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STUDIES IN ENZYME HISTOCHEMISTRY WITH PARTICULAR REFERENCE TO ORAL TISSUES*

I. PRINCIPLES OF OXIDATIVE METABOLISM AND DEHYDROGENASE HISTOCHEMISTRY

by

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INTRODUCTION

The oxidative cell metabolism provides the energy required for biological function. Any interference with the series of biochemical events responsible for the production of energy will alter cell physiology and may lead to cell injury or death.

Modern biochemical research has clarified many of the key reactions involved in the normal oxidative cell metabolism. This has lead to investigations of the metabolism under pathological conditions in order to obtain an understanding on the biochemical level of the pathogenesis of various disorders.

In terms of techniques, there are several different approaches to studies of the kind. Among them are enzyme histochemical methods.

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In order to evaluate the energy metabolism of certain oral tissues, a series of investigations based on enzyme histochemistry has been carried out in our laboratories. The general principles of the techniques employed are discussed in the article here presented, while succeeding publications will deal with the various experiments undertaken.

OUTLINE OF OXIDATIVE METABOLISM

In the intermediary cell metabolism carbohydrates, fats and proteins are more or less interconvertible through the set of reactions indicated in Figure 1*.

The intermediary, or energy, metabolism is usually subdivided into three sequences of biochemical events: glycolysis; the citrate cycle (Krebs' cycle); and oxidation by way of the pentose-phosphate shunt.

Oxidation in these systems (Fig. 1) frequently involves the removal of hydrogen from metabolic breakdown products (dehydrogenation). A hydrogen atom may be considered an association of an electron with a proton ($H \leftrightarrows e^- + H^+$). The oxidative reactions in question, hence, involve a removal of electrons (de-electrogenation). A released electron must be coupled

* The following abbreviations have been used throughout this report: ADP, adenosine diphosphate; ATP, adenosine triphosphate; GDP, guanosine diphosphate; GTP, guanosine triphosphate; NAD, oxidized nicotinamide adenine dinucleotide; $NADH_2$, reduced nicotinamide adenine dinucleotide; NADP, oxidized nicotinamide adenine dinucleotide phosphate; $NADPH_2$, reduced nicotinamide adenine dinucleotide phosphate; DH, dehydrogenase; G-3-P DH, glyceraldehyde-3-phophate DH; LDH, lactate DH; ICDH, isocitrate DH; α-KGDH, α-ketoglutarate DH-complex; GDH, glutamate DH; SDH, succinate DH; MDH, malate DH; G-6-P DH, glucose-6-phosphate DH; 6-P-G DH, 6-phosphogluconate DH; α-GPDH, α-glycerophosphate DH; P, inorganic phosphate; CoQ, coenzyme Q; Cyt., cytochrome; Cyt. ox., cytochrome oxidase. In addition, some abbreviations are explained in the text.

Fig. 1. Central, energy-producing pathways. The figure gives an abbreviated outline of the glycolytic pathway (–•–•–•–•→), the citrate cycle (·····→) and the pentose-phosphate shunt (– – – – – – →). Connections with other main metabolic pathways are also indicated. Energy-yielding or energy-consuming steps are marked by circled numbers. (Modified from ref. 24).

to an atom or a molecule, which thereby becomes reduced. In this manner, dehydrogenation reactions represent critical oxidative steps in the cell metabolism. Such reactions are catalyzed by enzymes called *dehydrogenases.* The dehydrogenases are composed from an apoenzyme, which is a protein, and a coenzyme, which is a much smaller, nitrogen-containing molecule. The hydrogen atoms from metabolic breakdown products are transferred to the coenzymes, which are either of the pyridinenucleotide type (NAD or NADP), or of the flavin type (e.g. succinate dehydrogenase). Within the cells, electrons are further transported to combine with oxygen by means of a series of intermediate electron acceptors and donors, the end product being water. This series constitutes the so-called electron transport chain (ETC, Fig. 2).

The transfer of electrons from one substance to another is accompanied by a release of energy in amounts corresponding to the difference between the *redox potentials** of the electron donor and acceptor. In order to preserve energy, the electron transport must take place in a stepwise fashion. At each point, it occurs by a coupling of phosphate to various nucleoside-diphosphates (*oxidative phosphorylation*) (21). Most frequently it takes place by an addition of phosphate to adenosinediphosphate (ADP) to form adenosinetriphosphate (ATP). The probable points of energy preservation in the ETC are indicated in Fig. 2.

One way of measuring energy, is in terms of the heat (cal.) liberated from (exergonic) or put into (endergonic) a system. Commonly, about 3000 cal./mole become released by breaking a chemical bond. When it comes to ATP, however, energy of the order of 7600—7800 cal./mole or more is stored in the "high-energy"-phosphate bonds of the molecule. ATP, thus, represents an energy store upon which the cells can draw whenever energy for biological function is required. In this case, ATP is converted to ADP, which can once more be phosphorylated to ATP by means of the ETC.

Ten ATP molecules are formed when one molecule of glucose is split into 2 molecules of pyruvate. Six of them derive from

* The reduction-oxidation (redox) potential is a measure of the ability of a substrate to accept or release electrons relative to that of a standard hydrogen electrode.

electron transport in the ETC (Fig. 1, step 3 & Fig. 2), while 2 originate at the substrate level by the conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate (Fig. 1, step 4). The remaining two stem from the dephosphorylation of phosphoenolpyruvate to enolpyruvate (Fig. 1, step 5). On the other hand, the conversion of glucose into glucose-6-phosphate (Fig. 1, step

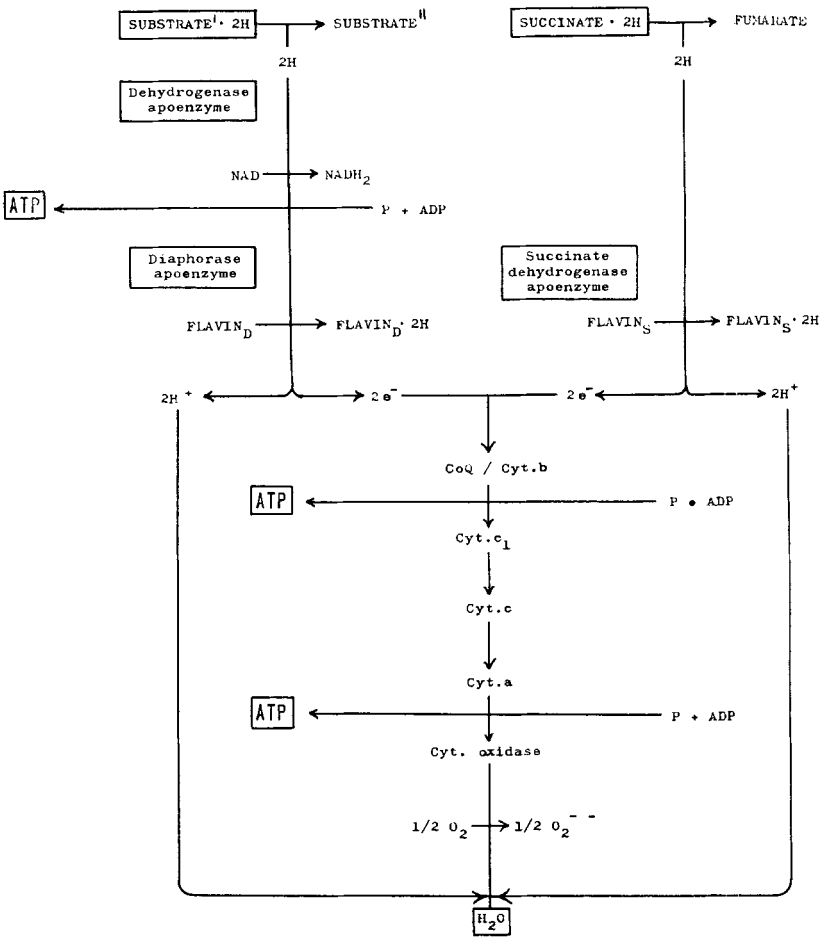


Fig. 2. The Electron Transport Chain (ETC). 3 ATP molecules are produced at each oxidative step in the energy pathways catalyzed by NAD-linked dehydrogenases. As seen from the figure, only 2 ATP-molecules are created when succinate is oxidized by the flavin-linked succinate dehydrogenase.

1) and of fructose-6-phosphate into fructose-1,6-diphosphate (Fig. 1, step 2) are endergonic processes, and the energy is furnished from ATP. Hence, the net production of energy in this part of the intermediary metabolism corresponds to the formation of 8 new ATP molecules.

In the citrate cycle, 12 new ATP molecules may become created when electrons from the oxidative steps no. 7, 8, 9, and 12, Fig. 1 are transported through the ETC. The hydrogen atoms attached to NADP after the dehydrogenation of isocitrate (Fig. 1, step no. 8) are probably transferred to NAD before coupling to the ETC (24). Reactions of this kind are catalyzed by enzymes called *transhydrogenases:*

$$NADPH_2 + NAD \overset{\text{transhydrogenase}}{\rightleftharpoons} NADH_2 + NADP$$

Electron transport from succinate to oxygen (Fig. 1, step 11) results in the production of 2 ATP molecules (Fig. 2), and the "substrate phosphorylation" at step 10, Figure 1, accounts for an additional high energy phosphate compound. Hence, a production of 15 new nucleosidetriphosphate molecules per molecule of oxidized pyruvate is theoretically possible. This equals 30 new nucleosidetriphosphate molecules created per molecule of glucose metabolized.

With regard to the pentose-phosphate shunt, it has been calculated (25) that complete oxidation of a glucose molecule can produce 12 molecules of $NADPH_2$, equivalent to 36 molecules of ATP. Subtracting one ATP molecule necessary for glucose phosphorylation, the net yield would be 35 ATP molecules. The efficiency of this pathway in terms of energy production *in vivo* is, however, at the moment hard to assess. It may be that one of the most important functions of this set of reactions is to furnish pentoses for nucleotide synthesis and reduced NADP, required for the synthesis of lipids.

The different ways of energy production through the three pathways in question make a study of them in various tissues interesting in relation to cellular activity. Investigations of the kind can be carried out by histochemical (see second part of this article) or biochemical methods. The former are concerned with

the localization in tissue sections of enzymatic activities. The latter involve measurement of O_2-consumption, CO_2-production, the activities of different enzymes in tissue slices, as well as the use of radioactive isotopes and other types of techniques.

Much effort has been devoted to investigations of the intracellular localization of oxidative enzymes. The biochemical approach has by and large been based on studies of subcellular fractions, as separated by ultracentrifugation (nuclear, mitochondrial and microsomal sediments, and the supernatant). Succinate dehydrogenase and the members of the ETC are supposedly confined to the mitochondria [8]. The localization of the other dehydrogenases is more uncertain, although some of the dehydrogenases of the citrate cycle are apparently not solely connected with the mitochondria, because they occur upon ultracentrifugation in the supernatant as well [8]. The main glycolytic activity upon ultracentrifugation is confined to the supernatant, which is also the case with the enzymes of the oxidative pentosephosphate cycle [8].

Recently, a tentative model for the correlation of mitochondrial structure and function with regard to energy metabolism has been proposed (Fig. 3, ref. [20, 22, 19, 51]).

In the model, the surface of the outer mitochondrial membrane is pictured as being studded with spherical granules. The dehydrogenation of the citrate cycle intermediates is presumably carried out within these granules. (The observed activity of some of these enzymes in non-mitochondrial cell fractions may, in part, be due to a release of such particles into the environment, caused by technical manipulations). In the electron microscope, an additional set of particles is seen. These appear to be attached to the surface of the inner mitochondrial membrane by means of short stalks [15]. They seem to correspond to the electron transport particles, ETP (or elementary particle, EP), postulated on biochemical grounds by *Green* and collaborators [7, 18, 4]. They may contain all members of the ETC (including the flavoproteins oxidizing $NADH_2$ and succinate), as well as those required for oxidative phosphorylation with ATP production [23]. This concept implies that the NAD-coenzymes have the additional important task of shuttling electrons from the outside particles to the

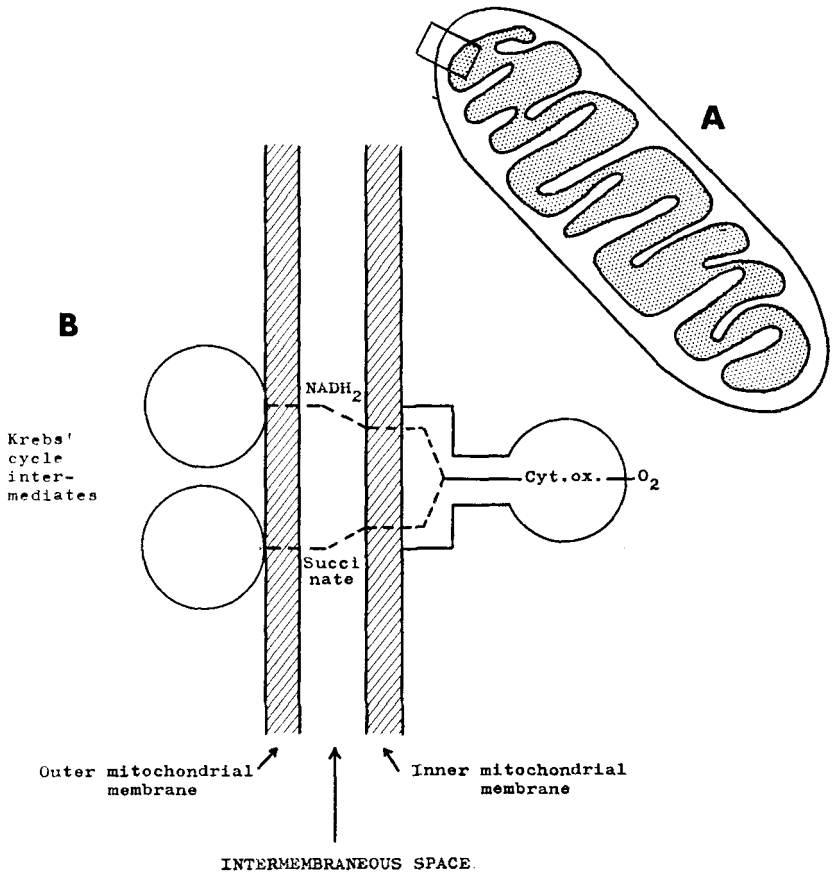


Fig. 3 A and B. A tentative model for mitochondrial structure and function. Framed area in "A" is given in larger magnification in "B". See text for further description. (Modified from ref. 22).

inside ones across the fluid-filled intermembranous space. In addition, it presupposes that succinate penetrates directly through the mitochondrial membranes, thus reaching the ETC.

As mentioned, the $NADH_2$ and $NADPH_2$ originating from citrate cycle reactions appear in most cases to be oxidized by mitochondrial systems. The intracellular mechanisms for the reoxidation of pyridinenucleotides, reduced extramitochondrially during glycolysis and by the pentose-phosphate cycle, are not well

established. As will be discussed, the site of oxidation of $NADH_2$ and $NADPH_2$ is an essential point in relation to the histochemical demonstration of the localization of dehydrogenase activity. The question of how and where these reduced nucleotides are reoxidized thus becomes of interest from a cytochemical point of view as well. There are several possibilities,

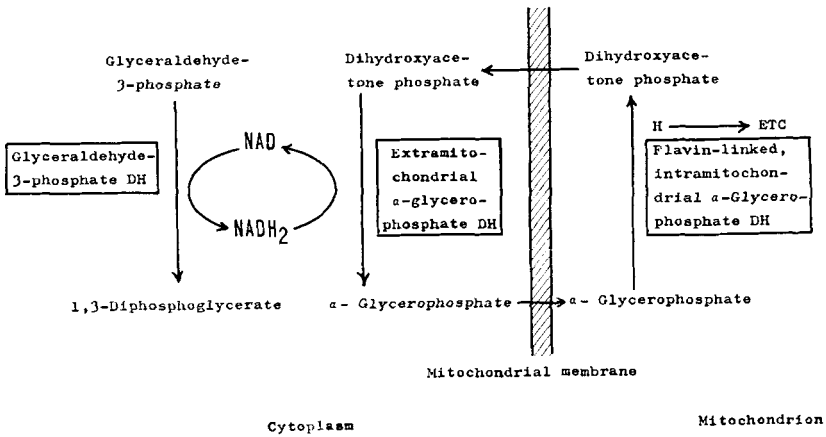
a) A direct transport of hydrogen from extramitochondrially reduced NAD to the ETC within the mitochondria, mediated by the appropriate enzyme located either outside or residing within the mitochondrial membrane. There is, however, experimental evidence contradicting a direct pathway [31, 56]. Moreover, an "intact" mitochondrial membrane is apparently impermeable to $NADH_2$ [5].

b) Hydrogen atoms from extramitochondrial $NADH_2$ may reach the ETC by way of intermediates serving as carriers of hydrogen across the mitochondrial membranes. At least three "shuttle systems" that can effect such a transfer have been suggested [5, 55]. Two of them are outlined in Fig. 4 a and b.

In the "*glycerophosphate shuttle*" (Fig. 4 a), it is supposed that dihydroxyacetone-phosphate (formed in equimolar amounts with glyceraldehyde-3-phosphate when fructose-1,6-diphosphate is split during glycolysis, see Fig. 1) will accept electrons from $NADH_2$ to form α-glycerophosphate by means of extramitochondrial α-glycerophosphate dehydrogenase activity. The glycerophosphate may permeate the mitochondrial membrane, and, when not utilized for the synthesis of lipids, serve as a substrate for the intramitochondrial, flavoprotein-linked α-glycerophosphate dehydrogenase which oxidizes its substrate by way of the ETC [5].

The second postulated pathway that will couple hydrogen from external $NADH_2$ to the ETC is the socalled "*aceto-acetate-β-hydroxybutyrate shuttle*" (Fig. 4 b, ref. [5]). Here extramitochondrial $NADH_2$ reduces acetoacetate to β-hydroxybutyrate under the influence of β-hydroxybutyrate dehydrogenase. However, this enzyme is, apparently, strictly localized within the mitochondria [32]. To explain the above reaction, it is assumed that there exists a hydroxybutyrate dehydrogenase, so situated as to permit an interaction with the extramitochondrial substrate, the

a) The "glycerophosphate shuttle."



b) The "aceto-acetate- -hydroxybutyrate shuttle."

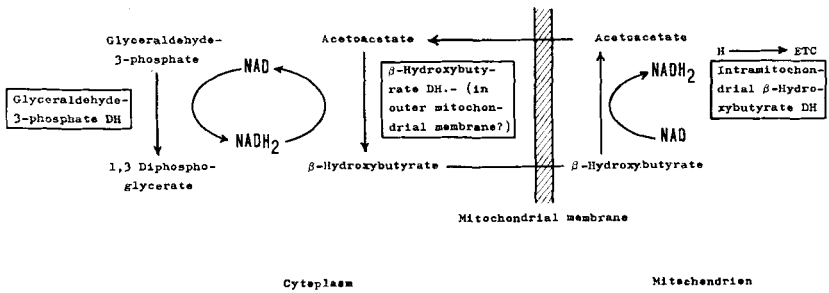


Fig. 4 a and b. Shuttle-mechanisms by which *extra*mitochondrially reduced NAD-molecules can be oxidized by an indirect coupling of their hydrogen atoms to the *intra*mitochondrial electron transport chain.

a) The "glycerophosphate shuttle".

b) The "acetoacetate-β-hydroxybutyrate shuttle".

most probable site of reaction being within the mitochondrial membrane. From there on, β-hydroxybutyrate may diffuse into the mitochondrium and serve as a substrate for the established, intramitochondrial β-hydroxybutyrate dehydrogenase, which in turn feeds electrons into the ETC (5).

c) $NADH_2$ can be oxidized by the reduction of pyruvate into lactate, the enzyme in question being $NADH_2$-dependent. However, a high rate of lactate accumulation in the presence of oxygen (aerobe glycolysis) is not considered a prominent feature of most normal cells.

d) By way of the oxalacetate-malate pathway [56, 55].

e) It is well established that reduced pyridinenucleotides, especially $NADPH_2$, are required for reductive cellular synthesis of fatty acids and steroids, the coenzymes thereby becoming oxidized.

f) As mentioned on page 477, $NADPH_2$ can also be oxidized by an enzymatically catalyzed transhydrogenation to NAD [30].

g) The extent to which $NADPH_2$ can be directly coupled to the ETC [28] is, at present, still subject to discussion. High activities of $NADPH_2$-diaphorase can, however, be demonstrated histochemically.

h) Finally, it has been established that $NADPH_2$ can be oxidized by gluthatione in a reaction catalyzed by the enzyme gluthatione reductase [50].

The relative significance in various tissues of the different mechanisms listed, is, at the moment, hard to assess.

PRINCIPLES OF DEHYDROGENASE HISTOCHEMISTRY

The ultimate goal of enzyme histochemistry is to establish the localization and degree of activity of enzymes under various conditions.

Enzyme histochemical techniques are almost exclusively based on a demonstration of enzyme *activity*. (Recently, however, *immuno*histochemical techniques have been developed for the localization of the enzymes themselves. The latter techniques will not be considered here.) An enzymatic reaction can be observed in a tissue section when a reaction product is insoluble and coloured (or fluorescent), or when it can be made to react with coupling substances so as to become coloured. When the coupling substance is included in the incubation medium, the technique is referred to as being of the "*simultaneous capture*" type. Otherwise it is classified as a "*postincubation*" coupling technique.

MTT

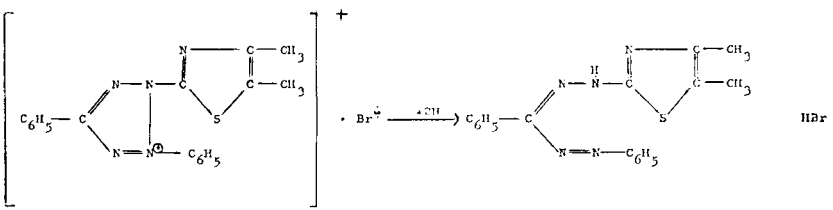


Fig. 5. Reduction of the colourless monotetrazolium salt, MTT (3—[4,5 dimethylthiazolyl-2]—2,5-diphenyl tetrazolium bromide), to its red formazan.

Nitro-BT

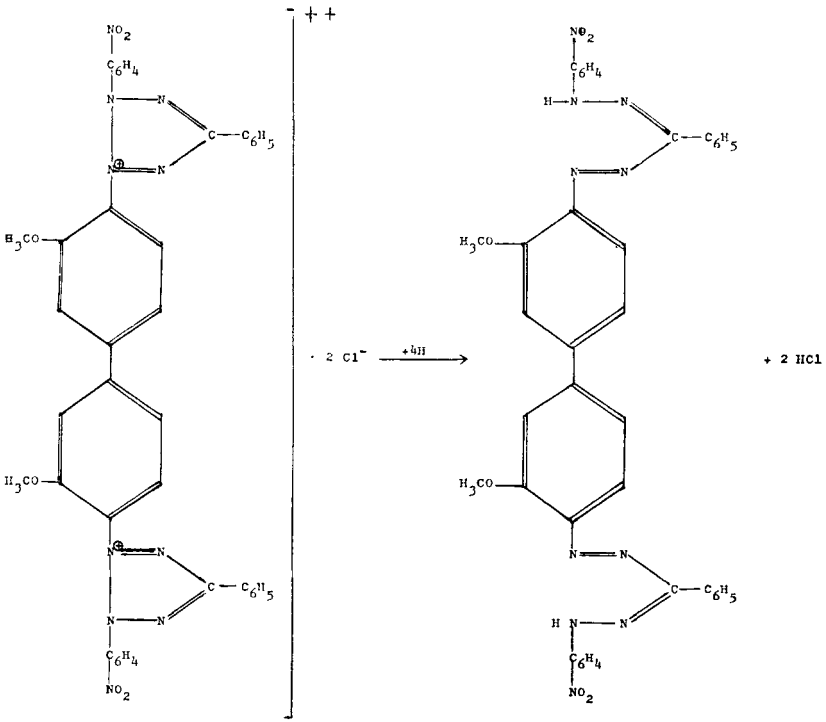


Fig. 6. Reduction of the uncloloured, ditetrazolium salt, Nitro-BT (2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-[3,3'-dimethoxy-4,4'-biphenylene] ditetrazolium chloride), produces blue diformazan.

For the demonstration of dehydrogenase activity, tissue sections are incubated in a medium containing the substrate, the coenzyme, other cofactors and an artificial electron acceptor (e.g. certain tetrazolium salts). When reduced the soluble tetrazoles become converted into insoluble dyes, called formazans [9, 10, 11, 12, 36, 37, 38, 57, 26].

Figures 5 and 6 show the reaction schemes of two common types av tetrazoles (the monotetrazolium salt, MTT, Fig. 5, ref. [3], and the ditetrazolium salt, Nitro-BT, Fig. 6, ref. [66]).

The tetrazoles employed in histochemistry accept hydrogen atoms indirectly by way of a *flavoprotein* catalyzed oxidation of the coenzymes linked to the apoprotein of the dehydrogenases (Fig. 2, ref. [9, 27, 28, 46, 13]). When these coenzymes are pyridine-nucleotides, the *flavoproteinenzymes* are usually designated $NADH_2$- or $NADPH_2$-*diaphorases* in histochemistry.

The point of interaction between the artificial electron acceptor and the electron transport chain is dependent upon the kind of tetrazolium salt employed [39, 63, 64, 33]. Some tetrazolium salts (e.g. MTT, INT = 2-p-iodophenyl-3-nitrophenyl-5-phenyltetrazolium chloride) accept electrons at two different points along the ETC [64]. The use of inhibitors (Amytal, Antimycin A, chlorpromazine, cyanides, azides) which block the electron flow at specific sites, have simplified studies of this kind [39, 64]. The most frequently used tetrazole at present is Nitro-BT which interacts at one point only along the ETC [64]; probably at the flavoprotein level.

From the above discussion, it follows that by enzyme histochemical methods, it is the site of diaphorase activity which is observed and not the exact localization of the dehydrogenase in question. However, the distance between a given dehydrogenase and its corresponding diaphorase appears in most cases to be well below the resolution limit of the light microscope (2000 Å) (Fig. 7 a).

Even though a dehydrogenase may convey hydrogen to more than one diaphorase, the picture of dehydrogenase localization as obtained in the light microscope is probably realistic (Fig. 7 b). Furthermore, if the diaphorase be not the rate limiting step in the transport of hydrogen atoms from substrate to tetrazolium

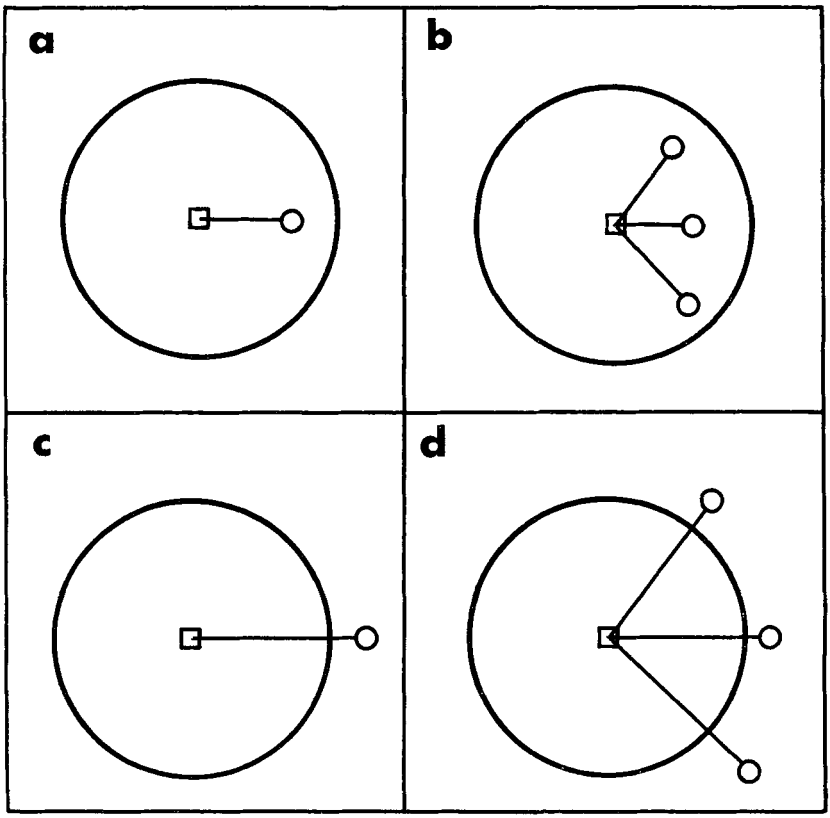


Fig. 7 a, b, c and d. Four relationships in the dehydrogenase-diaphorase systems. Dehydrogenases are marked by squares, and diaphorases by small circles. Around the points of dehydrogenase activity are drawn circles with a 2000 Å radius, equivalent to the resolution limit of the light microscope. See text for discussion.

salt, an indication of the degree of dehydrogenase function is obtained.

On the other hand, if dehydrogenase and diaphorase (s) are separated by more than 2000 Å (e.g. in systems where $NADH_2$ is produced extramitochondrially), misleading results with regard to dehydrogenase localization at the cellular level are obtained (Fig. 7 c). This is perhaps so also when it comes to activity evaluations (Fig. 7 d).

Farber & Bueding [12] suggested that the requirements for tissue diaphorase in histochemical techniques could be overcome

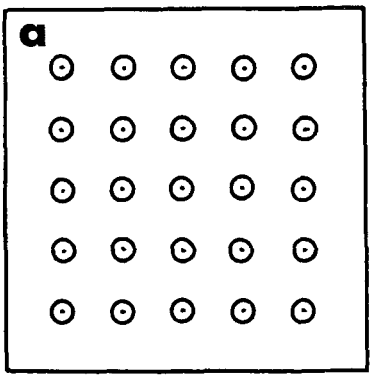
by including an intermediate electron transferring agent in the incubation medium for which purpose they utilized phenazine methosulfate. The use of this or similar compounds in histochemistry has found some application (6, 35), but the intermediate electron transferring agents have as yet not been extensively studied in this connection.

All of the sites of $NADH_2$-diaphorase activity within a tissue section are presumably shown when reduced NAD is utilized as substrate (Fig. 8 a), provided the postulated *"permeability barrier"* to $NADH_2$ in "intact" mitochondria (ref. 5 and page 480) is destroyed during the preparation of the sections. A *restricted* distribution pattern is registered when the specific substrate for a given NAD-linked dehydrogenase is employed (Fig. 8 b and c).

This is part of the basis for the assumption that it ought to be possible by means of histochemical methods to differentiate between the distribution of various NAD- and NADP-linked dehydrogenases (69, 37, 38, 43). It should, however, be kept in mind that it is actually the localization of the associated diaphorase, and not that of a given dehydrogenase, which is observed.

In the light microscope, it is difficult to establish whether or not the formazan particles are deposited within cellular organelles, unless the distribution of these organelles is confirmed by traditional techniques (6). Even so, problems may arise since controls must be carried out on alternate sections. The eventual establishment of the exact intracellular localization of enzymes along with the desirability for interpreting ultrastructures in terms of functional significance, have recently lead to investigations combining electron microscopy with enzyme staining (27, 61). Commonly, such procedures have encompassed incubation of fresh frozen or formalinfixed tissues followed by fixation in osmium tetraoxide. Recently, *Sabatini et al.* (54) pointed out the advantages of certain aldehyde fixatives for the combined

Fig. 8 a, b and c. Differences in the histological distribution patterns of formazans as observed, a) with $NADH_2$ as substrate for $NADH_2$-diaphorase; b) with substance "A" as substrate for the NAD-linked "A"-dehydrogenase; and c) with substance "B" as substrate for the NAD-linked "B"-dehydrogenase. Dots indicate complexes between various dehydrogenases and $NADH_2$-diaphorases. Reactive complexes under the three different conditions mentioned above, are marked by circled dots.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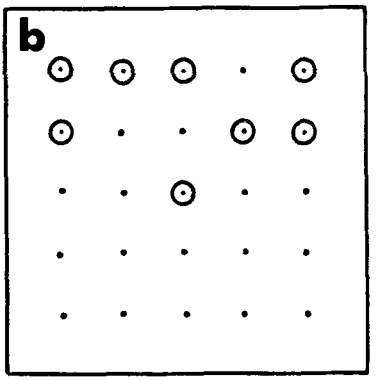
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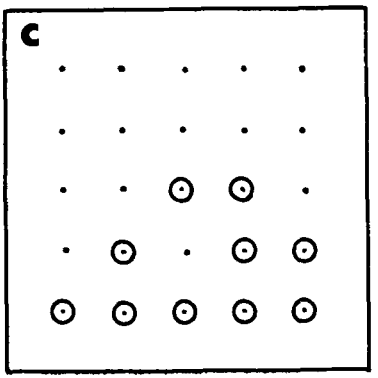
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study of ultrastructure and enzyme activity, and they, as well as others (2, 71, 59, 58, 60, 1, 14), have described the localization of formazan particles produced by dehydrogenase-diaphorase activity in relation to ultrastructural elements.

*

So far, the difficulties involved in establishing the precise localizations of dehydrogenases have been discussed on the assumption that the many technical hazards involved be carefully controlled. However, artifacts may arise during: a) sectioning; b) fixation; c) incubation; and d) postincubation treatment.

In oxidative enzyme histochemistry, sections are usually cut in a cryostat (— 20° to — 25° C) after initial freezing of the tissue specimen (fixed or fresh) in liquid nitrogen (— 196° C) or in isopentane precooled by liquid nitrogen. Subsequently, the sections are either freeze-dried, freeze-substituted, air-dried after melting, chemically fixed or directly transferred to the incubation medium. Both freezing and thawing may cause disruption of mitochondria and displacement of dehydrogenases (70).

There has been much discussion about advantages and disadvantages of chemical fixation as combined with enzyme histochemical methods. There are three points to consider: a) preservation of enzyme activity; b) retention of enzyme localization; and c) preservation of cellular structure (48). *Pearse* (45) recommended the use of unfixed, cryostat-cut sections for dehydrogenase histochemistry. *Novikoff* (41) and *Walker & Seligman* (67), on the other hand, emphasized the advantages of prefixed sections. If enzyme localization is intended at the histological level only, the use of unfixed sections may be satisfactory and, of course, necessary when an enzyme under investigation is strongly inhibited by chemical fixatives. The fact that many enzymes exist in multiple molecular forms (isozymes, 34), introduces the additional problem of differential inhibition by fixation (48). Another point is, that some enzymes are at the same time soluble (lyoenzymes), as well as firmly particlebound (desmoenzymes). *Novikoff* (41) has demonstrated the importance of such findings with respect to false localization in enzyme histochemical investigations.

At present, there are no general rules with regard to the useful-

ness of chemical fixation in combination with enzyme histochemistry.

Histochemical procedures for the demonstration of dehydrogenase activity involve the use of high substrate concentrations as compared to the physiological ones. This is to overcome diffusion barriers, to maintain adequate substrate levels throughout the incubation period, and to force the reaction to proceed primarily as a dehydrogenation. It has, however, been shown that high substrate levels inhibit the activities of certain isozymes (49, 16, 65). The implications of such findings to enzyme histochemistry have, so far, not been adequately investigated. Another aspect of this problem is that high concentrations of, for instance, isocitrate (for the demonstration of isocitrate dehydrogenase activity) might serve to produce high concentrations of substrates for other oxidative steps in the citrate cycle as well. If so, this would seriously influence the selective demonstration of isocitrate dehydrogenase. On the other hand, the histochemical distribution pattern of isocitrate dehydrogenase apparently remains about the same in sections where succinate dehydrogenase has been inhibited (by malonate) as compared to sections where no special precautions are taken. How to interpret an observation of this kind is difficult, although it may be speculated that lack of an adequate supply of cofactors be among the determining factors.

Some of the properties of the tetrazoles added to the incubation media have already been discussed. In addition, these substances must not only be sufficiently soluble in the substrate solutions, but also have a molecular size and charge so as to allow for rapid penetration into the cells and organelles. Furthermore, the tetrazoles (or formazans) must, of course, not inhibit the activity of the enzymes under investigation. Even the more recently developed tetrazolium salts, like MTT or Nitro-BT, probably capture but one tenth of the available electrons in the ETC (47). The flow of electrons to the artificial electron acceptor can be increased by creating a block in the ETC at a point nearer to the oxygen end of the chain than the point of tetrazolium interaction. Such an inhibition can be effected by several substances (see page 484), some of which are commonly included in the incubation media.

There is a definite possibility that an unspecific reduction of tetrazolium salts to formazans may occur during the histochemical reaction procedure, especially in solutions of pH around 9.0. It may, however, occur even at physiological pH (Fig. 9).

Fig. 9. "Nothing dehydrogenase "-effect in the parakeratinized, epithelial zone of a section from human gingiva, incubated at pH= 7.4 in a Nitro-BT medium, devoid of dehydrogenase substrates.

In the case shown in Fig. 9, Nitro-BT was employed as electron acceptor. The incubation medium was devoid of substrate and was adjusted to pH 7.4 with phosphate buffer. This effect is referred to as "nothing dehydrogenase", because it is believed that hydrogen is transferred to the tetrazoles from SH-groups within the tissue (44). A positive reaction under such conditions

may, however, also be due to endogenous substrates. The finding that blocking of SH-groups by means of N-ethyl maleimide interferes with tetrazole reduction, supports the assumption that sulfhydrylgroups are important for the "nothing dehydrogenase" effect (44).

When reduced, the tetrazoles ought to be adequately coloured and non-crystalline. Moreaver, they should be completely insoluble in biological fluids and possess a general affinity (or "substantivity") for proteins. Monoformazans do not adequately satisfy these requirements. Improvements can, however, be achieved by utilizing their ability to form metallic complexes by chelation (42). *Pearse* (45) claimed to have devised such a capture reaction "which prevents crystallization and which presumably also prevents diffusion of the free formazan". The mentioned procedure was strongly criticized by *Novikoff et al.* (40), who concluded that "The deposition of the formazan of MTT-Co^{++} is determined in large part by physicochemical factors other than enzyme localization." On the other hand, these authors preferred the use of Nitro-BT staining, which gave satisfactory intracellular localization of oxidative enzymes in the light microscope (40).

The diformazan from Nitro-BT exhibits a bright blue colour. It is not uncommon, however, that a red tinge interferes. Whether this is due to monotetrazolium salt contamination of the Nitro-BT preparations, or is caused by incomplete reduction of the ditetrazole, has not yet been settled (36, 62). Another undesired feature of the reaction is the tendency of the Nitro-BT formazans to become deposited on lipid-aqueous surfaces (40). To overcome this effect, Tetra-Nitro-BT (TNBT) was introduced in 1961 by *Rosa & Tsou* (52). Their investigations with TNBT for the cytochemical localization of succinate dehydrogenase (53) indicate that TNBT is particularly useful in studies involving the localization of enzymes in cells containing considerable amounts of lipids. The advantages of TNBT for the demonstration of several dehydrogenases have recently been emphasized (68, 69).

QUANTITATIVE ENZYME HISTOCHEMISTRY

Only semiquantitative estimates of enzyme activity are possible with the techniques of enzyme histochemistry. Evaluations

are usually based on light microscopical observations of the density of the formazan reaction in a section. One way to circumvent quantitation by subjective, visual impressions is to extract the formazan deposits from the sections and then measure their colour intensities spectrophotometrically [29]. Another approach which has been more frequently applied is to quantitate enzyme activity by means of microchemical studies of sections adjacent to those used for qualitative enzyme histochemistry [17].

EVALUATION OF OXIDATIVE METABOLISM AS REVEALED BY ENZYME HISTOCHEMICAL METHODS

Provided adequate control measures are taken, histochemical methods may indicate fairly the localization of a given enzyme, and to some extent also its activity. However, the *in vitro* conditions employed, differ considerably from those existing *in vivo*. Hence, great care must be exercised in interpreting histochemical results in terms of enzymatic activity and, especially metabolic significance. Moreover, the activity of a given enzyme changes throughout the lifecycle of the cell. Thus, negative observations with regard to the activity of a specific dehydrogenase in a tissue section constitute no proof of the absence of the enzyme. Repeated negative results may merely indicate inadequate techniques. Optimal conditions for tissue preparation and histochemical procedure must be established individually for each different dehydrogenase. Accordingly, comparative evaluations of the activities of different enzymes are subjected to a great many errors.

In spite of the above objections, oxidative enzyme histochemistry may certainly render valuable biological information in terms of correlating structure and function.

SUMMARY

A short outline of oxidative metabolism is presented, stressing some of the features pertinent to dehydrogenase histochemistry. Various aspects of the problems involved in demonstrating en-

zyme activity in relation to tissue structure are discussed. Artifacts may arise during sectioning, fixation, incubation, and post-incubation treatment. A further difficulty emerges from the fact that when no intermediate electron carrier is included in the incubation medium, the spot visualized by the histochemical reaction is the localization of an associated enzyme, a diaphorase, and not specifically that of the dehydrogenase in question. On the other hand, somewhat inconsistent and confusing results have been obtained with various intermediate electron carriers.

Only semiquantitative estimates of enzyme activity are possible when the evaluations are based on observations of the density of the reaction product in a section. Employing standardized conditions, valuable information regarding variations in the activity of a certain enzyme among different cells and tissues, may be obtained. However, considering the many technical hazards involved, the proper foundation is still lacking for comparing the activities of different enzymes to each other.

RÉSUMÉ

ÉTUDES SUR L'HISTOCHIMIE DES ENZYMES, PLUS PARTICULIÈREMENT EN CE QUI CONCERNE LES TISSUS BUCCAUX

I. PRINCIPES DU MÉTABOLISME OXYDATIF ET HISTOCHIMIE DES DÉSHYDROGÉNASES

L'auteur présente un bref aperçu du métabolisme oxydatif, en soulignant certains des traits relevant de l'histochimie des déshydrogénases. Divers aspects des problèmes soulevés par la mise en évidence de l'activité enzymatique en ce qui concerne les tissus font l'objet d'une discussion. Des artéfacts peuvent se produire au cours de la coupe, au cours des traitements de fixation, d'incubation et de post-incubation. Une difficulté supplémentaire résulte du fait que, lorsque le milieu d'incubation ne contient pas de transporteur d'électrons intermédiaire, l'endroit indiqué par la réaction histochimique est la localisation d'une enzyme associée, une diaphorase, et non pas de manière spécifique celle de la déshydrogénase en question. Mais d'autre part, les résultats obtenus avec divers transporteurs d'électrons intermédiaires étaient contradictoires et déroutants.

Seules des estimations semi-quantitatives de l'activité enzyma-

tique sont possibles lorsque les évaluations sont basées sur des observations de la densité du produit de la réaction dans une coupe. En employant des conditions standardisées, on peut obtenir des renseignements de valeur au sujet des variations de l'activité d'une enzyme donnée dans différentes cellules et dans différents tissus. Cependant, vu le nombre de hasards impliqués du point de vue technique, il manque encore une base appropriée pour la comparaison entre les activités de différentes enzymes.

ZUSAMMENFASSUNG

ENZYMHISTOCHEMISCHE STUDIEN MIT BESONDERER BERÜCKSICHTIGUNG DER MUNDGEWEBE

I. PRINZIPIEN DES OXYDATIVEN STOFFWECHSELS UND DER DEHYDROGENASEHISTOCHEMIE

Hier wird ein kurzer Grundriss des oxydativen Stoffwechsels gegeben, inbesonders einige der Sonderheiten, die zur Dehydrogenasehistochemie pertinent sind. Es werden verschiedene Aspekte der Probleme diskutiert, die mit der Demonstration von Enzymaktivität in Relation zur Gewebsstruktur verbunden sind. Artefakte können bei Herstellung von Schnitten entstehen, durch Fixation, Inkubation und während der Postinkubationsbehandlung. Wenn kein intermediater Elektronenträger im Inkubationsmedium vorhanden ist, liegt eine zuzätzliche Schwierigkeit vor: Die histochemische Reaktion zeigt nämlich die Lokalisation eines assosiierten Enzyms, einer Diaphorase, und nicht spezifisch die Lokalisation der gesuchten Dehydrogenase. Andererseits sind etwas unregelmässige Resultate durch Zusatz verschiedener intermediate Elektronenträger erreicht worden.

Nur semiquantitativen Estimaten der Enzymaktivität sind möglich, wenn die Evaluierung auf Betrachtung der Dichte des Reaktionproduktes basiert ist. Durch Anwendung standardisierter Bedingungen können wertvolle Aufklärungen der Variationen in der Aktivität eines bestimmten Enzyms in verschiedenen Zellen und Geweben gewonnen werden. In Anbetracht der vielen technischen Schwierigkeiten fehlt aber die richtige Grundlage für eine vergleichende Auswertung der Aktivitäten verschiedener Enzyme zu einander.

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