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# *Trichomonas vaginalis*: Subcellular Localization of NAD$^+$- and NADP$^+$-linked Malic Enzymes

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## Introduction

Although *Trichomonas vaginalis* lacks a functional tricarboxylic acid cycle (Read, 1957; Wellerson *et al.*, 1960), it contains a high malate dehydrogenase activity (Tanaka, 1971; Brugerolle and Metenier, 1973). Physiological functions of this enzyme in trichomonad flagellates are still unknown. Therefore, we have investigated several enzymes involved in L-malate metabolism, and found that *T. vaginalis* contained at least two types of NAD$^+$-linked malate dehydrogenases, differing in subcellular localization and enzymatic properties (Tanabe *et. al.*, 1976). The stoichiometric analysis indicated that the particulate enzyme was a NAD$^+$-linked malic enzyme and the soluble one was a NAD$^+$-linked malate dehydrogenase (Tanabe *et al.*, 1979).

Tanabe and Asami (1975) also demonstrated the presence of NADP$^+$-linked malic enzyme in the crude extract of this parasite, and preliminary characterization indicated that NAD$^+$- and NADP$^+$-linked malic enzymes might be different; these two enzymes were different in enzymatic properties and elution profile on Sephadex G-100 chromatography (Tanabe, unpublished data).

This communication deals with subcellular localization of NAD$^+$- and NADP$^+$-linked malic enzymes in *T. vaginalis*.

## Materials and Methods

*Parasite:* *T. vaginalis* (strain 73) was cultivated stationarily in a Erlenmeyer flask containing 120 ml of the Asami medium (Asami, 1952). Detailed procedures of cultivation and harvesting of the parasites have been described previously (Tanabe, 1979).

The parasites were washed and finally suspended in 5 mM Tris-Cl, pH 7.4 containing 250 mM sucrose and 1 mM 2-mercaptoethanol at a ratio of 1 ml of packed cell per 9 ml of the Tris-buffered sucrose and disrupted for 2 min using a glass homogenizer with a Teflon pestle.

*Chemicals:* Crystalline lactic dehydrogenase from rabbit muscle and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO. USA). NAD$^+$ and NADP$^+$ were obtained from Oriental Yeast Co. (Tokyo, Japan). Other chemicals were of the highest purity commercially available.

*Enzyme assay:* NAD$^+$- and NADP$^+$-linked malic enzymes were assayed at room temperature either by measuring the initial velocity of NAD$^+$ or NADP$^+$ reduction spectrophotometrically with Hitachi Perkin Elmer 139 type spectrophotometer, or by enzymatic determination of the reaction product (pyruvate) using the neutralized supernatant fluid of perchloric acid extracts of the assay mixture.

The standard assay mixture of NAD$^+$-

linked malic enzyme contained 2 mM L-malate, 330 $\mu$M $NAD^+$, 1 mM $MnCl_2$, 5–20 $\mu$g of the enzyme protein and 66.7 mM glycine buffer, pH 8.6 in a final volume of 3.0 ml. The standard assay mixture of $NADP^+$-linked malic enzyme contained 1 mM L-malate, 100 $\mu$M $NADP^+$, 1 mM $MnCl_2$, 30–150 $\mu$g of the enzyme protein and 66.7 mM imidazole buffer, pH 6.2 in a final volume of 3.0 ml.

One unit of the enzyme was defined as the amount needed for reduction of 1 micromole of $NAD(P)^+$ or formation of 1 micromole of pyruvate per min.

*Other assays:* Protein was determined by Folin-phenol procedure (Lowry *et al.*, 1951) using bovine serum albumin as a standard. Pyruvate was determined by enzymatic method using lactic dehydrogenase (Czok and Lamprecht, 1974).

*Isolation of subcellular fractions:* The homogenate of the parasites was fractionated by differential centrifugation as follows: a nuclear fraction (600 g×7 min sediment), a large granule fraction (13,000 g×10 min sediment), a small granule fraction (105,000 g×90 min sediment) and a final supernatant fraction (105,000 g×90 min supernatant). All manipulations were done at 4 C.

*Sucrose density gradient:* Only the 13,000 g sediment was analyzed by this technique. A 13.5 ml discontinuous sucrose gradient (65, 60, 58, 55, 50, 45, 40, 30 and 20 %) was prepared in 5 mM Tris-Cl buffer, pH 7.4 containing 5 mM 2-mercaptoethanol. Approximately 1 ml of the suspended 13,000 g sediment was placed on the gradient and was centrifuged at 72,000 g for 3 hours at 4 C with a swinging bucket rotor (Hitachi RPS25-3 type, Hitachi, Tokyo, Japan). Each band was isolated by a density gradient fractionator (ISCO, Nebraska, USA), and was used for enzyme assay. All procedures were done at 4 C.

*Electron microscopical observation:* Subcellular fractions were fixed with buffered glutaraldehyde, and then collected and washed by centrifugation. The pellets were postfixed in 1 % osmium tetroxide buffered with 100 mM cacodylate, pH 7.4. All samples were dehydrated and then embeded in Epon. Thin sections were made with Poter-Blum MT 2-B ultramicrotome using a diamond knife, and were observed with Hitachi HU-12AS electron microscope following staining with uranyl acetate and lead nitrate.

## Results

*Subcellular distribution of $NAD^+$- and $NADP^+$-linked malic enzymes:* A typical distribution pattern of $NAD^+$- and $NADP^+$-linked malic enzymes in *T. vaginalis* is summarized in Table 1. The activities of $NAD^+$- and $NADP^+$-linked malic enzymes were primarily associated with the 13,000 g sediment (the large granule) fraction. The specific

Table 1 Subcellular distribution of $NAD^+$-and $NADP^+$-linked malic enzymes after differential centrifugation of *Trichomonas vaginalis* homogenate

| Fraction | Total volume (ml) | Total protein (mg) | $NAD^+$-linked malic enzyme | $NADP^+$-linked malic enzyme |
|---|---|---|---|---|
| | | | $\mu$moles of pyruvate formed/min/mg protein | |
| Whole homogenate | 12 | 36.5 | 0.53 | 0.17 |
| 600g sediment | 5 | 11.0 | 0.42 | 0.19 |
| 13,000g sediment | 6 | 6.6 | 1.26 | 0.37 |
| 105,000g sediment | 6 | 3.2 | 0.02 | 0.02 |
| 105,000g supernatant | 10 | 14.6 | 0.04 | 0.01 |

The enzymatic activity was assayed by enzymatic determination of pyruvate using neutralized supernatant fluid of perchloric acid extracts of the assay mixture, after enzymatic reaction was conducted for 2 min. Details of assay are given in the text.

activities of NAD$^+$- and NADP$^+$-linked malic enzymes in this fraction increased 1.8-fold and 2.1-fold respectively. Based on this finding, the 13,000 g sediment was used for further analysis.

The large granule fraction isolated by differential centrifugation was further fractionated by isopycnic centrifugation in a discontinuous sucrose gradient. A typical distribution pattern of the components in the 13,000 g sediment was schematically represented in Fig. 1. Nine bands and a pellet were isolated and designated as P-I to P-X. It is shown in Fig. 2 that most activities of NAD$^+$- and NADP$^+$-linked malic enzymes were primarily associated with P-VII, P-VIII and P-IX.

Electron microscopical observations exhibited presence of hydrogenosome-like dense

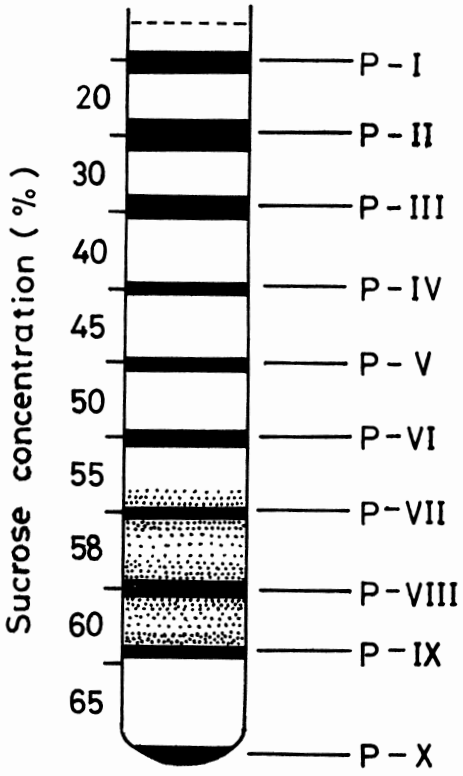


Fig. 1 Distribution of the components after discontinuous sucrose gradient centrifugation of the large granule fraction of *Trichomonas vaginalis*.

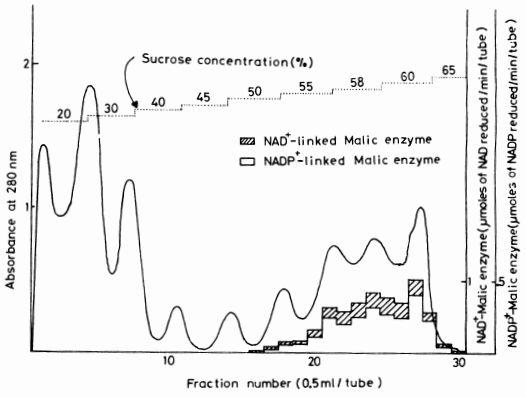


Fig. 2 Distribution of NAD$^+$- and NADP$^+$-linked malic enzymes after discontinuous sucrose gradient centrifugation of the large granule fraction of *Trichomonas vaginalis*.

The enzymatic activity was assayed by spectrophotometric method. Details of fractionation and assay are given in the text.

granules, membranous structures, small vesicles and particles containing various inclusion bodies in the 13,000 g sediment fraction (Photo. 1). It was also demonstrated that P-VII, P-VIII and P-IX mainly consisted of the hydrogenosome-like granules (Photo. 2). High magnification analysis of hydrogenosome-like granule demonstrated that this granule was limited by a single unit membrane and filled with a finely granular matrix.

*Latency of NAD$^+$- and NADP$^+$-linked malic enzymes:* In Table 2 are shown the results of study on latency of NAD$^+$- and NADP$^+$-linked malic enzymes in the large granule fraction. The activities of these two enzymes were very low when the particles were osmotically protected by 250 mM sucrose. In contrast, addition of Triton X-100 resulted in marked increase of these enzymatic activities. The specific activities of NAD$^+$- and NADP$^+$-linked malic enzymes increased 1.9-fold and 2.5-fold respectively. Sonic oscilation of the large granule fraction also resulted in marked increase of the activities of NAD$^+$- and NADP$^+$-linked malic enzymes. However, Triton X-100 failed to increase the activities of these enzymes in the large

Table 2 Effect of Triton X-100 on $NAD^+$- and $NADP^+$-linked malic enzymes in the large granule fraction osmotically protected in 250 mM sucrose

| | Concentration of Triton X-100 in reaction mixture (%) | $NAD^+$-linked malic enzyme | $NADP^+$-linked malic enzyme |
|---|---|---|---|
| | | $\mu$moles of $NAD(P)^+$ reduced/min/mg protein | |
| Control assay mixture | | 0.83 | 0.21 |
| + Triton X-100 | 0.1 | 1.59 | 0.51 |
| + Triton X-100 | 0.3 | 1.52 | 0.41 |
| + Triton X-100 | 0.5 | 1.51 | 0.38 |

The enzymatic activity was assayed by spectrophotometric method. Control assay mixture was the same as the standard assay mixture given in the text except for adding 250 mM sucrose.

Table 3 Effect of sonic oscilation on $NAD^+$- and $NADP^+$-linked malic enzymes in the large granule fraction osmotically protected in 250 mM sucrose

| | Concentration of Triton X-100 in reaction mixture (%) | $NAD^+$-linked malic enzyme | $NADP^+$-linked malic enzyme |
|---|---|---|---|
| | | $\mu$moles of $NAD(P)^+$ reduced/min/mg protein | |
| Large granule fraction | 0 | 0.82 | 0.25 |
| | 0.1 | 1.53 | 0.53 |
| Large granule fraction + sonic oscilation | 0 | 1.28 | 0.39 |
| | 0.1 | 1.25 | 0.42 |

The enzymatic activity was assayed by spectrophotometric method. Details of assay are given in the text and the legend to Table 2.

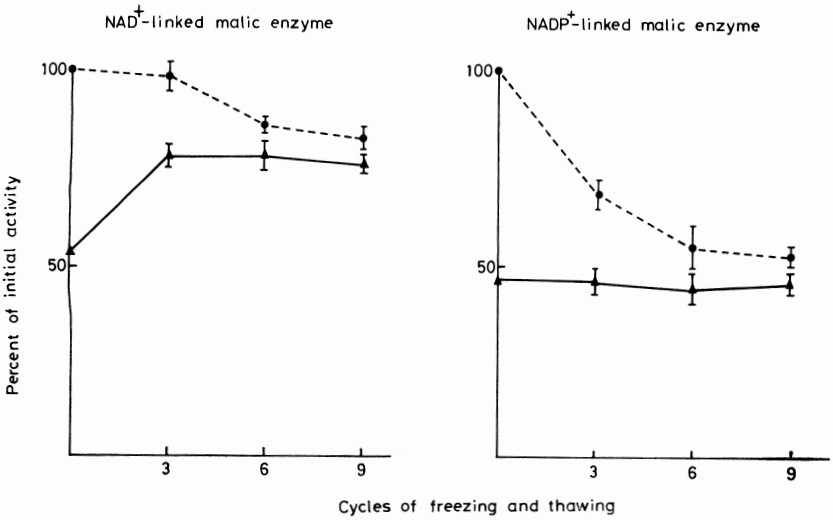


Fig. 3 Effect of freezing and thawing on $NAD^+$- and $NADP^+$-linked malic enzymes in the large granule fraction osmotically protected in 250 mM sucrose.

The enzymatic activity was assayed by spectrophotometic method. Details of assay are given in the text. Total activity (dotted line) was determined in presence of 250 mM sucrose and 0.1 % Triton X-100, and free activity (solid line) was assayed in presence of 250 mM sucrose.

granule fraction previously treated with sonic oscilation (Table 3).

As shown in Fig. 3, repeated freezing and thawing resulted in gradual decrease of the total activities of $NAD^+$- and $NADP^+$-linked malic enzymes, which were assayed in presence of 250 mM sucrose and 0.1% Triton X-100, in the large granule fraction. In contrast, the free activities of these enzymes, which were assayed without Triton X-100, were not changed ($NADP^+$-linked malic enzyme) or increased ($NAD^+$-linked malic enzyme) by this treatment. Moreover, it was also found that Triton X-100 had no effect on $NAD^+$- and $NADP^+$-linked malic enzyme activities when the large granule fraction was treated with nine cycles of freezing and thawing.

These results suggest that $NAD^+$- and $NADP^+$-linked malic enzymes are contained in a membrane-limited particle sensitive to detergent, freezing and thawing, or sonic oscilation and that the membrane of this particle seemed intact even after isolated by differential centrifugation.

## Discussion

Although electron microscopical observations have demonstrated that *T. vaginalis* and other trichomonad flagellates lack mitochondria, the trichomonads contained microbody-like granules in their cytoplasm (Nielsen *et al.*, 1966; Müller, 1973). Physiological functions of this cytoplasmic organelle had been unknown until Müller and Lindmark first reported on the biochemical characterization of this organelle in *Trichomonas foetus* (Müller, 1973; Lindmark and Müller, 1973). They isolated this granule by differential and isopycnic centrifugation of *T. foetus* homogenate prepared in 250 mM sucrose, and demonstrated the presence of hydrogenase as well as other enzymes in this granule. They called, therefore, this granule a hydrogenosome. Lindmark *et al.* (1975) also demonstrated the existence of hydrogenase, pyruvate synthase and $NAD(P)^+$-linked malic enzyme in the microbody-like granules in *T. vaginalis*, and suggested that this granule was probably identical with the hydrogenosome in *T. foetus*.

Tanabe and Asami (1975) have demonstrated that $NAD^+$- and $NADP^+$-linked malic enzymes are associated with the large granule fraction of *T. vaginalis*. The purified $NAD^+$-linked malic enzyme could not utilize $NADP^+$ as a coenzyme (Tanabe *et al.*, 1979). Moreover, there were marked differences between $NAD^+$- and $NADP^+$-linked malic enzymes with respect to the optimal pH, solubility in ammonium sulfate solution, sensitivity to heavy metal ions, effect of divalent cations and elution profile on Sephadex G-100 chromatography (Tanabe and Asami, 1975; Tanabe, unpublished data). These findings suggest that these two enzymes are different.

Present studies indicate that $NAD^+$- and $NADP^+$-linked malic enzymes are mainly present in the hydrogenosome-like granule of *T. vaginalis*. Moreover, these two enzymes exhibited structure-bound latency which can be suppressed by Triton X-100. Sonic oscilation and repeated freezing and thawing also could suppress their latency. Lindmark and Müller (1973) observed that Triton X-100 or repeated freezing and thawing markedly suppressed the latency of pyruvate synthase and $NAD(P)^+$-linked malic enzyme in the hydrogenosome of *T. foetus*. These evidences, therefore, suggest that the hydrogenosome-like granule containing $NAD^+$- and $NADP^+$-linked malic enzymes is limited by a membranous structure that appears to function as a permeable barrier to L-malate or $NAD(P)^+$. This is further confirmed by electron microscopical observation that the hydrogenosome-like granule is limited by a single unit membrane. All of these findings also indicate the presence of the hydrogenosome in *T. vaginalis* as demonstrated by Lindmark *et al.* (1975).

Čerkasovová *et al.* (1973) demonstrated that the isolated hydrogenosome from *T. foetus* was able to reduce oxygen upon addition of either pyruvate or malate and $NAD^+$. It is suggested that L-malate is converted to pyruvate by malic enzyme and thus pyruvate

becomes the final substrate for oxygen uptake (Müller, 1976). In addition, it has been also shown that the aerobic metabolism of pyruvate appears to be linked with a substrate level phosphorylation (Čerkasovovà and Čerkasov, 1976; Čerkasov *et al.*, 1978). It is, therefore, likely that $NAD^+$- and $NADP^+$-linked malic enzymes may participate in regulating the pyruvate level functioning in energy conservation in the hydrogenosome of trichomonad flagellates.

## Summary

*Trichomonas vaginalis* contains two types of malic enzymes, i. e., $NAD^+$- and $NADP^+$-linked malic enzymes, differing in enzymatic properties and elution profile on Sephadex G-100 chromatography. The activities of $NAD^+$- and $NADP^+$-linked malic enzymes were largely associated with the 13,000 g sediment fraction isolated by differential centrifugation of *T. vaginalis* homogenate prepared in 250 mM sucrose. Isopycnic centrifugation of 13,000 g sediment clearly indicated that $NAD^+$- and $NADP^+$-linked malic enzymes were mainly present in the high density sucrose fractions (P-VII, P-VIII and P-IX). Electron microscopical observation clearly demonstrated that these three fractions mainly consisted of the hydrogenosome-like granules. These evidences suggest that $NAD^+$- and $NADP^+$-linked malic enzymes are probably located in the hydrogenosome-like granule in *T. vaginalis*.

$NAD^+$- and $NADP^+$-linked malic enzymes exhibited structure-bound latency which can be suppressed by a non-ionic detergent, Triton X-100. Sonic oscilation and repeated freezing and thawing also could suppress their latency. These findings suggest that the particles containing $NAD^+$- and $NADP^+$-linked malic enzymes are limited by a membranous structure that appears to function as a permeable barrier to L-malate or $NAD(P)^+$.

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## 腟トリコモナスの NAD$^+$-linked malic enzyme および NADP$^+$-linked malic enzyme の細胞内局在性について

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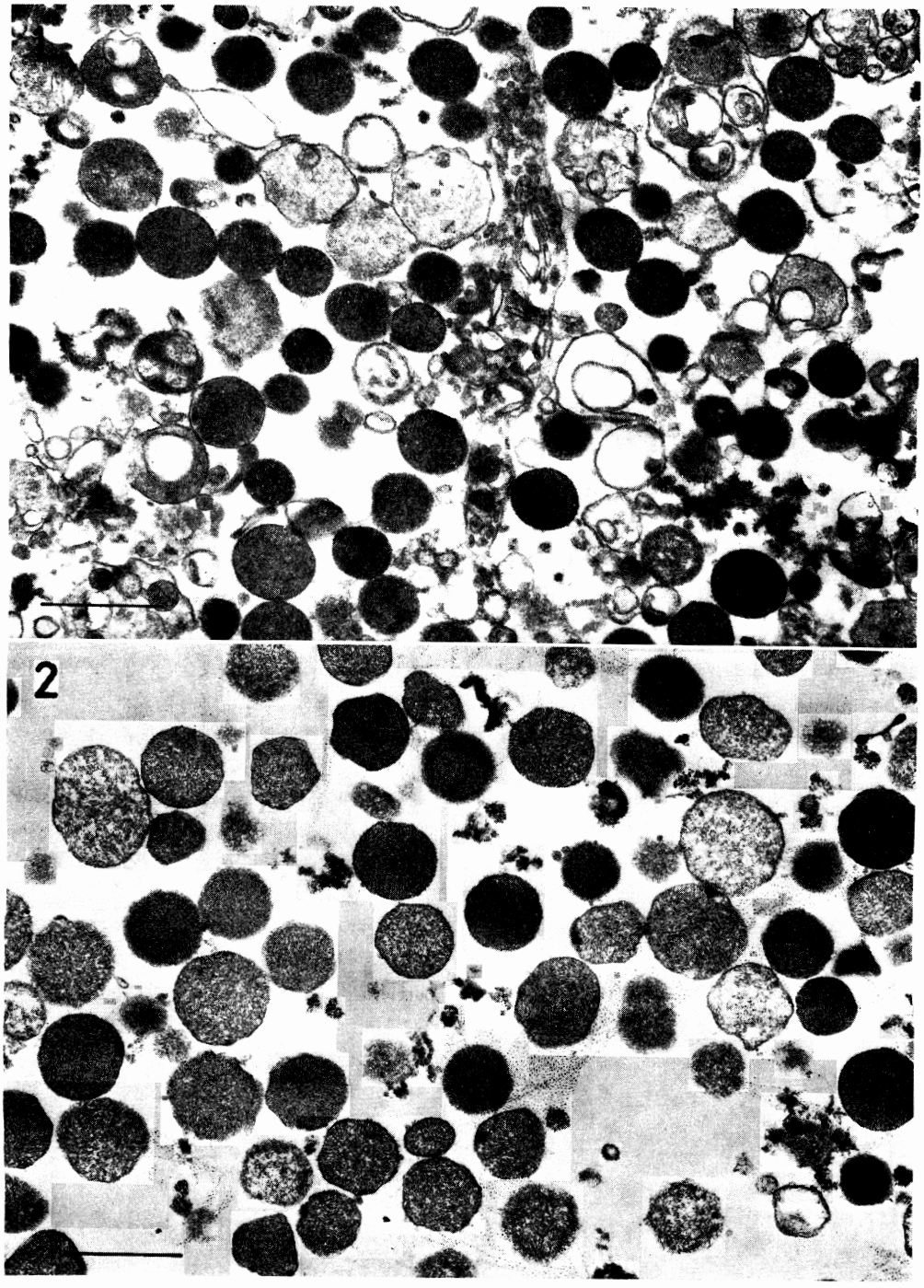
腟トリコモナスのリンゴ酸代謝に直接関連する酵素である NAD$^+$-linked malic enzyme（以下 NAD-ME と略）と NADP$^+$-linked malic enzyme（以下 NADP-ME と略）の細胞内局在性を検討し，以下の成績を得た．

(1) NAD-ME および NADP-ME は共に遠心分画法で得た 13,000 g 沈渣分画（大顆粒分画）に主として局在していた．この大顆粒分画をさらに discontinuous ショ糖密度勾配遠心法（65, 60, 58, 55, 50, 45, 40, 30, 20 %）により分画したところ，10 画分（ショ糖濃度の低い方から P-I, P-II, -----, P-X）が分離された．両酵素は共に P-VII, P-VIII および P-IX 画分にのみ検出され，しかもほぼ同一の分布様式を示した．P-VII, P-VIII および P-IX 画分の超微形態学的観察から，両酵素が共に腟トリコモナスの細胞質に存在する hydrogenosome-like dense granule に局在することが示唆された．

(2) 大顆粒分画中の NAD-ME および NADP-ME 活性は共に等張反応液中では低いが，反応液への Triton X-100 の添加は著明にその活性を増大した．また大顆粒分画を音波あるいは凍結融解処理することによつても両酵素活性の増大が見出された．このことは，NAD-ME および NADP-ME を含む細胞質内顆粒が permeable barrier として機能する膜様構造物により限界されていることを示唆しているものと考えられた．




Photos. 1 and 2 Electron micrographs of the large granule fraction, and P-VIII fraction isolated by isopycnic centrifugation.

Scale marker represents 1 $\mu$m. (1) The 13,000 g sediment fraction after differential centrifugation. (2) The P-VIII fraction isolated by isopycnic centrifugation.