# EVALUATION OF TISSUE ENZYME REACTIONS IN THE MYOCARDIUM

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Succinate dehydrogenase activity in the rat myocardium was assessed with reference to the type of diformazan deposition: "linear" in the control, or as small or large granules in the experimental series. Correlations were established between the type of diformazan deposit and activity of the enzyme determined from the rate of decoloration of methylene blue. The need for extreme caution in the assessment of results obtained by scanning cytophotometry of sections stained in the reaction with nitro-BT is demonstrated.

Great difficulties arise in the evaluation of the results of the reaction with nitro-blue tetrazolium (nitro-BT) in the myocardium because the reaction product, formazan, may differ in character depending on the experimental conditions [2, 3, 5, 15, 16, 19].

In the normal myocardium the formazan deposits have the appearance of lilac blue bands ("linear" formazan), against the background of which the small dark blue granules are difficult to distinguish (Fig. 1a). In response to various procedures acting on the myocardium the linear form of the deposits is often changed to one of fine or coarse granules (Fig. 1b, d). These changes in the character of the formazan deposits in the myocardium have been observed by many writers but the significance of this phenomenon in the assessment of the level of enzyme activity has not yet been sufficiently convincingly explained [1, 2, 5, 12, 15, 16, 19, 22-24].

Existing indirect methods of measuring enzyme activity do not completely satisfy the pathomorphologist. Spectrophotometric assessment of dye extracted from the tissue gives the overall response but does not permit any analysis of topographical variations in enzyme activity. This overall response includes not only active enzyme located at the appropriate locus in the mitochondria, but also a diffusing enzyme [13] giving intensive formazan formation, as is observed during injury and coagulation necrosis of the myocardial cells [2, 20].

The method of scanning cytophotometry requires verification. Ryumshina [7], for example, obtained the highest indices of enzyme activity of the liver cells by this method when she administered radon waters of the maximal (for her experiments) radioactivity, whereas the other indices (total loss of glycogen by the same cells and the initial stages of atrophy in them) were evidence of metabolic disturbances.

These considerations suggested that a comparative quantitative assessment should be made of succinate dehydrogenase (SDH) activity in the rat myocardium by two methods: by the method of scanning cytophotometry and also by a method in which enzyme activity was judged from the time of conversion of methylene blue into its colorless form depending on the level of enzyme activity [8].

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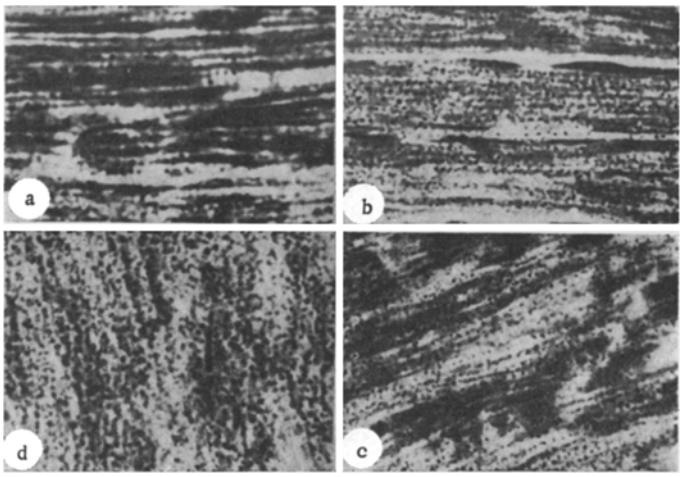


Fig. 1. Various forms of formazan deposits in the myocardium of the control and experimental rats: a) linear form of formazan – control; b) increase in number of fine formazan granules while retaining linear background in experiment with hypoxia; c) the same 1 h after injection of adrenalin; d) coarse formazan granules in the adrenalin model of myocardial injury.

## EXPERIMENTAL METHOD

Semenov's method was modified by introducing preliminary staining of the sections with 0.05% methylene blue solution for 30 sec, followed by rinsing out the unfixed dye in three portions of phosphate buffer. The incubation medium containing 10% sodium succinate solution (1 ml) in 5 ml 0.067 M phosphate buffer (pH 7.6) was thus applied to a previously stained frozen section. The dynamics of decolorization was recorded in the central areas of the section by a probing cytophotometer connected to a galvanometer. The values were read and recorded every 30 sec and the reaction was regarded as complete if the galvanometer reading did not change during 1.5 min.

Experiments were carried out on male rats weighing 220–250 g which were killed by decapitation. In a series of frozen sections 10 $\mu$ in thickness, one section was first treated by the reaction with nitro-BF for SDH [21] to obtain information regarding the character of the formazan deposits; when satisfactory differences had been obtained in the character of the formazan by comparison with the control, the reaction of decolorization of methylene blue was carried out in some sections (the rate of this reaction also was determined), while in other sections the reaction with nitro-BT was carried out to determine the activity of the enzyme from the optical density of the formazan, using a scanning cytophotometer by Morozov's technique [4]. All numerical results were subject to statistical analysis.

## EXPERIMENTAL RESULTS

Large granules of formazan (Fig. 1d) were obtained in the adrenalin model of myocardial injury (three rats) 24 h after intramuscular injection of 1 :1000 adrenalin solution in a dose of 0.2 ml/100 g body weight. It was always combined with deposition of lipids in the same cells, so that the probe could be accurately centered on these areas in sections stained homogeneously with methylene blue, for the drops of lipids refract light strongly.

Fine formazan granules with retention of the linear background (Fig. 1b) were obtained in the model of hypoxic hypoxia (three rats) produced by keeping rats for 2 h in an air-tight 1.5-liter exsiccator with the addition of small portions of air. In these experiments there was no need to specially select the areas for the reaction was uniform throughout the sections. Similar fine granules of formazan (Fig. 1c) were obtained after injection of the same dose of adrenalin into the animals but with sacrifice of the animals 1 h after the injection (two rats). Three healthy rats served as the control.

| Series | No. of expts. | Decolorization time (in min) |
|---|---|---|
| Control | 24 | 12,4 ± 0,5 |
| Hypoxia | 37 | 9,0 ± 0,18 |
| Adrenalin | 18 | 17,4 ± 0,48 |

| Series | No. of expts. | Decrease in intens. of light beam (cnvntnl. units) |
|---|---|---|
| Control | 25 | 94,4 ± 0,38 |
| Hypoxia | 24 | 88,8 ± 0,41 |
| Adrenalin 24 h | 25 | 75,8 ± 0,64 |
| Adrenalin 1 h | 25 | 89,6 ± 0,48 |

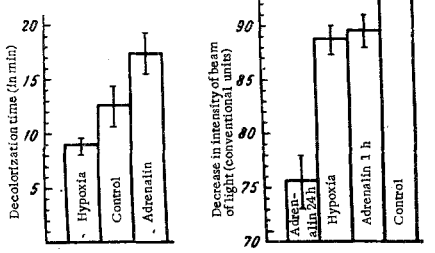





Fig. 2. Results of assessment of enzyme activity from decolorization time (a) and from optical density of formazan measured by decrease in intensity of a beam of light (in conventional units; b).

Identical results in the form of decrease in SDH activity were obtained by the two methods only for the coarsely granular formazan. For the other two forms of formazan the measurements gave directly opposite results: the highest SDH activity as reflected in the shortest time of decolorization of methylene blue in the hypoxic model, and as reflected in the results of scanning photometry in the control animals (Fig. 2a, b). It had to be decided which result was the most reliable. The method of assessment of enzyme activity by the rate of the decolorization reaction must be regarded as more accurate because this reaction, which directly reflects the level of enzyme activity, took place under optical control. The reaction with nitro-BT, however, was virtually uncontrolled, and the reaction product in the control sections ("linear" formazan) is lilac blue in color as a result of incomplete reduction of the ditetrazolium molecule, which is regarded as an indication of lower activity of the enzyme [6, 10, 18]. The results obtained by scanning cytophotometry regarding the highest enzyme activity in the control cannot therefore be regarded as reliable. High SDH activity detected in the myocardial sections in hypoxic hypoxia, on the other hand, was confirmed indirectly by the analogous character of the formazan deposits in hypoxia and 1 h after injection of adrenalin. Adrenalin has both an inotropic and a chronotropic action on the myocardium, the latter requiring higher expenditure of energy. Under moderately hypoxic conditions this temporary increase in SDH activity is evidently a compensatory reaction, as Tsagareli has noted [11]. Even total anoxia is accompanied by a transient increase in the contractile strength of the heart, followed by depression [25]. The compensatory increase in enzyme activity may perhaps take place through the intervention of catecholamines as a component of the nonspecific adaptation reaction.

The greatest scatter of the values of the decolorization time of methylene blue was obtained in the control experiments, which calls for an explanation. If Fig. 1a is compared with Fig. 1b, it can be seen that the tissue enzyme characteristics of the myocardial sections in the control and experimental series were very similar, but the main structural feature correlating with acceleration of the decolorization reaction was the presence of fine dark blue granules, the number of which rose steadily in all muscle cells during hypoxic hypoxia. Similar granules were visible in the control sections, and indeed in some cells there were more, but in others fewer of them. This demonstrates the different levels of enzyme activity even in two neighboring cells, a feature in harmony with views regarding the heterogeneity of the myocardium [9, 17].

This enzymic heterogeneity is clearly visible when β-hydroxybutyrate is used as the reaction substrate, whereas the reaction for SDH with nitro-BT gives a picture of uniform intensity of staining throughout the section. The test described above, with evaluation of enzyme activity from the rate of the decolorization reaction, thus provides a more objective means of determining the reaction component responsible for the enzymic and, consequently, the energetic and functional heterogeneity of the myocardium.

The results show the need for a more cautious assessment of the values obtained by the usual method of scanning cytophotometry, at least where the myocardium is concerned. Either different methods will have to be developed, or different principles embodied in the optical instruments used, in order that more precise information on enzyme histochemistry can be obtained.

## LITERATURE CITED


1. K. A. Gornak and E. F. Lushnikov, Arkh. Pat., No. 1, 14 (1963).
2. K. M. Danilova, Arkh. Pat., No. 4, 27 (1969).
3. K. M. Danilova, M. I. Klibaner, and V. N. Baranov, Arkh. Pat., No. 1, 23 (1971).
4. Yu. E. Morozov, Tsitologiya, No. 5, 690 (1970).
5. N. A. Probatova, Fatty Degeneration of the Myocardium in the Light of Modern Data, Author's Abstract of Candidate's Dissertation, Moscow (1965).

6. N. T. Raikhlin and N. A. Probatova, Arkh. Pat., No. 8, 74 (1969).
7. T. A. Ryumshina, Histochemistry of Oxido-Reduction Enzymes in the Liver during Internal Administration of Radon Water, Author's Abstract of Candidate's Dissertation, Moscow (1970).
8. V. E. Semenov, Z. Zellforsch. Abt. Histochem., 22, 305 (1935).
9. A. I. Strukov and V. S. Paukov, Arkh. Pat., No. 4, 3 (1969).
10. Yu. M. Torchinskii, Vopr. Med. Khimii, No. 3, 230 (1958).
11. Z. G. Tsagareli, Morphology of the Heart in General Hypoxia, Author's Abstract of Doctoral Dissertation, Erevan (1971).
12. N. M. Cherpachenko and R. I. Sokolova, Arkh. Pat., No. 2, 23 (1970).
13. F. A. Bruns et al., Klin. Wschr., 39, 342 (1961).
14. J. L. Cox et al., Am. Heart J., 76, 650 (1968).
15. J. Dusek et al., Am. J. Path. 62, 321 (1971).
16. V. Ferrans et al., Am. Heart. J., 68, 71 (1964).
17. V. Fischer et al., Am. J. Physiol., 211, 301 (1966).
18. W. Gabler et al., Acta Histochem. (Jena), 36, 295 (1970).
19. A. Hecht, Einführung in Experimentelle Grundlagen Moderner Herzmuskelpathologie, Jena (1970), p. 191.
20. A. Hecht and D. Schneider, Exp. Path., 1, 137 (1967).
21. M. M. Nachlas et al., J. Histochem. Cytochem., 5, 420 (1957).
22. N. Niles et al., Lancet, 1, 963 (1964).
23. E. A. Schenk and A. J. Moos, Circulat. Res., 18, 605 (1966).
24. B. Strehler et al., Exp. Geront., 1, 301 (1966).
25. E. Woods and J. Richardson, Am. J. Physiol., 196, 203 (1959).