[Jap. J. Parasit., Vol. 28, No. 5, 355-361, 1979]

Alcohol Dehydrogenase Activities in *Trichomonas vaginalis*

TSUTOMU TAKEUCHI, SEIKI KOBAYASHI, MASANOBU TANABE, YOSHIMASA KANEDA, SACHIO MIURA, MITSUKI MASUDA AND KEIZO ASAMI

Department of Parasitology, School of Medicine, Keio University Shinjuku, Tokyo 160, Japan

(Received for publication; May 8, 1979)

Although a number of enzymes of glycolysis have been identified in *Trichomonas vaginalis* (von Brand, 1973), a putative anaerobe causing human trichomoniasis, no enzymes involved in alcohol metabolism have been detected. This has been compatible with previous observation that trichomonads produced organic acids such as pyruvate, lactate, acetate and succinate but not ethanol as glycolytic endproducts in addition to CO_2 (Müller, 1976; Gutteridge and Coombs, 1977), although a tiny difference of pattern of endproduct formation has been observed between *T. vaginalis* and *T. foetus* (Gutteridge and Coombs, 1977).

Recent works on alcohol metabolism in *Entamoeba histolytica* (Reeves *et al.*, 1971; Lo and Reeves, 1978), another putative anaerobe causing human amoebiasis, however, led us to study alcohol metabolism of *T. vaginalis*. Similar characteristics of metabolic pathways between *E. histolytica* and trichomonads such as functioning of ferredoxin in various enzymatic reactions (Weinbach *et al.*, 1976; Reeves *et al.*, 1977; Takeuchi, unpublished observation) as well as absence of aerobic respiratory enzyme systems (Lwoff, 1951; Weinbach *et al.*, 1977; Read, 1957; Wellerson *et al.*, 1959; Jírovec and Petru, 1968) also prompted us to investigate if *T. vaginalis* had a metabolic pathway of alcohol production similar to that of *E. histolytica*.

In the present communication, we report results of identification and partial characterization of alcohol dehydrogenases of *T. vaginalis*.

Materials and Methods

Parasite: *T. vaginalis* (73 strain) was grown in the Asami medium (Asami, 1952) as described by Tanabe (1979). After 24 hour-cultivation at 37 C, the parasites were harvested and washed twice in tris-buffered sucrose (0.2 M tris-HCl, pH 7.4 containing 0.25 M sucrose) by centrifugation at 500 g for 5 min. Finally the parasites were suspended in the tris-buffered sucrose so that the protein concentration was approximately 4 mg/ml. All of these procedures were done at 4 C.

Reagents: NAD^+, $NADP^+$, NADH, NADPH and palmitoyl CoA were supplied by the Sigma (St. Louis, Mo.). Pentachlorophenol (PCP) was obtained from the Tokyo Kasei Co. (Tokyo, Japan). Other chemicals were of the highest purity commercially available.

Assay procedures: An enzyme activity of alcohol dehydrogenase(s) was assayed by determining the initial velocity of either NAD(P)$^+$ reduction or NAD(P)H oxidation spectrophotometrically.

The assay mixture for $NAD(P)^+$ reduction contained 25 mM substrate (mainly isopropanol), 1 mM $NAD(P)^+$, the enzyme preparation (0.3–0.5 mg protein) and 70 mM HEPES pH 7.4 in a final volume of 1 ml. After the endogenous reduction of the nucleotide, which was practically zero, was recorded at 20 C, the enzymatic reaction was initiated by adding substrate. Absorbance change at 340 nm was continuously monitored with the Hitachi 556 recording spectrophotometer (Hitachi Co., Tokyo, Japan).

The assay mixture for NAD(P)H oxidation contained 0.25 mM NAD(P)H, 20 mM of either acetaldehdye or acetone, the enzyme preparation (0.3–0.5 mg protein) and 70 mM HEPES pH 7.4 in a final volume of 1 ml. The enzymatic NAD(P)H oxidation was also started by adding susbtrate, after the endogenous oxidation was recorded in the same manner as above. The enzymatic activity was calculated based on the extinction coefficient of NAD(P)H minus $NAD(P)^+$ at 340 nm, i. e., $e=6.22\ mM^{-1},\ cm^{-1}$.

Protein concentration was determined either by the spectrophotometric method of Layne (1957) or by Lowry's folin-phenol procedure (Lowry *et al.*, 1951).

Enzyme preparation: Immediately after harvested, trichomonads were disrupted by homogenization using a glass homogenizer loosely fitted with a teflon pestle for approximately 4 min, and the homogenate was centrifuged at 15,000 g for 20 min. Ammonium sulfate was added to the isolated supernatant fluid to make 40% saturation with stirring. After stirred for 60 min, the sample was centrifuged at 15,000 g for 15 min, and the supernatant fluid was brought to 90% saturation of ammonium sulfate in the same manner as above. The resulting pellet was isolated by centrifugation as above, dissolved in 3 ml of 50 mM tris-HCl, pH 7.4 and dialyzed against 1 liter of the same buffer overnight. The dialyzed fraction was used as the enzyme preparation. All of these procedures were done at 4 C.

Results

Functioning of alcohol dehydrogenase(s) in *T. vaginalis* is evident as demonstrated in Table 1. Among the alcohols tested, isopropanol elicited $NADP^+$ reduction but not NAD^+ reduction, whereas methanol, ethanol and n-propanl did not stimulate either NAD^+ or $NADP^+$ reduction. On the other hand, both acetaldehyde and acetone enhanced NADH as well as NADPH oxidation. Particularly, NADH oxidation elicited by ace-

Table 1 Activity of alcohol dehydrogenases in *Trichomonas vaginalis*

Assay mixtures	$NAD(P)^+$ reduced or NAD(P)H oxidized
	μmole/mg protein/min
NAD^+ 1 mM, ethanol 25 mM	0
NAD^+ 1 mM, isopropanol 25 mM	0
NAD^+ 1 mM, methanol 25 mM	0
NAD^+ 1 mM, n-propanol 25 mM	0
$NADP^+$ 1 mM, ethanol 25 mM	0
$NADP^+$ 1 mM, isopropanol 25 mM	0.066
$NADP^+$ 1 mM, methanol 25 mM	0
$NADP^+$ 1 mM, n-propanol 25 mM	0
NADH 0.25 mM, acetaldehyde 20 mM	0.050
NADH 0.25 mM, acetone 20 mM	0.210
NADPH 0.25 mM, acetaldehyde 20 mM	0.021
NADPH 0.25 mM, acetone 20 mM	0.029

Values are averages of at least two independent determinations.

tone was much faster than other activities. These findings indicate that both NAD^+- and $NADP^+$-dependent alcohol dehydrogenase are functional in *T. vaginalis*. Moreover, it is likely that NAD^+- and $NADP^+$-dependent isopropanol dehydrogenase activities are catalyzed by at least two independent enzymes, since only $NADP^+$ was reduced by isopropanol, while both NADH and NADPH were oxidized by adding acetone. Thus, it is probable that the equilibrium of NAD^+-dependent isopropanol dehydrogenase much favors NADH oxidation, and the $NADP^+$-dependent enzymatic reaction possibly favors $NADP^+$ reduction. The data on methanol activation of NADPH oxidation by either acetaldehyde or acetone (Table 3), which will be discussed later in this communication, suggest that these two reactions are catalyzed by different enzymes. Thus, it is likely that *T. vaginalis* has at least three independent alcohol dehydrogenases, i. e., $NADP^+$-isopropanol dehydrogenase, $NADP^+$-ethanol dehydrogenase and NAD^+-isopropanol dehydrogenase. It is not known, however, if NADH oxidation by acetaldehyde is also catalyzed by a different enzyme. Based on these findings, the present communication primarily concerns with $NADP^+$-dependent alcohol dehydrogenases.

Inhibition studies of these activities indicated that both $NADP^+$-ethanol and $NADP^+$-isopropanol dehydrogenase were extremely sensitive to palmitoyl CoA. Addition of 0.2 mM palmitoyl CoA almost abolished these enzyme activities (Table 2). Various transient metal-chelators were also inhibitory to $NADP^+$-dependent alcohol dehydrogenases. Among the chelators tested, bathophenanthroline inhibited the activity of these enzymes by 60–70 % at 1 mM. Salicylaldoxime and o-phenanthroline were less inhibitory than bathophenanthroline. Pentachlorophenol, a potent uncoupler of mitochondiral oxidative phosphorylation, also inhibited these enzyme activities. PCP at 0.5 mM almost abolished the enzyme activities. Inhibitions by these compounds were observed at other concentrations of isopropanol, i. e., 12.5 mM and 8.5 mM.

Table 2 Inhibition of $NADP^+$-dependent alcohol dehydrogenases of *Trichomonas vaginalis* by palmitoyl CoA, transient metal-chelators and PCP

Assay mixtures	$NADP^+$ reduced or NADPH oxidized
	μmole/mg protein/min
$NADP^+$ 1 mM, isopropanol 25 mM	0.066
+ Palmitoyl CoA 0.2 mM	0
+ Bathophenanthroline 1 mM	0.029
+ o-Phenanthroline 5 mM	0.044
+ Salicylaldoxime 5 mM	0.029
+ PCP 0.5 mM	0
NADPH 0.25 mM, acetaldehyde 20 mM	0.029
+ Palmitoyl CoA 0.2 mM	0
+ Bathophenanthroline 1 mM	0.009
+ o-Phenanthroline 5 mM	0.009
+ Salicylaldoxime 5 mM	0.020
+ PCP 0.5 mM	0.009

o-Phenanthroline and salicylaldoxime were dissolved in ethanol, and PCP in methanol. Effect of ethanol added together with salicylaldoxime was negligible, since only 10 ul of this compound (0.5 M solution) was added. Under this condition, the enzyme activity was not affected. In other experiments, effect of alcohols was subtracted from the data.

Effect of various alcohols including isopropanol is also of interest in order to elucidate the catalytic properties of these enzymes. As was demonstrated in Table 3, addition of methanol at high concentrations activated $NADP^+$ reduction elicited by isopropanol as well as NADPH oxidation by acetone, but not NADPH oxidation by acetaldehyde. As was mentioned earlier in this communication, this finding suggests that NADPH oxidation elicited either by acetone or acetaldehyde is catalyzed by different enzymes. Other alcohols, i. e., ethanol and n-propanol, also enhanced the forward and the reverse reaction of $NADP^+$-isopropanol dehydrogenase, but were slightly less effective than methanol. On the other hand, isopropanol was potently inhibitory to both NADPH oxidation by acetaldehyde and that by acetone. This might be a kind of product inhibition. It is still interesting, however, that isopropanol also inhibits NADPH oxidation elicited by acetaldehyde, although the finding mentioned above suggest that NADPH oxidation by acetaldehyde or acetone is catalyzed by different enzymes. Regarding the possibility that methanol is oxidized in the presence of isopropanol, it is unlikely that isopropanol activates methanol oxidation, since methanol did not stimulate $NADP^+$ reduction at any concentrations tested. The following kinetic analysis on the effect of methanol on $NADP^+$-isopropanol dehydrogenase seems to confirm this.

Kinetic analysis of $NADP^+$-isopropanol dehydrogenase revealed that Km values for $NADP^+$ and isopropanol were 0.45 mM and 8.5 mM respectively. Vmax was approximately 0.09 μmole $NADP^+$ reduced/mg protein/min at the present assay conditions. Substrate inhibition by high concentrations of isopropanol was, however, observed. This

Table 3 Effect of non-metabolized alcohols on $NADP^+$-dependent alcohol dehydrogenases of *Trichomonas vaginalis*

Assay mixtures	$NADP^+$ reduced or NADPH oxidized
	μmole/mg protein/min
$NADP^+$ 1 mM, isopropanol 25 mM	0.066
+ methanal 10%	0.100
+ methanol 20%	0.132
+ ethanol 5%	0.105
+ ethanol 10%	0.113
+ n-propanol 10%	0.083
NADPH 0.25 mM. acetone 20 mM	0.029
+ methanol 10%	0.083
+ methanol 20%	0.066
+ isopropanol 0.5%	0.016
+ isopropanol 10%	0
+ ethanol 10%	0.050
NADPH 0.25 mM, acetaldehyde 20 mM	0.025
+ methanol 5%	0.028
+ methanol 10%	0.028
+ ethanol 5%	0.030
+ ethanol 10%	0.030
+ isopropanol 5%	0.003
+ isopropanol 10	0

Percentages of alcohols stand for final concentrations. These alcohols were present in the assay mixture before the reaction was started by adding substrates.

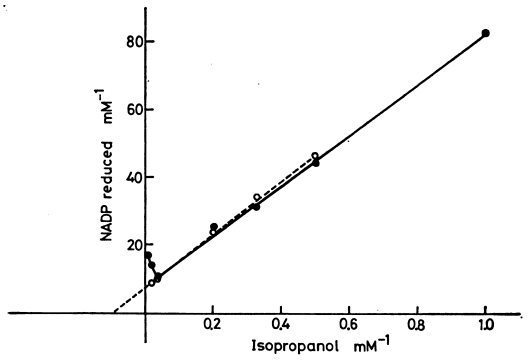

Fig. 1 Kinetic Analysis of NADP$^+$-isopropanol dehydrogenase of *Trichomonas vaginalis*. Solid line represents data from the assay conducted in the absence of methanol, and dotted line with open circle stands for data in the presence of 10% methanol. Methanol was added to the assay mixture before the reaction was started by adding isopropanol. Other details as in the text.

was apparent at more than 20 mM isopropanol. Kinetic assay in the presence of methanol at 10% suggested that this substrate inhibition of NADP$^+$-isopropanol dehydrogenase was recovered by methanol. Fig. 1 demonstrates that in presence of methanol the substrate inhibition of the enzyme activity disappeared, although Km for isopropanol was not affected. Km for NADP$^+$ was also not affected by methanol.

Discussion

Previous investigations performed in order to identify endproducts of glycolysis of trichomonads indicated that organic acids like acetate and succinate, and carbon dioxide were mainly produced. No enzymes involved in alcohol metabolism have been identified, although *E. histolytica*, which has been found to have several basic metabolic pathways in common with trichomonads, exhibited high alcohol dehydrogenase activities. The present findings on alcohol dehydrogenase in *T. vaginalis*, however, presented cogent evidences on functioning of several alcohol dehydrogenases in this parasite, and thus confirmed further similarity between trichomonads and *E. histolytica* particularly in that a high NADP$^+$-dependent isopropanol dehydrogenase was functional (Reeves *et al.*, 1971). These observations also raised a possibility that ethanol and possibly isopropanol might be endproducts of glycolysis of this parasite, although previous studies have not identified these compounds.

The observation that NAD$^+$- and NADP$^+$-dependent isopropanol dehydrogenase reaction are catalyzed by different enzymes seems to be compatible with that in *E. histolytica* (Takeuchi, unpublished observation). Lo and Reeves (1978) also postulated NAD$^+$- and NADP$^+$-dependent alcohol dehydrogenase in amoeba catalyzed independent reactions. Moreover, functioning of distinct NADP$^+$-ethanol and NADP$^+$-isopropanol dehydrogenase, which was postulated in this study, seems to be compatible with alcohol dehydrogenases previously investigated in various materials. For instance, the horse liver alcohol dehydrogenase catalyzes only oxidation of primary alcohols (Sund and Theorell, 1963), and secondary chain alcohol dehydrogenase is sometimes involved in a different entity (Okunuki, 1966).

Inhibition of the trichomonad NADP$^+$-alcohol dehydrogenases by various transient metal-chelators suggests that these enzymes are metallo-enzymes. This is also compatible with the previous observations that alcohol dehydrogenases contain zinc as its prosthetic group (Brändén *et al.*, 1975). It is not known, however, what kind of metal is involved in the catalysis of the trichomonad enzymes. The potent inhibitory effect of bathophenanthroline may indicate that iron rather than zinc is involved in the catalysis of these enzymes. Inhibition by palmitoyl CoA has also been observed with other enzymes such as glucose-6-phosphate dehydrogenase (Kawaguchi and Bloch, 1974). The mechanism of PCP inhibition is not known at present, and to our knowledge the trichomonad alcohol dehydrogenase seems to be the first one which was found to be sensitive to this uncoupling agent except for mitochondrial oxidative phosphorylation.

This mechanism is now under investigation in our laboratory.

Recovery of $NADP^+$-isopropanol dehydrogenase from substrate inhibition, which was observed in the presence of high concentration of methanol, seems to be interesting in order to elucidate the catalytic mechanism of this enzyme. To our knowledge, no alcohol dehydrogenases have been found to be enhanced by non-metabolized alcohols other than the amoebal alcohol dehydrogenase (Takeuchi, submitted for publication). Further characterization of the effect of these non-metabolized alcohols on the trichomonads alcohol dehydrogenases is also being conducted and will be presented elesewhere.

Abstract

Alcohol dehydrogenase activity of *Trichomonas vaginalis* was isolated and concentrated by centrifugation and ammonium sulfate precipitation. Among several alcohols tested, only isopropanol elicited $NADP^+$ reduction, while no alcohols stimulated NAD^+ reduction. NADH and NADPH oxidation were enhanced by acetaldehyde as well as by acetone. NADH oxidation by acetone was much faster than other enzyme activities. Thus, NAD^+-isopropanol dehydrogenase reaction much favors acetone reduction suggesting that NAD^+-isopropanol and $NADP^+$-isopropanol dehydrogenase are different enzymes. $NADP^+$-isopropanol dehydrogenase was sensitive to palmitoyl CoA, transient metal-chelators and pentachlorophenol (PCP). Palmitoyl CoA and PCP almost abolished the enzyme activity at 0.2 mM and 0.5 mM respectively. $NADP^+$-ethanol dehydrogenase as determined by NADPH oxidation was also inhibited by these compounds. Kinetic analysis of $NADP^+$-isopropanol dehydrogenase indicated that Km values for isopropanol and $NADP^+$ were 8.5 mM and 0.45 mM respectively. Vmax was approximately 0.09 μmole $NADP^+$ reduced/mg protein/min. Substrate inhibition of $NADP^+$-isopropanol dehydrogenase was evident at more than 20 isopropanol. Investigations on the effect of non-metabolized alcohols on $NADP^+$-ethanol and $NADP^+$-isopropanol dehydrogenase indicated that only $NADP^+$-isopropanol dehydrogenase was activated by methanol, ethanol and n-propanol at higt concentrations. This finding suggests that $NADP^+$-ethanol and $NADP^+$-isopropanol dehydrogenase are also different enzymes. Further kinetic analysis on the effect of methanol on $NADP^+$-isopropanol dehydrogenase showed that the substrate inhibition by isopropanol was recovered by methanol.

References

1) Asami, K. (1952): Bacteria-free cultivation of *Trichomonas vaginalis*. Kitasato Arch. Exp. Med., 25, 149–156.
2) von Brand, T., (1973): Biochemistry of parasites. 2nd ed., Academic Press, New York, 124 pp.
3) Brändén, C-I, Jörnvall, H., Eklund, H. and Furugren, B. (1975): Alcohol dehydrogenases. The Enzymes, 3rd. ed., vol. 11, Academic Press, New York, 156 pp.
4) Gutteridge, W. E. and Coombs, G. H. (1977): Biochemistry of Parasitic Protozoa. Macmillan Press, London, 45–48.
5) Jírovec, O. and Petru, M. (1968): *Trichomonas vaginalis* and trichomoniasis. Advances in Parasitology (Dawes, B., ed.), Academic Press, New York, Vol. 6, 117–188.
6) Kawaguchi, A. and Block, K. (1974): Inhibition of glucose-6-phosphate dehydrogenase by palmitoyl CoA. J. Biol. Chem., *249*, 5793–5800.
7) Layne, E. (1957): Spectrophotometric and turbidmetric methods of measuring proteins. Methods in Enzymology, Academic Press, New York, Vol. 3, 447–454.
8) Lo, H. and Reeves, R. E. (1978): Pyruvate-to-ethanol pathway in *Entamoeba histolytica*. Biochem. J., 171, 225–230.
9) Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951): Protein measurement with folin-phenol reagent. J. Biol. Chem., 193, 265–275.
10) Lwoff, M. (1951): Nutrition of parasitic ameba. Biochemistry and Physiology of Protozoa (Lwoff, A., ed.), Academic Press, New York, 235 pp.
11) Müller, M. (1976): Carbohydrate and energy metabolism of *Tritrichomonas foetus*. Bio-

chemistry of Parasites and Host-parasite Relationships (van den Bossche, H., ed.), North-Holland Publishing Co., 3-14.
12) Okunuki, K. (1966): Secondary alcohol: NAD oxidoreductase. Enzyme Handbook, (Okunuki, K. *et al.*, eds.), Asakura Shoten, Tokyo, 62 pp.
13) Read, C. P. (1957): Comparative studies on the physiology of trichomonad protozoa. J. Parasit., 43, 385-394.
14) Reeves, R. E., Montalvo, F. E. and Lushbaugh, T. S. (1971): Nicotinamide-adenine dinucleotide phosphate-dependent alcohol dehydrogenase; The enzyme from *Entamoeba histolytica* and some enzyme inhibitors. Int. J. Biochem., 2, 55-64.
15) Reeves, R. E., Warren, L. G., Susskind, B. and Lo, H. (1977): An energy conserving pyruvate-to-acetate pathway in *Entamoeba histolytica*, J. Biol. Chem., 252, 726-731.
16) Sund, H. and Theorell, H. (1963): Alcohol dehydrogenases. The Enzymes (Boyer, P. *et al.*, eds.), 2nd ed., Academic Press, New York, 25-83.
17) Tanabe, M. (1979): *Trichomonas vaginalis*: NADH oxidase activity. Exp. Parasit. (in press)
18) Weinbach, E. C., Diamond, L. S., Claggett, C. E. and Kon, H. (1976): Iron-sulfur proteins of *Entamoeba histolytica*. J. Parasit., 62, 127-128.
19) Weinbach, E. C., Harlow, D. R., Claggett, C. E. and Diamond, L. S. (1977): *Entamoeba histolytica*: Diaphorase activities. Exp. Parasit., 41, 186-197.
20) Wellerson, R., Dosher, G. and Kupferberg, A. B. (1959): Metabolic studies on *Trichomonas vaginalis*. Ann. N. Y. Acad. Sci., 83, 253-258.

膣トリコモナスにおけるアルコール脱水素酵素活性について

竹内　勤　小林正規　田辺将信　金田良雅　三浦左千夫　増田光喜　浅見敬三
（慶大医学部寄生虫学教室）

膣トリコモナスにおけるアルコール脱水素酵素活性について研究を行い以下の結果を得た．

1) 活性は 15,000 g×20 min の上清にみられ，40-90 %の硫酸アンモニウム分画により殆んど回収された．

2) 数種のアルコールのうちイソプロパノールのみが $NADP^+$ を還元した．NAD^+ はどのアルコールによつても還元されなかつた．一方，NADPH の酸化はアセトアルデヒド，アセトン両者によつて促進され，NADH の酸化も同様であつたが，アセトンによる NADH の酸化は極めて早く，他の5～10倍の活性を示した．

3) $NADP^+$-イソプロパノール脱水素酵素活性はパルミチル CoA，Transient metal のキレーター，及びペンタクロロフェノールによつて強く阻害された．又逆反応によつて観察した $NADP^+$-エタノール脱水素酵素活性もこれらの化合物により阻害された．

4) $NADP^+$-イソプロパノール脱水素酵素活性はメタノール，エタノール，n-プロパノール等が高濃度に存在する場合，順，逆両反応とも促進された．一方，$NADP^+$-エタノール脱水素酵素活性はこれらのアルコールによつて影響されなかつた．イソプロパノールは両者の逆反応を阻害した．

5) $NADP^+$-イソプロパノール脱水素酵素においては Km NADP＝0.45 mM，Kmisopropanol＝8.5 mM．及び Vmax＝0.09 μmole $NADP^+$ reduced/mg protein/min であつた．又イソプロパノールが 20 mM 以上になると基質阻害が見られた．

6) メタノールの活性化作用についての実験の結果，低濃度の基質を用いた際は活性化作用がみられず，結局イソプロパノールによる基質阻害がメタノールによつて回復されることが判つた．

7) 以上の結果，NAD^+-イソプロパノール，$NADP^+$-イソプロパノール脱水素酵素反応は各々異る酵素で触媒され，又 $NADP^+$-エタノール脱水素酵素反応も $NADP^+$-イソプロパノール脱水素酵素とは異る酵素で触媒されるらしいことが判つた．