A Quantitative Cytochemical Method for the Measurement of $\beta$-Hydroxyacyl CoA Dehydrogenase Activity in Rat Heart Muscle

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Summary. Although cytochemical methods exist for measuring dehydrogenases that act on substrates involved in the production of chemical energy from sugars, virtually no methods exist for measuring the dehydrogenases that act on fatty acids. Yet the oxidation of fatty acids accounts for over 60% of the oxidative activity of cardiac muscle. Consequently a new quantitative cytochemical method, based on a new substrate (DL-S-$\beta$-hydroxybutyryl-N-acetyl cysteamine), has been developed for measuring the activity of hydroxy-acyl coenzyme A dehydrogenase, which is the penultimate step of the $\beta$-oxidation of fatty acids to acetyl-coenzyme A that is used in the Krebs' cycle. Menadione or phenazine methosulphate is used as the intermediate hydrogen-acceptor, with neotetrazolium chloride as the final acceptor. The medium contains nitroprusside, ostensibly to react with any cysteamine liberated by hydrolysis of the substrate. As a control, cysteamine is substituted for the substrate. The concentrations of reactants have been optimized for cardiac muscle; the reaction is linear with thickness of the sections and with time of reaction from 15 to 60 min.

Introduction

Many cytochemical methods, some of them quantitative, have been developed for oxidative metabolism based on glucose as the primary energy-source. However, many types of cell may derive half their energy from the oxidation of fatty acids. In well oxygenated cardiac muscle, fatty acids have been shown to be the preferred substrate (Neely et al. 1972; Neely and Morgan 1974). Generally their oxidation accounts for 60%–70% of the oxidative activity of cardiac muscle, but this can be markedly increased in certain conditions (Neely et al. 1972). However, fatty acid oxidation becomes suppressed, with a concomitant stimulation of glycolysis, in hypoxic or anoxic hearts (Neely and Morgan 1974). Thus, it is of some importance to be able to measure fatty acid oxidation in cardiac muscle, especially in the biopsies that are currently being taken...
for the cytochemical monitoring of myocardial function during prolonged open-heart surgery. (e.g. Čanković-Darracott et al. 1978).

A key enzyme-system in the oxidation of fatty acids is the oxidation by $\beta$-hydroxyacyl-coenzyme A dehydrogenase (Mahler 1971). In conventional biochemistry, this enzyme activity is measured by the reversal of the reaction, namely by the reduction (by NADH) of aceto-acetyl-coenzyme A. Because this substrate is very expensive, cysteamine is often used instead of coenzyme A: the aceto-acetyl-cysteamine is about 5 times less efficient as a substrate but this can be compensated for by increasing the concentration of the substrate accordingly. However, this reaction, which involves the oxidation of NADH, cannot be linked to a cytochemical chromogenic reaction.

To overcome this problem, the more normal substrate, namely the hydroxybutyryl derivative, has been synthesized, with cysteamine in place of coenzyme A. This $\beta$-hydroxybutyryl cysteamine substrate is liquid, and about 90–95% pure. Hydroxyacyl dehydrogenase should mediate the reaction:

$$\text{hydroxybutyryl cysteamine} + \text{NAD}^+ \rightarrow \text{acetoacetylcysteamine} + \text{NADH}.$$

The reducing equivalents from NADH formed in this reaction can be transferred, directly or indirectly, to a tetrazolium salt.

This communication describes the use of this substrate, the problems involved in its use, and proposes a new cytochemical procedure for measuring $\beta$-hydroxyacyl-coenzyme A dehydrogenase activity.

Materials and Methods

Albino Wistar rats (about 300 g) of either sex were killed by cervical dislocation, and the heart was removed. The left ventricle was cut into pieces up to 5 mm$^3$ and were chilled in n-hexane ("free from aromatic hydrocarbons" grade, from BDH, boiling range 67°–70° C) at -65° to -70° C. Sections (8 μm) were cut on a Bright's cryostat, with a cabinet temperature of -25° to -30° C. The knife was further cooled to -70° C by packing the haft with solid carbon dioxide ice. The procedures have been described fully by Chayen et al. (1973a).

All the reactions were carried out in a medium containing 30% polyvinyl alcohol (30 g GO4/140 PVA in 100 ml of 0.05 M glycyl-glycine buffer, pH 8.0). The PVA was obtained from Wacker-Chemie, GmbH, Postfach 1, D-8000 München 11, West Germany. N-glycyl-glycine, cysteamine hydrochloride (mercaptoethylamine HCl) and sodium nitroprusside were obtained from BDH. Menadione (Vitamin K$_3$; 2 methyl 1,4-naphthoquinone), phenazine methosulphate (PMS), neotetrazolium chloride (NT) and both preparations of carnitine, were obtained from Sigma. NAD$^+$ was obtained from Boehringer Mannheim GmbH. DL-$\beta$ hydroxybutyryl-N acetyl-cysteamine was synthesized for this study: it is currently available from Sigma.

The basic reaction medium with various concentrations of substrate, coenzyme, etc., was poured into small Perspex rings surrounding the sections in order to retain the reaction medium over the section. All reactions were carried out at 37° C in an atmosphere of moist oxygen-free nitrogen, since oxygen competes with NT (Altman 1970). The medium was gassed with oxygen-free nitrogen and allowed to equilibrate to 37° C before use. When it was necessary to adjust the pH of the reaction medium, the volumes of NaOH or HCl that were added were noted so that equivalent volumes of distilled water could be added to the other reaction media to ensure that there were no differences in substrate or coenzyme concentrations.

The amount of coloured formazan in 10 fields of each of duplicate sections was measured by scanning and integrating microdensitometry using the Vickers M86 microinterferometer and microdensitometer (Vickers Instruments Ltd., Haxby Road, York, England). The complete width of the cardiac muscle fibre, in longitudinal section, was measured. The machine settings were: x 40 objective with a scanning spot of 0.5 μm diameter in the plane of the section and light of 585 nm wavelength. The mask size was A5 so that the area scanned for each measurement...