Macroscopic enzyme histochemistry in myocardial infarction: use of coenzyme, cyanide, and phenazine methosulphate

NW DERIAS, CWM ADAMS

From the Department of Pathology, Guy's Hospital Medical School, St Thomas's Street, London SE1 9RT

SUMMARY Transversely sectioned human heart slices, obtained at necropsy from normal subjects and from cases of recent myocardial infarction, were stained with the nitroblue tetrazolium (NBT) dehydrogenase macroreaction for the gross identification of recent myocardial infarction. The addition of nicotinamide adenine dinucleotide (NAD) to the incubating medium greatly improved the sensitivity of the method, while addition of cyanide caused just a modest improvement. Addition of the electron transfer mediator phenazine methosulphate (PMS) resulted in false non-selective staining and obscured areas of recent myocardial damage.

The NBT-dehydrogenase macroreaction is used in many centres for the macroscopic identification of early myocardial infarction at necropsy. The test depends on a negative reaction with NBT caused by loss of dehydrogenase activity. We recently modified it by adding the coenzyme nicotinamide adenine dinucleotide (NAD) and cyanide to the incubating medium and it was found that dehydrogenase activity was maintained longer post-mortem and early infarcts were easier to detect.

Phenazine methosulphate (PMS) is frequently used in microscopic dehydrogenase histochemistry to increase the reaction velocity of the tetrazolium reaction by acting as an intermediary electron acceptor, obviating the need for the relevant endogenous diaphorase (tetrazolium reductase). Phenazine methosulphate accepts an electron directly from the reduced coenzyme (or flavoprotein) and donates it to the tetrazolium salt without the need for NADH-tetrazolium reductase, which is an integral part of the electron transfer chain and can limit the rate of transfer of electrons to the tetrazolium salt.

The purpose of this paper is to compare the relative efficacy of coenzyme (NAD), cyanide and PMS in increasing the reliability of the NBT macroreaction for the post-mortem diagnosis of myocardial infarction.

Material and methods

Transversely-sectioned heart slices of approximately 1 cm thickness were taken at necropsy from subjects who had died from recent myocardial infarction or from primary causes unconnected with the heart. A total of 145 hearts were examined, comprising 108 cases of myocardial infarction, 14 road traffic accidents, 10 malignant disease, 8 bronchopneumonia, 3 chronic renal failure and 2 homicides. Death followed within 12 hours of the onset of clinical symptoms, while the death-necropsy interval varied from 2-72 hours.

Heart slices were rinsed in cold running water to remove traces of blood, and were then incubated in the NBT incubating medium described for the dehydrogenase macroreaction with and without added substrate. A sufficient volume of incubating medium was used to cover completely the heart slice to a depth of 2 cm and, thus, to prevent atmospheric oxygen from competing for the liberated electrons. The stock NBT incubating solution was NBT (0.5 mg/ml; 100 ml) and 0.2 M Tris-HCl buffer (pH 7.4; 100 ml). The pH of the solution was adjusted to 7.1 with 0.2 M Tris without HCl, and was stored at 4°C. Exogenous (added) substrates were sodium β-hydroxybutyrate (12.7% wt/vol), sodium lactate (12.5% vol/vol) or sodium succinate (67.5% wt/vol). These were added to the stock solution in the ratio 5:12 (vol/vol). The term endogenous substrate implies that the reaction depended on tissue substrate—for example, a mixture of the respiratory carboxylic acids. NAD and sodium cyanide were added respectively at concentrations of 100 and 114 mg per 100 ml of incubating medium. PMS was added at a concentration of 10 mg per 100 ml.
Incubation was carried out at 37°C for 20 min; in the last case incubation was in absolute darkness as PMS is light-sensitive. The heart slices in their containers were gently agitated with a mechanical shaker to prevent stagnation of the reagent and corresponding staining artefacts.

Results

Normal myocardium stained dark blue with our standard NBT method, with or without added substrate, but no reaction was obtained unless coenzyme or cyanide or, better, both were added. The Table and Fig. 1 show the relative effects of adding NAD, CN or specific substrate to the stock NBT incubating solution in various permutations. Although CN improved the reaction on its own, it only caused a modest improvement to the increased reaction obtained after adding NAD alone. The recognition of myocardial infarction was easier and more reliable with the complete incubating medium—that is, with added NAD and CN (Fig. 2). The special case of succinoxidase must be mentioned (Table, Fig. 1). This system does not require NAD or NADP and, in this instance, the addition of CN alone allowed a strong NBT reaction to be obtained.

When PMS was added to the NBT incubating media, with either endogenous (tissue) or exogenous (added) substrate, false-positive macrostaining was seen in the heart slices. Such false staining was detected by comparison with the adjoining (mirror-image) heart slice incubated in a similar incubating media without PMS. Recent myocardial infarction

<table>
<thead>
<tr>
<th>Serial number</th>
<th>NBT incubating medium</th>
<th>Colour of reaction product</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SNIS</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>SNIS + NAD</td>
<td>+ + +</td>
</tr>
<tr>
<td>3</td>
<td>SNIS + cyanide</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>SNIS + cyanide + NAD</td>
<td>+ + +</td>
</tr>
<tr>
<td>5</td>
<td>SNIS + lactate</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>SNIS + lactate + cyanide</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>SNIS + lactate + NAD</td>
<td>+ + +</td>
</tr>
<tr>
<td>8</td>
<td>SNIS + lactate + cyanide + NAD</td>
<td>+ + +</td>
</tr>
<tr>
<td>9</td>
<td>SNIS + succinate</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>SNIS + succinate + cyanide†</td>
<td>+ + +</td>
</tr>
</tbody>
</table>

*Serial number refers to the numbered samples in Fig. 1. † Succinoxidase does not require NAD.

Fig. 1 Staining of heart slices with various NBT incubating media. (The numbers refer to the serial numbers of media in the Table).
was clearly seen with the standard method but was somewhat obscured with PMS (Fig. 3).

Discussion

This paper is concerned with the addition of co-enzyme (NAD), cyanide (CN) and phenazine methosulphate (PMS) to a nitroblue tetrazolium (NBT) incubating medium for the detection of myocardial infarction at necropsy.

The results clearly show that the addition of NAD greatly increases the stainability of normal heart and, thus, allows greater contrast in comparison with areas of enzyme loss in myocardial infarction. In a previous study, we found that cyanide had an additional beneficial effect, but only a modest additional effect was apparent here in the tests summarised in Fig. 1 and the Table. However, in the instance of succinoxidase requiring no coenzyme, cyanide greatly increased the reaction intensity of normal myocardium. Cyanide, as stated above, inhibits cytochrome oxidase in the late stages of dehydrogenation or tissue oxidation. Electrons are, thus, diverted from oxygen towards the tetrazolium salt.

The use of phenazine methosulphate to circumvent
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coenzyme-tetrazolium reductase systems in the tissue led to marked non-specific staining and is of no practical value. Addition of PMS seemed to result in the accelerated transfer of electrons in solution thus allowing soluble dehydrogenases (see above) to reduce NBT in the incubating medium with resulting false positive staining of the heart slice by the deposited formazan pigment. This view is supported by the observation that the colour of the NBT-PMS incubating medium turned dark blue during incubation. However, when the dehydrogenase macroreaction was allowed to take place first and the PMS was added in a second stage, no colour change was observed in the NBT incubating medium. In the latter case, tissue tetrazolium reductases had completed the dehydrogenation reaction and no electrons were available on subsequent addition of PMS.

The beneficial effect of adding NAD to the incubating medium implies that NAD may be normally lost during post-mortem changes in human heart muscle. By contrast, the specific dehydrogenases and coenzyme (NAD)-tetrazolium reductase seem to survive post-mortem in normal heart muscle in adequate amounts for at least 7 days, provided that the corpse is stored at 4°C.4 From the results with PMS, it is apparent that most dehydrogenases are leached into the medium during incubation of normal myocardium, in that the reaction takes place in the incubating medium rather than the tissue. The enzyme or group of enzymes that is leached out in vivo from the infarcted heart yet is retained post-mortem in normal heart is, therefore, unlikely to be a specific dehydrogenase. It is much more likely to be the tissue coenzyme-tetrazolium reductase and, in the examples cited, this would be NAD-tetrazolium reductase. This enzyme is known to be sited in mitochondria and, together with succinoxidase, is organelle-bound; it is not readily leached out as are the cell-sap enzymes. Thus, the NBT macroreaction (with added NAD) may well depend on the loss of NAD-tetrazolium reductase from damaged intracellular organelle sites in infarcted myocardium.

References


Requests for reprints to: Professor CWM Adams, Department of Pathology, Guy's Hospital Medical School, St Thomas's Street, London SE1 9RT, England.
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N W Derias and C W Adams

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