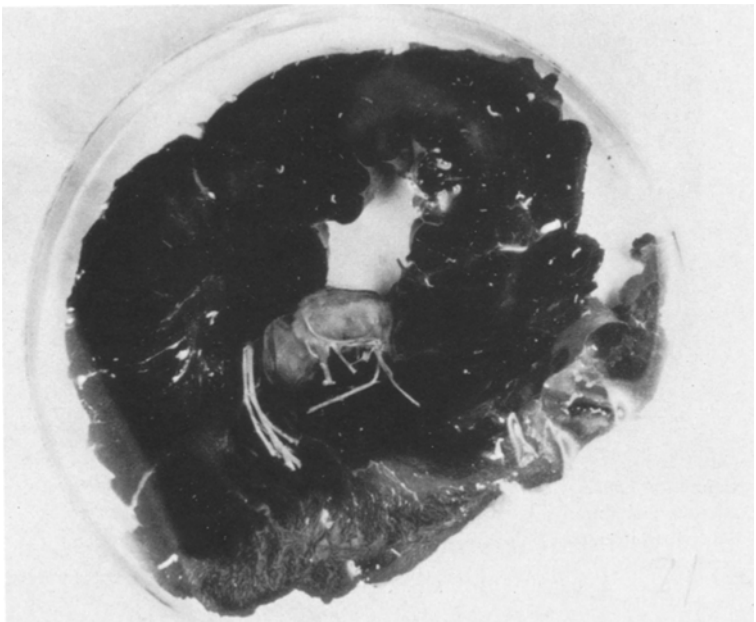

Fig. 5. NADH was added to the slice of Fig. 4. Previously non stained tissue became almost stained

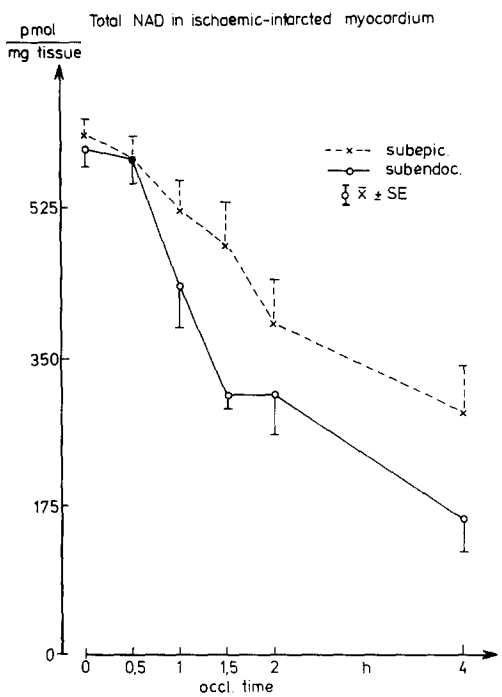

Fig. 6. Kinetics of total NAD, the sum of NAD and NADH, in the ischaemic-infarcted myocardium of six dogs. × represents subepicardial samples ($\bar{x}$+S.E.), ○ subendocardial samples ($\bar{x}$−S.E.)

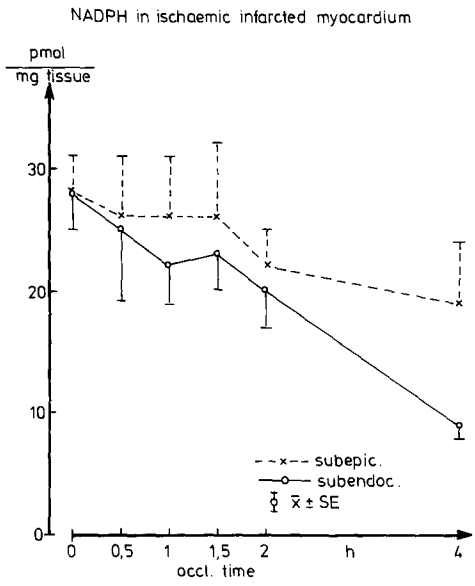

Fig. 7. Kinetics of NADPH in the ischaemic-infarcted myocardium of six dogs

the infarct size. After 24 h of ischaemia the situation became more complex. The addition of NAD and NADP "repaired" only a small portion of the infarcts. NADH, NADPH, and succinate led to an almost complete staining of the infarctions (Figs. 4, 5) which became entirely stained when PMS was added.

The effect of 20 h of autolysis was tested in one dog. Heart slices of this dog were only faintly stained by NBT and the septal infarction was not clearly identifiable. The addition of NAD, however, rendered the previously well perfused myocardium stained, whereas the infarcted region remained unstained.

Kinetics of Coenzymes in the Infarcted Myocardium

Total NAD, the sum of NAD and NADH, remained unaltered within the first half hour of ischaemia (Fig. 6). A significant decrease started in the infarcted subendocardium 0.5–1 h after occlusion of the LAD. After 2 h NADPH kinetics resembled those of total NAD (Fig. 7). Loss of NADPH, however, occurred somewhat later. In contrast NADH increased at first, remained elevated for 1 h and began to decline thereafter (Fig. 8).

Histochemical Infarct Size Compared to Ultrastructurally Determined Size

In these experiments the transmural propagation of infarcts with and without reflow was evaluated by histochemistry and electron microscopy. After 6 h occlusion followed by 90 min reflow the transmural extent of these infarcts, assessed by these two techniques, yielded almost identical results. After 6 h of ischaemia without reflow ultrastructural signs of irreversibility sometimes occurred in the infarcted subepicardium, these were not identified by the NBT stain.

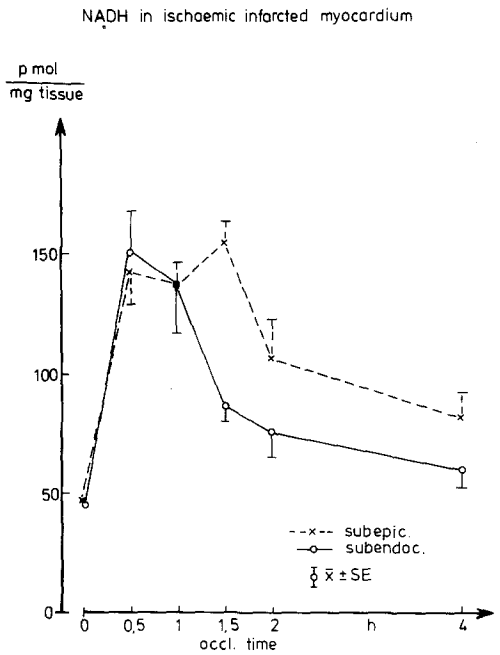

Fig. 8. Kinetics of NADH in the ischaemic-infarcted myocardium of six dogs

Discussion

Theoretical Background of Tetrazolium Stains

Tetrazolium salts are chemically characterized by a ring structure, which contains one carbon and four nitrogen atoms, one of which is quarternary (Nineham 1955). Mono-tetrazoles (like MTT) contain one heterocyclic ring, whereas double- or di-tetrazoles have two characteristic rings. Mono-tetrazoles are converted to formazans by the uptake of two electrons and one proton. This reduction is combined with opening of the ring, which leads to the colored formazan (Altman 1972). Which intracellular systems are able to reduce tetrazolium salts? First it has to be stated that in general tetrazolium salts are reduced by redoxsystems having a lower redoxpotential than the dye itself. In this regard the most important biological systems are NAD/NADH, NADP/NADPH, flavoproteins, cytochrome$_b$, cytochrome$_{b5}$, and cytochrome$_{P450}$ (Altman 1972). Without the intervention of an enzyme system which can react with oxygen or an electron carrier (PMS) neither NADH nor NADPH will react with tetrazoles. In a test tube, which contains NADH (NADPH), suitable substrates and a corresponding dehydrogenase, MTT does not become reduced. The reason must be that dehydrogenases have binding sites for the substrate and for the coenzyme. As tetrazoles are not bound at the substrate site, and as no free electrons are available to be picked up, the dye does not become reduced. When however NADH is oxidized by an oxidase, free electrons can be picked up by the tetrazole, which becomes reduced. Such enzymes are called NADH or NADPH

diaphorases. Enzymes of the respiratory chain and the cytochrome$_{P450}$ pathway belong, among other lesser defined hydrogen pathways, to this enzyme system.

Activities of Oxidoreductases in Infarcted Myocardium

The experiment of a 4 h occlusion at high oxygen consumption clearly indicated that the tested dehydrogenases were not responsible for the different staining behaviour of normal and infarcted myocardium. Enzyme activities did not decrease (Fig. 2), although histochemistry demonstrated a large infarction (Fig. 1). It was also shown that at that stage of infarction the addition of reduced or oxidized coenzymes or succinate stained the previously nonstained myocardium. We conclude from these observations that dehydrogenases were still working in infarcts, as they reduced NAD(NADP) to NADH(NADPH) and that the diaphorases were still active, as they led to formazan production. After 24 h of ischaemia the situation is different. Direct measurements of dehydrogenase activities showed a 75% loss of activity. Accordingly the addition of NAD or NAD plus lactate to the incubation medicum only partly "repaired" the biochemical lesion. A better staining of infarctions was achieved with NADH (Fig. 5) and succinate. Total staining, however, could only be reached when PMS was also present. PMS is an artificial electron carrier, which can by-pass the diaphorases. The addition of lactate never altered the staining behaviour. We gather that at that stage of infarction loss of coenzymes is joined by markedly decreased activity of dehydrogenases and diaphorases.

Loss of Coenzymes in Infarctions

Nachlas and Shnitka (1963), and Ramkisson (1966) explained the basic mechanism of the NBT stain by alterations in dehydrogenase activity, as clearly indicated by the almost identical titles of their studies. Both of them, however, considered that loss of coenzymes and substrates may also be important in early infarcts. Measurements of tissue dehydrogenase activities or of tissue coenzyme concentrations were not performed in normal or in infarcted myocardium identified by the tetrazolium stain. This was done in our study, at different stages of ischaemia and infarction. In general infarcts can be diagnosed by the NBT stain 4–6 h after occlusion of a coronary artery. At that time total NAD has decreased from about 600 pmoles/mg frozen tissue (f.t.) to 160 pmoles/mg f.t. in the infarcted subendocardium and to 300 pmoles/mg f.t. in the infarcted subepicardium. At the same time the sum of NADH and NADPH is about three to four times lower in the infarcted subendocardium and two to three times lower in the infarcted subepicardium than in "normal" myocardium. Myocardium remains unstained, if total NAD drops to about 250 pmoles/mg fresh weight and NADH plus NADPH to 80 pmoles/mg fresh weight. Subepicardial values are higher than subendocardial ones, reflecting that more myocytes of the ischaemic subepicardium remain viable (Jennings and Reimer 1979) and do not lose coenzymes. In well perfused myocardium NADH concentration is equal to or lower than NADPH concentration. When this "normal" myocardium is stained in NBT solution, it is no longer "normal" but ischaemic.

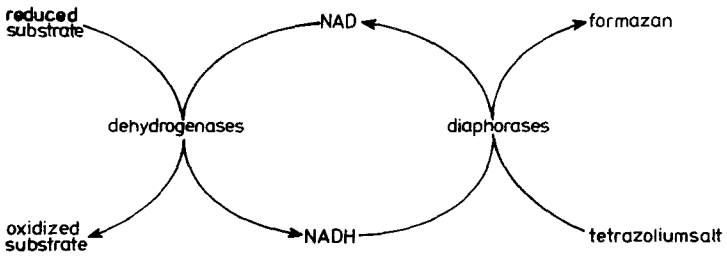

Fig. 9. An enzymatic cycle as the basis of the NBT stain for identifying myocardial infarction

Under this condition NADH rises and thus becomes more important for the staining mechanism than NADPH, which remains almost unaltered. The speed at which ischaemic myocardium becomes irreversibly infarcted and thus loses coenzymes, depends on the left ventricular oxygen consumption and collateral blood flow at the time of and shortly after the occlusion (Müller et al. 1980). Irreversible cell injury occurs rapidly when a coronary artery is occluded at a high oxygen consumption and low collateral blood flow. This phenomenon is reflected by the fact that the NBT stain sometimes indicates infarctions after only 3 h and sometimes 6–8 h after experimental coronary occlusion, provided no reflow was allowed.

Change of the Composition of NBT Stain Following Autolysis

Pathologists sometimes face the problem of how to exclude myocardial infarction in hearts with long lasting autolysis. To study this problem we produced a 24 h infarction in one dog, killed the animal and kept it for another 20 h at room temperature, before the heart was stained with NBT solution. In this case the slices were only stained very faintly. If NAD was added, however, "normal" myocardium became regularly stained and the infarction became clearly visible. If NAD had not have caused a staining of the noninfarcted myocardium, we would have added NADH or succinate. The danger that infarctions become stained with these two compounds is somewhat higher than with NAD, which first has to be converted to NADH, before it is able to reduce NBT by means of tissue diaphorase.

Biochemical Mechanism of NBT Stain

We believe that the basis of tetrazolium stains for infarctions is an enzymatic cycling. Our concept is presented in Fig. 9. Localization of the involved reactions is somewhat difficult. The final reaction of the cycle, oxidation of NADH and production of formazan by means of diaphorases, takes place on the cut surfaces of the heart slices. These oxidases are mainly membrane-bound and do not diffuse into the incubation medium. If PMS and NADH is added to the NBT solution, formazan is also produced in the incubation medium (Wenk 1970), as the diaphorases are by-passed. The other "participants" of the cycle however can diffuse (Altman 1972), so that the reduction of NAD to NADH can happen on the cut surface of a slice as well as in the incubation medium.

Due to the high dilution in the incubation medium the latter process appears to be negligible, provided that exogenous coenzymes are not added.

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