IX. EXPERIMENTS ON NADH OXIDATIONS

CHAPTER-IX

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Introduction:

The earlier experimental data indicating inhibition of respiration of S.faecalis cells (Chapter-V) and not the GAP-DH activity (Chapter-VI) by SRC-820, suggested the possibility that this substance may be acting at the stage of reoxidation of niacinamide coenzymes, NADH and NADPH (Cf.Seth et al, 1964; Parmar and Seth, 1965) or even at the later stages of the respiratory chain. It was thought of interest, therefore, to study the oxidation of NADH and NADPH in the extracts of S.faecalis.

S.faecalis lacks cytochrome and the reoxidation of NADH was believed to be accomplished by the conversion of pyruvate to lactate in presence of lactate dehydrogenase. It has recently been reported that S.faecalis contains atleast five distinct and separable flavoprotein enzymes that catalyzes the oxidation of NADH (Dolin, 1961). These are: (i) NADH-oxidase, (ii) NAD^{+} peroxidase, (EC.1.11.1.1), (iii) Cytochrome C-reductase (iv) diaphorase that can use artificial electron acceptors such as 2,6-dichlorophenolindophenol,

ferricyanide and a series of quinones and (v) menadione reductase. NADPH is not a substrate for any of these enzymes.

NAD^{+}-Peroxidase (EC.1.11.1.1), it was reported, can not be measured aerobically using H_2O_2 in crude extracts (Dolin, 1955). In the present investigation, only the activities of (i) NADH-oxidase and (ii) diaphorase were studied.

An extract prepared from slow-vacuum dried cells of S. faecalis (see Materials and Methods) were used as the source of these enzymes and the influence of SRC-820 on their activities are presented in this chapter.

In preliminary experiments, it was found that the cell-extracts were able to oxidize reduced NAD. The activity was followed spectrophotometrically at 340 nm according to the method of Weber and Kaplan (1954).

Dialysis of the extract in cold for 24 hours resulted in loss of activity. Dialysis for a short period, 3 to 4 hours was then carried out and the activity was followed as before. An attempt was made to stabilize the gradual loss in activity during dialysis by adding 3 mg Bovine Albumin (Fraction-V)

per ml of extract but it was found that added albumin did not stabilize the activity. However, to avoid any risk of denaturation due to low protein concentration of the extract during dialysis, albumin was added in the extract in all experiments. Care was also taken to complete one set of experiment as quickly as possible after dialysis was over and during this period no appreciable loss in activity could be seen. Similar observations have also been reported with respect to NADH-oxidase (Lightbown and Kogut, 1959; see also, Dolin, 1961). NADH-oxidase activity in Krebs-Ringer phosphate buffer, pH 7.5, was found to be considerably less than that observed in phosphate buffer, pH 7.5.

A. NADH-Oxidase:

The enzyme activity was found to be proportional to the amount of cell-extracts taken. Extracts containing an amount equal to 1.6 mg of dry cells, it was found, could oxidase 0.07 umoles of NADH per minute (Cf. Dolin, 1953). The influence of different concentrations of NADH ranging from 1.67×10^{-5} M to 6.7×10^{-5} M was studied. Activity of NADH oxidase was found to increase with increase in the concentration of NADH. The optimum pH for activity was found to be pH 7.0. It should be mentioned that no oxidation of NADPH

(phosphate buffer, pH 7.5) took place under these identical conditions (Cf.Weber and Kaplan, 1954).

1. Effect of SRC-820 on NADH-Oxidase:

Using a concentration of NADH equal to 3.3×10^{-5} M, the effect of different concentrations of SRC-820 ranging from 3.3×10^{-3} M to 10×10^{-3} M was studied (Fig.20). As can be seen from Fig.20, the percentage inhibition increases with increase in SRC-820 concentration amounting to 35% with the highest concentration of SRC-820 (10×10^{-3} M).

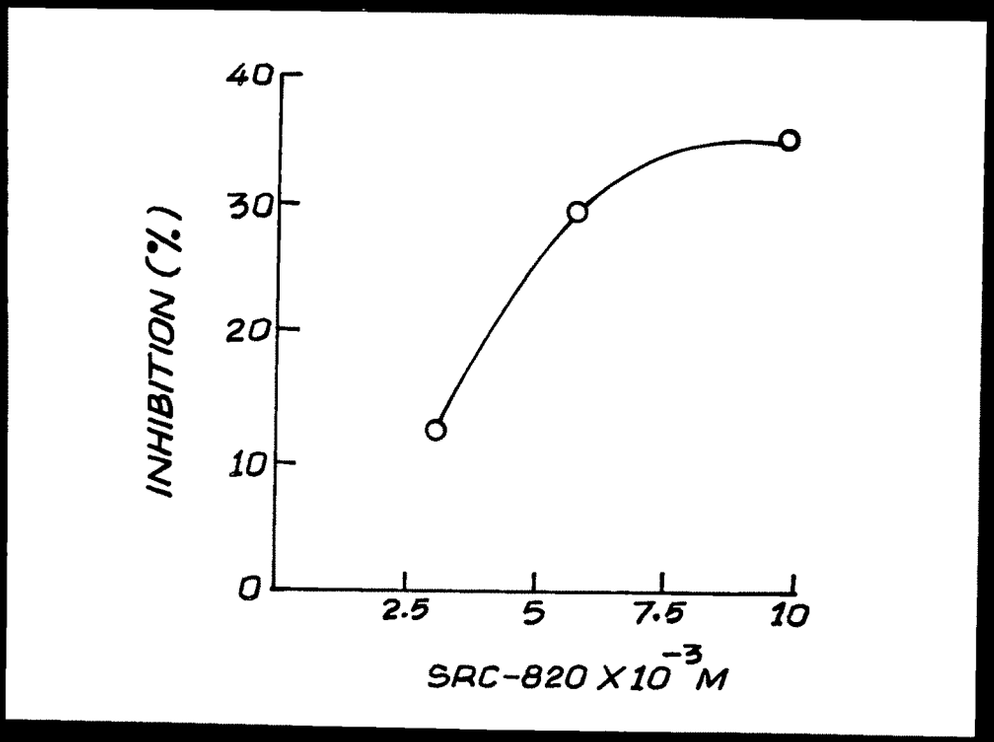
Inhibition due to SRC-820, it was found, could be overcome by increasing the substrate concentration.

2. Effect of p-Chloromercuribenzoate (pCMB):

pCMB was found to inhibit NADH-oxidase activity. At a concentration of 5×10^{-4} M, 100% inhibition was observed and at a concentration of 2.5×10^{-6} M about 40% inhibition in the activity was seen (Fig.21). In another experiment, the effect of pCMB (2.5×10^{-6} M) in presence of SRC-820 (3.3×10^{-3} M) was studied. SRC-820 alone at this level exerted about 19% inhibition whereas in presence of pCMB, the inhibition was found to be higher (about 76%, Fig.22). This effect of SRC-820 is

Fig. 20

Effect of SRC-820·HCl on NADH-oxidase.



Assay System:

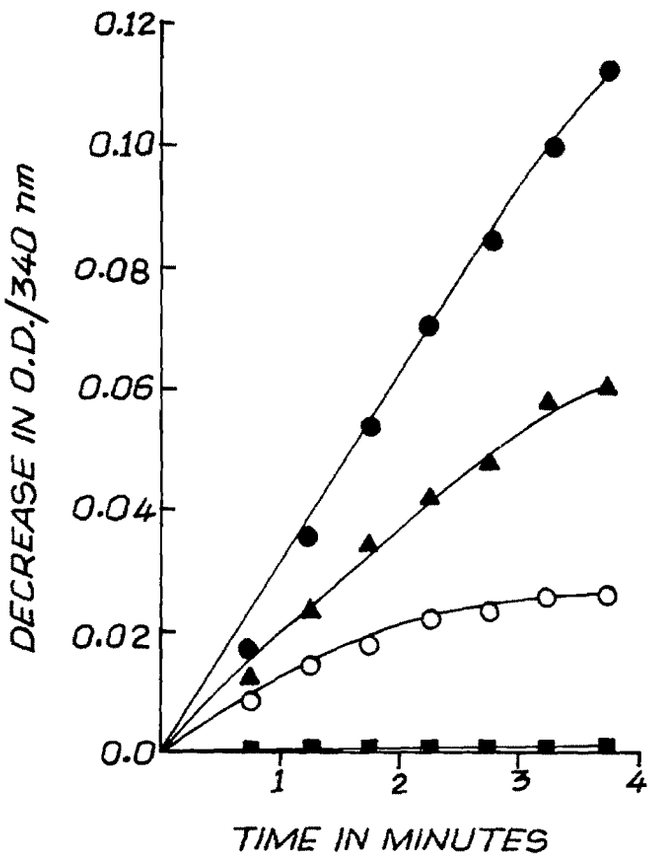
Reaction mixture contained in a total volume of 3 ml (in µmoles): K-phosphate buffer, pH 7.5, 300; NADH, 0.1; 3 hr. dialysed cell-extract (sup.) in 0.1 M K-phosphate buffer, pH 7.5 containing bovine albumin-fraction V (3 mg/ml), 0.1 ml equivalent to 0.2 mg of slow-vacuum dried cells and SRC-820 as shown in figure.

Reaction started by addition of cell-extract.

Blanks did not contain cell-extracts but bovine albumin.

Fig. 21

Effect of p-Chloromercuribenzoate on NADH-oxidase



Assay System:

Reaction mixture contained in a total volume of 3 ml (in μmoles): K-phosphate buffer, pH 7.0, 100; NADH, 0.2; 3 hr. dialysed cell-extract (sup.) in 0.001 M K-phosphate buffer, pH 7.0, containing bovine albumin-fraction V (3 mg/ml), 0.2 ml equivalent to 0.12 mg of slow-vacuum dried cells and pCMB as following:

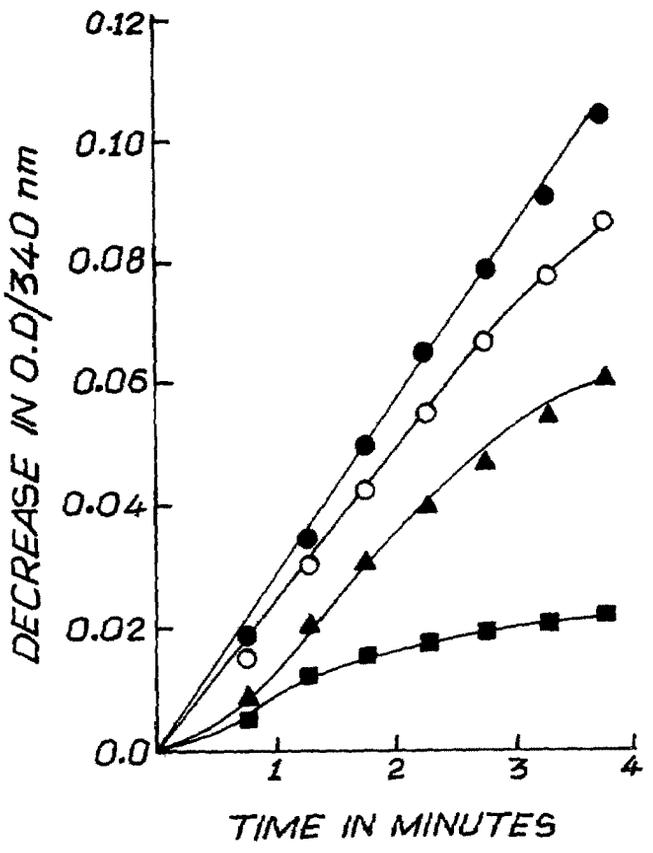
●—● Control; ▲—▲ 2.5×10^{-6} M;

○—○ 5×10^{-6} M and ■—■ 5×10^{-5} M

Reaction started by the addition of NADH.

Fig. 22

Effect of pCMB and SRC-820·HCl on NADH-oxidase.



Assay System:

Reaction mixture contained in a total volume of 3 ml (in µmoles): K-phosphate buffer, pH 7.0; 100; NADH, 0.2; 3 hr. dialysed cell-extract (sup.) in 0.001 N K-phosphate buffer, pH 7.0, containing bovine albumin-fraction V (3 mg/ml), 0.2 ml equivalent to 0.12 mg of slow-vacuum dried cells. The concentrations of pCMB and SRC-820·HCl were 0.0075 µmoles and 10 µmoles respectively, when present.

●—● Control (9); O—O With SRC-820·HCl (3); ▲—▲ With pCMB (3) and ■—■ With pCMB + SRC-820·HCl (3).

Figures in parentheses represent number of experiments.

quite opposite to that observed in the case of iodoacetate in GAP-Dehydrogenase (Chapter-VI).

The effect of pre-incubation of extract with L-cysteine on inhibition due to pCMB was studied next. L-cysteine protects the enzyme activity from pCMB (Fig.23).

Iodoacetate was found to exert its inhibitory effect only at very high concentration (1.2×10^{-3} M). At this concentration, about 47% inhibition in activity could be seen. In this case also, inhibitory effect of iodoacetate was found to be increased in presence of SRC-820 (3.3×10^{-3} M).

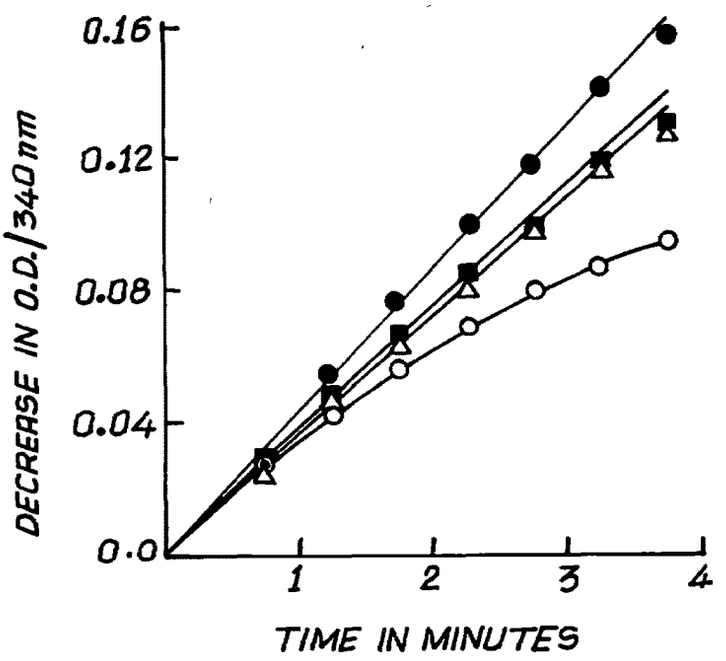
3. Effect of 8-Hydroxyquinoline (HQ):

The effect of 8-hydroxyquinoline, a well known metal chelator, on the activity of NADH-oxidase was studied. 8-hydroxyquinoline (6.6×10^{-4} M) inhibited the activity of NADH-oxidase by about 19%. In presence of SRC-820 (3.3×10^{-3} M), this inhibition was found to be higher (50%).

Molybdenum and calcium (both at 6.7×10^{-4} M) and NaF (2×10^{-3} M) did not have any effect on the activity of NADH-oxidase.

Fig. 23

Effect of L-cysteine on inhibition of NADH-oxidase by pCMB



Assay System:

Reaction mixture contained in a total volume of 3 ml (in μmoles): K-phosphate buffer, pH 7.0, 100; NADH, 0.2; 3 hr. dialysed cell-extract (sup.) in 0.001 M K-phosphate buffer, pH 7.0, containing bovine albumin fraction-V (3 mg/ml), 0.2, equivalent to 0.12 mg of slow-vacuum dried cells. The concentration of both L-cysteine and pCMB were 0.0075 μmole, when present.

●—● Control; ○—○ With pCMB; △—△ With L-cysteine and ■—■ With L-cysteine + pCMB.

B. Diaphorase:

Diaphorases are flavoproteins capable of catalyzing the oxidation of reduced pyridinenucleotides [NAD(P)H] by artificial or model electron acceptors such as methylene blue, 2,6-dichlorophenol-indophemol (DCIP) or ferricyanide. They are, however, not capable of reacting directly with molecular oxygen. Most of these enzymes have FMN or FAD as the prosthetic group.

Diaphorase obtained from some organisms was reported to contain FMN as a firmly bound prosthetic group and NADPH cannot replace NADH as the electron donor (Dolin, 1961; Walker and Kilgour, 1965).

Diaphorase Activity:

Oxidation of NADH and NADPH was followed in presence of DCIP by measuring the decrease in O.D. at 600 nm according to the method of Mahler (1955). Absorption due to indophenol dye (DCIP) was found to be linear at 600 nm, with a dye concentration range of 1.6×10^{-6} M to 4×10^{-5} M and the final concentration of DCIP used in the assay system was 4×10^{-5} M.

It was mentioned earlier that no activity could be observed spectrophotometrically with NADPH as

substrate. However, oxidation of NADPH takes place in presence of DCIP. Catalytic amounts of NAD (3.3×10^{-6} M) added to this system did not stimulate the oxidation of NADPH. SRC-820·HCl (1×10^{-2} M) has no effect on NADPH:DCIP diaphorase activity.

The activity of diaphorase when carried out in Krebs-Ringer phosphate, pH 7.5, it was found to be more by about 20% than phosphate buffer (5×10^{-2} M), pH 7.5. This is in contrast to the results obtained with NADH-oxidase, while in tris buffer (5×10^{-2} M), the activity was higher by 50% than in phosphate buffer. Above this level (5×10^{-2} M) of tris, no appreciable increase in activity could be seen. Here too, the results are quite in contrast to that obtained with NADH-oxidase.

It was also found that added molybdenum (3.3×10^{-4} M) did not affect the activity.

3. Effect of SRC-820·HCl, Quinazoline-4-one (QZ·HCl) and β-picoline on the activity of NADH:DCIP-Diaphorase:

SRC-820·HCl and QZ·HCl both at the level of 1×10^{-2} M exert no effect on this enzyme activity; β-picoline (1×10^{-2} M), however, exerted a slight inhibitory effect (about 13%).

S U M M A R Y

1. An extract prepared from slow-vacuum dried cells of S.faecalis catalyzes the oxidation of NADH.
2. Dialysis of extract for a period of about 24 hr results in loss of activity.
3. Optimum pH for oxidation of NADH is around 7.0.
4. Oxidation of NADH is less in Krebs-Ringer phosphate buffer than in phosphate buffer.
5. SRC-820·HCl (10^{-2}M) inhibits competitively the oxidation of NADH by about 35%.
6. p-Chloromercuribenzoate (pCMB) inhibits NADH-oxidase activity. An inhibition of about 40% was seen at 2.5 X 10^{-6}M pCMB. Inhibition due to pCMB (2.5 X 10^{-6}M) was found to be higher (about 76%) when SRC-820 (10^{-2}M) was also present.
7. Iodoacetate inhibits (47%) only when present at very high concentration (1.2 X 10^{-3}M). Here too, SRC-820 exerts a similar effect as in the case of pCMB.
8. 8-Hydroxyquinoline (HQ) (6.7 X 10^{-4}M) inhibits NADH-oxidase activity by about 19%. When both SRC-820 (3.3 X 10^{-3}M) and HQ are present, the inhibitory effect is increased.

9. Added Mo^{2+} or Ca^{2+} (both at 6.7×10^{-4} M) or NaF (2×10^{-3} M) has no effect on NADH-oxidase activity.

10. Oxidation of NADPH cannot be demonstrated spectrophotometrically at pH 7.5.

11. Oxidation of both NADH and NADPH can be demonstrated using 2,6-dichlorophenolindophenol (DCIP) method.

12. Presence of catalytic amounts of NAD^{+} (3.3×10^{-6} M) does not affect the oxidation of NADPH by this method.

13. Activity of diaphorase is higher in Tris buffer than in phosphate buffer or Krebs-Ringer phosphate buffer.

14. SRC-820·HCl and Quinazoline-4-one·HCl (QZ) (both at 10^{-2} M) has no effect on diaphorase activity whereas β-picoline (10^{-2} M) has a weak inhibitory effect.