Glycerol Oxidation and Triose Reduction by Pyridine Nucleotide-linked Enzymes in the Fission Yeast *Schizosaccharomyces pombe*

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The fission yeast *Schizosaccharomyces pombe* has been shown to produce four separate pyridine nucleotide-linked glycerol dehydrogenases (or triose reductases), distinguished by differences in their coenzyme specificity (NAD^+ or NADP^+) and oxidation product (dihydroxyacetone or glyceraldehyde). Evidence for four separate activities was obtained by heat inactivation studies, comparison of cells grown under different conditions, and separation and partial purification of the enzymes. One enzyme, a glycerol: NAD^+ 2-oxidoreductase is repressed by glucose but not induced by glycerol and appears to function primarily in glycerol catabolism. The second, a glycerol: NADP^+ 2-oxidoreductase is stimulated by growth on glucose and appears to function as a dihydroxyacetone reductase involved in glycerol synthesis. The third has the properties of a glycerol: NADP^+ oxidoreductase, while the fourth is, in fact, alcohol dehydrogenase (alcohol: NAD^+ oxidoreductase) which possesses weak activity as a glycerol: NAD^+ 1-oxidoreductase.

INTRODUCTION

The fission yeast *Schizosaccharomyces pombe* can use glycerol as its sole source of carbon and energy. Until recently it has been generally assumed that all yeasts which utilize glycerol, first convert it to *sn*-glycerol 3-phosphate by glycerol kinase, and then oxidize this product to dihydroxyacetone phosphate by a glycerol-3-phosphate dehydrogenase, as has been shown for several yeasts (Bergmeyer *et al.*, 1961; Gancedo *et al.*, 1968; Sprague & Cronan, 1977). We have shown that a different mechanism operates in *S. pombe*, which first oxidizes glycerol to dihydroxyacetone (DHA) by an NAD^+ -linked glycerol dehydrogenase, and then phosphorylates the triose by a specific DHA kinase (May & Sloan, 1981; May *et al.*, 1982). Our initial studies with two strains of *S. pombe* have now been extended to a further 37 strains, each of which possessed glycerol dehydrogenase but no glycerol kinase (G. Vasiliadis & J. W. May, unpublished results). This alternative route of glycerol utilization has also been reported to occur in some bacteria (Lin, 1976), and in several yeasts in addition to *S. pombe* (Babel & Hofmann, 1982). The discovery of this alternative pathway for glycerol utilization in *S. pombe* prompted us to undertake a more detailed study of its glycerol metabolism. We now present evidence for not one, but four, distinct pyridine nucleotide-linked enzymes which can catalyse the oxidation of glycerol and the reduction of one or both of the trioses, DHA and glyceraldehyde (GLD).

METHODS

Organism. The organism used was *S. pombe* strain 972 h^- , maintained as described previously (May & Sloan, 1981).

Growth of cells and preparation of cell extracts. Methods for growing and harvesting cells and preparing cell extracts have been described (May & Sloan, 1981; May *et al.*, 1982). Larger batches of cells were prepared by

Abbreviations: DHA, dihydroxyacetone; GLD, DL-glyceraldehyde; ADH, alcohol dehydrogenase; GDH, glycerol dehydrogenase (specific enzymes numbered 1 to 4); *gdh*, glycerol oxidizing activity (specific reactions are numbered *gdh1*, oxidation at C_1 , NAD^+ -linked; *gdh2*, oxidation at C_2 , NAD^+ -linked; *gdh3*, oxidation at C_1 , NADP^+ -linked; *gdh4*, oxidation at C_2 , NADP^+ -linked).

growth in one or more (up to six) 14 l fermentation vessels (Quickfit) containing 12 l Edinburgh minimal medium no. 2 (Mitchison, 1970). For some experiments, the carbon and energy source in the medium was modified by replacing the glucose with 1% (v/v) glycerol. Each vessel was inoculated with 120 ml starter culture and incubated in a water bath at 30 °C for 70 h with aeration (1 litre air min⁻¹) and stirring (220 r.p.m.).

Enzyme assays. Enzyme activities were measured at 20 °C in cuvettes containing a total volume of either 3.0 ml or 1.0 ml. Pyridine nucleotide-linked dehydrogenase activities were measured by determining the initial rates of reduction of NAD⁺ or NADP⁺, or oxidation of NADH or NADPH, by following changes in A_{340} with a Perkin Elmer, model 552, double-beam recording spectrophotometer. A value of $6.22 \times 10^{-3} \text{ l mol}^{-1} \text{ cm}^{-1}$ was used for the molar absorption coefficients of NADH and NADPH at 340 nm. Specific activities are given as nmol substrate used min⁻¹ (mg protein)⁻¹.

Glycerol dehydrogenases. Dehydrogenation of glycerol may occur at either C₁ or C₂ producing GLD or DHA respectively and it may be linked to either NAD⁺ or NADP⁺, the reactions possible being:

- (1) Glycerol + NAD⁺ $\rightleftharpoons$ GLD + NADH + H⁺ (*gdh1*)
- (2) Glycerol + NAD⁺ $\rightleftharpoons$ DHA + NADH + H⁺ (*gdh2*)
- (3) Glycerol + NADP⁺ $\rightleftharpoons$ GLD + NADPH + H⁺ (*gdh3*)
- (4) Glycerol + NADP⁺ $\rightleftharpoons$ DHA + NADPH + H⁺ (*gdh4*)

It is not possible to distinguish between (1) and (2) or between (3) and (4) by measuring glycerol oxidation; to do this the reverse reaction, reduction of GLD or DHA, must be measured. Since a proton is involved in these reactions the equilibrium is affected by pH, a high pH favouring glycerol removal and a low pH favouring its formation, but glycerol formation is favoured thermodynamically, the equilibrium constants for these reactions being about 10⁻¹² [e.g. 2.4×10^{-12} calculated for reaction (2) by McGregor *et al.* (1974)]. The activities represented by equations (1) to (4) will be designated *gdh1*, *gdh2*, *gdh3* and *gdh4* respectively, while specific enzymes catalysing predominantly one of these activities will be designated GDH1, GDH2, GDH3 or GDH4. Such enzymes may not necessarily display exclusive specificity for one substrate or one coenzyme.

Glycerol oxidation linked to either NAD⁺ or NADP⁺ was measured by a modification of the method of Ruch *et al.* (1974). Oxidation was favoured by buffering the system at pH 9.5 and including hydrazine to trap the product. The reaction mixture contained the enzyme preparation, 0.6 mM-NAD⁺, 2 mM-(NH₄)₂SO₄, 10 mM-glycerol, 60 mM-hydrazine hydrate and 40 mM-glycine/KOH buffer (pH 9.5).

Triose reduction linked to NADH, though favoured by low pH, could be measured in crude extracts only at pH 9 or higher because of the presence of an NADH oxidase system, which was very active in less alkaline solutions. It was not possible to circumvent the NADH oxidation at pH 6 by the addition of cyanide since this also inhibited triose reductase activity. The assay system contained the enzyme preparation, 80 mM-K₂CO₃/KHCO₃ buffer (pH 9.0), 0.3 mM-NADH and 5 mM-DHA or GLD. The system for measuring triose reduction linked to NADPH contained the enzyme preparation, 50 mM-potassium hydrogen phthalate/KOH buffer (pH 6.0), 0.3 mM-NADPH and 5 mM-DHA or GLD.

Glycerol oxidase. Glycerol oxidase activity was measured by the method of Uwajima *et al.* (1979) which is based on a colorimetric determination of the H₂O₂ formed.

Alcohol dehydrogenase. Alcohol dehydrogenase (ADH) could be measured by the same method as for NAD⁺-linked glycerol oxidation except that glycerol in the assay mixture was replaced by 50 mM-ethanol. It was more commonly measured in the reverse, thermodynamically favoured, direction by a method similar to that used for measuring triose reductase, the assay system containing the enzyme preparation, 80 mM-carbonate buffer (pH 9.0), 0.3 mM-NADH and 5 mM-acetaldehyde.

Protein. The protein content of crude extracts and partly purified preparations was measured by the biuret method (Koch & Putnam, 1971). For solutions which contained little or no UV-absorbing material other than protein, the protein content was assayed spectrophotometrically by measuring A_{214} (Waddell, 1956). Bovine serum albumin was the reference protein for each method.

Electrophoresis. Enzyme preparations were monitored by disc gel electrophoresis following the general method of Davis (1964) using an anionic system at pH 8.3 and 7.5% (w/v) polyacrylamide gel. After electrophoresis, the gels were stained either for protein with Coomassie brilliant blue R, or for enzyme activity with a mixture containing 100 mM-carbonate buffer (pH 9.0), 0.15 mM-NAD⁺ or NADP⁺, phenazine methosulphate (0.025 mg ml⁻¹), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 0.25 mg ml⁻¹) and 50 mM-glycerol or ethanol.

Heat treatment of extracts. This was done in a thermostatically controlled circulating water bath maintained at the desired temperature. The extract (2.0 ml) in a glass tube was immersed in the bath and gently stirred with a magnetic stirrer. After 10 min the tube was rapidly cooled in ice and any precipitate removed by centrifugation.

Enzyme fractionation methods. All steps were done at 0–4 °C.

Step 1. Ethodin treatment. Crude extracts were treated with 6,9-diamino-2-ethoxyacridine lactate (ethodin) to precipitate nucleic acids (Thorner & Paulus, 1971). A 2.5% (w/v) solution in 20 mM-Tris/HCl (pH 7.6) was added

dropwise with stirring to the extract (contained in 100 mM-Tris/HCl, pH 7.6) to a final concentration of 50 mg ethodin to 1 g protein, and stirred overnight. The heavy yellow precipitate was removed by centrifugation at 27 000 g for 30 min leaving a clear yellow solution.

Step 2. Ammonium sulphate precipitation. The supernatant solution from step 1 was fractionated by stepwise precipitation with increasing concentrations of $(\text{NH}_4)_2\text{SO}_4$. Solid $(\text{NH}_4)_2\text{SO}_4$ was added slowly with stirring to the desired concentration and the solution was stirred gently for 30 min. The precipitate was removed by centrifugation. The process was repeated by adding more solid $(\text{NH}_4)_2\text{SO}_4$ to the measured volume of supernatant solution, the amount required to give the next higher chosen concentration being determined by reference to Wood's tables (Wood, 1976). The precipitate was again removed by centrifugation. The most useful steps for fractionation were found to be 1.8 M-, 2.5 M- and 3.8 M- $(\text{NH}_4)_2\text{SO}_4$. Precipitates were dissolved in a small volume of 100 mM-Tris/HCl (pH 7.6). Before testing for enzyme activity solutions were desalted and freed from residual ethodin by passage through a column of Sephadex G-25.

Step 3. Ion exchange chromatography. A column of DEAE-cellulose (Whatman DE 52; 1.5 × 20 cm or other appropriate size) was equilibrated with 20 mM-Tris/HCl (pH 8.0). The sample was applied to the column, and then eluted with stepwise increasing concentrations of KCl (0–1.5 M) in the same buffer. The eluate was collected in 2.0 ml fractions.

Step 4. Gel permeation chromatography. Fractions from step 3 which had enzyme activity were pooled and concentrated to 1.5 ml by ultrafiltration through a membrane filter in a stirred cell (Cell 8050, Diaflo membrane PM 30; Amicon). The sample was loaded on to a Sephadex G-200 column (2.0 × 75 cm) previously equilibrated with 20 mM-Tris/HCl (pH 8.0), eluted with the same buffer and collected in 2.0 ml fractions.

Purification of ADH. ADH from *S. pombe* was isolated and purified from glucose grown cells harvested in the stationary phase by the rapid purification method of Scopes *et al.* (1981), in which most of the cell protein is denatured with a high concentration of ethanol; a purification of 110-fold was achieved.

Chemicals. DHA, GLD, 6,9-diamino-2-ethoxyacridine lactate (ethodin) and Tris were from Sigma; NAD^+ (grade II), NADH (grade II), NADP^+ and NADPH were mainly from Boehringer, with some samples from Sigma; Sephadex gel permeation media were from Pharmacia. Other chemicals were of analytical reagent grade when available and were from BDH or Ajax (Sydney, Australia).

RESULTS

Types of glycerol dehydrogenases

All four types of pyridine nucleotide-linked GDH activity were found in *S. pombe*, i.e. *gdh1*, *gdh2*, *gdh3* and *gdh4*, but we were unable to detect any glycerol oxidase.

Heat stability of enzymes

A comparison was made of the degree of inactivation of the different enzyme activities in extracts which had been heated for 10 min at temperatures up to 80 °C. Representative results are shown in Table 1. The greatest stability was shown by *gdh1* and ADH, both of which retained activity at 80 °C. The *gdh3* activity was the most labile, being partly inactivated at 50 °C and completely inactivated at 60 °C, while *gdh2* and *gdh4* showed little loss of activity at 60 °C but complete inactivation at 70 °C. These results suggest the presence of at least three separate enzymes.

Effect of different growth conditions

The results obtained in a series of experiments in which a comparison was made of enzyme activities in cells grown on either glucose or glycerol, and harvested either during exponential growth or after the culture had reached the stationary phase are shown in Table 2. In cultures grown to the stationary phase on glucose, no glucose remained, but in cultures grown to the stationary phase on glycerol, much glycerol still remained. Glucose-stationary cells (B) and glycerol grown cells, whether exponential or stationary (C) did not differ in their enzyme activities. In comparison to cells (B) and (C), glucose-exponential cells (A) showed very strong (97%) repression of *gdh2* activity, no change in *gdh1*, *gdh3* or ADH and a small (17%) increase in *gdh4*. The NAD^+ -linked glycerol oxidation rate (which includes both *gdh1* and *gdh2*) in cells (A) relative to cells (B) or (C) was consistent with strong repression of *gdh2* while the NADP^+ -linked glycerol oxidation rate (which includes both *gdh3* and *gdh4*) showed a 200% increase in contrast to the relatively small increase (in *gdh4* only) shown by measurements in the reverse direction.

Table 1. *Heat inactivation of glycerol dehydrogenases in cell extracts*

Extracts were held at the specified temperature for 10 min (see Methods). The values equivalent to 100% were 31, 65, 26, 110 and 1500 nmol substrate used min⁻¹ (mg protein)⁻¹ for *gdh1*, *gdh2*, *gdh3*, *gdh4* and ADH respectively.

Activity type	Assay system (substrate/coenzyme)	Oxidoreductase activity remaining (unheated extract = 100)			
		50 °C	60 °C	70 °C	80 °C
<i>gdh1</i>	GLD/NADH	75	60	40	10
<i>gdh2</i>	DHA/NADH	85	80	0	0
<i>gdh3</i>	GLD/NADPH	60	0	0	0
<i>gdh4</i>	DHA/NADPH	90	70	0	0
ADH	Acetaldehyde/NADH	100	100	80	40

Table 2. *Effect of growth conditions on glycerol dehydrogenases*

The oxidoreductase activities were determined in extracts of cells grown on glucose and harvested during the exponential phase (A), of cells grown on glucose and harvested during the stationary phase when glucose was not detectable (B), and of cells grown on glycerol (C). With the latter, specific activities did not change with time of harvesting.

Activity type	Assay system (substrate/coenzyme)	Oxidoreductase activity [nmol substrate used min ⁻¹ (mg protein) ⁻¹]			Activity ratio, A:B
		A	B	C	
<i>gdh1</i>	GLD/NADH	28	26	24	1.1
<i>gdh2</i>	DHA/NADH	1.5	50	50	0.03
<i>gdh3</i>	GLD/NADPH	17	18	17	1.0
<i>gdh4</i>	DHA/NADPH	105	90	90	1.2
<i>gdh1</i> + <i>gdh2</i>	Glycerol/NAD ⁺	3	27	28	0.1
<i>gdh3</i> + <i>gdh4</i>	Glycerol/NADP ⁺	15	5	4	3.0
ADH	Acetaldehyde/NADH	1600	1600	1600	1.0

These results, which show that *gdh2* and *gdh4* activities cannot be due to the same enzyme, taken in conjunction with the relative heat stabilities, make it necessary to postulate the presence of four separate GDH enzymes.

Separation of enzyme activities

Cells containing all four GDH activities were obtained either by growth on glycerol or by growth on glucose to the stationary phase; the latter method gave more rapid growth and a higher cell density. Extracts prepared from such cells were fractionated as described in Methods through step 1 (ethodin treatment) and step 2 (successive precipitation with 1.8M, 2.5M and 3.8M-(NH₄)₂SO₄) to yield three fractions; Table 3 shows the distribution of activities in these fractions. All the *gdh2* activity and most of the *gdh4* activity were found in fraction I (1.8M); *gdh1* and ADH showed highest activity in fraction II (2.5M) with much lower activity in fraction I, while *gdh3* activity was highest in fraction III (3.8M) but with some appearing in fractions I and II. It is important to bear in mind that specific activities of different enzyme reactions cannot be compared directly when the assay conditions differ (e.g. *gdh2* and *gdh4*).

Further purification of fraction I was achieved by chromatography on DEAE-cellulose (step 3). Elution with increasing concentrations of KCl produced a fraction possessing only *gdh2* and *gdh4* activity. Gel permeation chromatography of this material (step 4) gave pure glycerol:NAD⁺ 2-oxidoreductase (GDH2); details of its purification and characterization are given in the accompanying paper (Marshall *et al.*, 1985), where it is shown that its activity with NADPH (*gdh4* activity) is about 30% of that with NADH.

Table 3. Separation of glycerol dehydrogenase activities by precipitation with $(\text{NH}_4)_2\text{SO}_4$

The extract was prepared from cells grown as in (B) (see Table 2). Fractions I, II and III were obtained by precipitation with 1.8 M-, 2.5 M- and 3.8 M- $(\text{NH}_4)_2\text{SO}_4$ respectively. Activities are mean values from three separate experiments.

Activity type	Assay system (substrate/coenzyme)	Oxidoreductase activity [nmol substrate consumed min ⁻¹ (mg protein) ⁻¹]			
		Crude extract	Fraction I	Fraction II	Fraction III
<i>gdh1</i>	GLD/NADH	24	17	79	0
<i>gdh2</i>	DHA/NADH	54	390	0	0
<i>gdh3</i>	GLD/NADPH	21	22	20	65
<i>gdh4</i>	DHA/NADPH	97	730	43	18
ADH	Acetaldehyde/NADH	1650	270	3700	13

The main activity in fraction III was *gdh3* together with some *gdh4*. Chromatography on DEAE-cellulose at pH 8.0 was not possible since neither activity could be adsorbed under these conditions. Using the cation-exchange system carboxymethyl-cellulose (Whatman, CM70), operated at pH 4.5 with 20 mM-acetate, both activities adsorbed but eluted in the same fraction and could not be separated. Gel permeation chromatography on Sephadex G-200 also failed to effect any separation. Through all these attempts at separation the activity ratio *gdh3*:*gdh4* remained constant at about 3:1, suggesting the presence of a single enzyme, glycerol:NADP⁺ oxidoreductase (GDH3).

The results described so far show that *gdh4* activity in fraction I is due to GDH2 and that in fraction III to GDH3 but do not yet account for activity in fraction II. Glucose-exponential cells were used for the separation of this activity since GDH2 is repressed under these conditions. Extracts were first brought to 1.6M- $(\text{NH}_4)_2\text{SO}_4$ to give a fraction I with negligible *gdh2* or *gdh4* activity; the $(\text{NH}_4)_2\text{SO}_4$ concentration was then increased to 2.5M and the precipitate containing *gdh4* activity was further purified by chromatography on DEAE-cellulose. The product contained only *gdh4* activity and was thus a glycerol:NADP⁺ 2-oxidoreductase (GDH4).

Relation between *gdh1* activity and ADH

The fourth activity *gdh1* and ADH behaved very similarly in all the fractionation procedures. They were both very stable when heated in crude extracts, and after purification through step 2 where they both precipitated mainly in fraction II, they again proved equally heat stable. When fraction II, obtained from glucose-stationary cells, was further purified by chromatography on DEAE-cellulose (step 3), the two activities eluted together. The active fractions were pooled and subjected to gel permeation chromatography on Sephadex G-200 (step 4), but again the two activities could not be separated. The purification was monitored by gel electrophoresis at each step followed by activity staining with NAD⁺ and either ethanol or glycerol as substrate. The final product showed a single protein band which possessed both ADH and *gdh1* activities.

An alternative purification of ADH was carried out by the rapid method of Scopes (1981), though we were not able to crystallize the enzyme. On electrophoresis this product gave one main protein band and faint, slower running bands, all of which possessed ADH and *gdh1* activities. Assays with this product showed that its specific activity when oxidizing GLD was about 3.5% of its activity with acetaldehyde, while it was inactive with DHA or with NADP⁺ as coenzyme (Table 4).

DISCUSSION

We reported initially that *S. pombe* produces an NAD⁺-linked glycerol dehydrogenase (May & Sloan, 1981). We have now shown that *S. pombe* produces three more enzymes with glycerol dehydrogenase/triose reductase activity linked to NAD⁺ or NADP⁺ but we have found no evidence for a glycerol oxidase such as has been reported to occur in the fungi *Aspergillus*,

Table 4. *Substrate specificity of purified ADH*

ADH was purified and rates of NADH-linked aldehyde (or ketone) reduction or NAD⁺-linked alcohol oxidation were measured as described in Methods. There was no activity when NADPH replaced NADH or NADP⁺ replaced NAD⁺. The values equivalent to 100% were 130 μmol substrate used min^{-1} (mg protein^{-1}) for aldehyde reduction and 200 μmol substrate used min^{-1} (mg protein^{-1}) for alcohol oxidation.

Aldehyde reduction		Alcohol oxidation	
Substrate	Relative activity	Substrate	Relative activity
Acetaldehyde	100	Ethanol	100
GLD	3.7	Glycerol	0.3
DHA	0		

Table 5. *Characteristics of the glycerol dehydrogenases of S. pombe*

Enzyme	Classification	Corresponding EC no.	Catalytic activities		Inducers or repressors	Role in glycerol metabolism
GDH1 (= ADH)	Glycerol:NAD ⁺ 1-oxidoreductase (a property of alcohol:NAD ⁺ oxidoreductase)	1.1.1.1	Acetaldehyde/NADH	100	None	Uncertain. Could be significant since ADH activity high.
			<i>gdh1</i>	3.7		
GDH2*	Glycerol:NAD ⁺ 2-oxidoreductase	1.1.1.6	<i>gdh2</i>	100	No induction by glycerol; strong repression by glucose.	Catabolic utilization of glycerol as carbon and energy source.
			<i>gdh4</i>	30		
GDH3	Glycerol:NADP ⁺ oxidoreductase	1.1.1.72 or 1.1.1.21	<i>gdh3</i>	100	None	Uncertain. May be a specific glycerol:NADP ⁺ oxidoreductase or a broad specificity aldehyde reductase.
			<i>gdh4</i>	25		
GDH4	Glycerol:NADP ⁺ 2-oxidoreductase	1.1.1.156	<i>gdh4</i>		Some induction by glucose	A specific DHA reductase involved in glycerol synthesis. Possible role in xerotolerance.

* For purification and characterization of GDH2 see Marshall *et al.* (1985).

Penicillium and *Neurospora* by Uwajima *et al.* (1979). The main characteristics of the four enzymes found are summarized in Table 5.

The enzyme we first reported (GDH2) is a glycerol:NAD⁺ 2-oxidoreductase (EC 1.1.1.6), the first enzyme in the pathway by which *S. pombe* utilizes glycerol as a carbon and energy source. It is subject to catabolite repression by glucose but not to glycerol induction; though we suggested initially that glycerol may have some inductive effect in strain 972 h⁻ (May & Sloan, 1981), this is not supported by later work where care was taken to ensure that glucose-stationary cells were only harvested when glucose was no longer detectable in the culture and the cells were fully de-repressed. The purification and characterization of this enzyme is described in the accompanying paper (Marshall *et al.*, 1985). A similar catabolic enzyme has been studied extensively in bacteria such as *Klebsiella aerogenes* (*Aerobacter aerogenes*) (McGregor *et al.*, 1974) and *Escherichia coli* (Tang *et al.*, 1979).

The enzyme GDH4 is a glycerol:NADP⁺ 2-oxidoreductase (EC 1.1.1.156). Glycerol appeared to exert no regulatory control on the production of this enzyme, but cells growing exponentially on glucose possessed higher activity than glucose-exhausted or glycerol-growing cells. This stimulation by glucose appeared to be relatively small if activity was measured by the rate of glycerol oxidation but considerably larger if measured by the rate of DHA reduction. This type of enzyme has been found in the fungi *Aspergillus niger* (Baliga *et al.*, 1962) and *Mucor javanicus* (Dutler *et al.*, 1977), and in the halophilic alga *Dunaliella parva* (Ben-Amotz & Avron, 1974). Its function in *Dunaliella* appears to be in the synthesis of glycerol, which has been shown to act as an osmoregulatory solute, thus enabling these organisms to tolerate high external solute concentrations (Ben-Amotz & Avron, 1973). Some xerotolerant yeasts such as *Saccharomyces rouxii* have also been shown to use glycerol as an osmoregulatory solute (Brown, 1978), when growing in the presence of either high salt concentrations or high sugar concentrations. It seems not unlikely that *S. pombe*, a yeast also able to grow in the presence of 50% glucose, may possess the same mechanism of xerotolerance, using GDH4 as a synthetic enzyme for glycerol production. If this were so, the apparent inductive effect of glucose on GDH4 may not necessarily be a specific glucose effect but an effect of increased osmotic pressure. Further work is in progress on the characterization of this enzyme, its regulation, and the mechanism of xerotolerance in *S. pombe*.

The properties of the enzyme GDH3 indicate that it is a glycerol:NADP⁺ oxidoreductase showing most activity as a GLD reductase but also showing considerable DHA reductase activity. Enzymes with similar properties have been isolated from several sources and they can be divided into two types. The first type, exemplified by the enzymes isolated from the fungus *Neurospora crassa* (Viswanath-Reddy *et al.*, 1978) and from rabbit muscle (Korman *et al.*, 1972) appears to be relatively specific for glycerol and trioses (EC 1.1.1.72). The second type is a much broader specificity aldehyde or aldose reductase (EC 1.1.1.21), such as has been obtained from the yeasts *Candida utilis* (Scher & Horecker, 1966) and a species of *Rhodotorula* initially isolated by enrichment culture on GLD (Sheys *et al.*, 1971), and from various mammalian tissues such as calf liver (Attwood & Doughty, 1971) and pig kidney (Bosron & Prairie, 1972). The limited information we have obtained so far about the properties of GDH3 is not sufficient to decide to which type it belongs or what its metabolic function may be.

The fourth enzyme possessing glycerol dehydrogenase activity was designated GDH1. This activity proved to be inseparable from ADH throughout various attempts at separation, while a purified preparation of ADH from *S. pombe* was found to reduce GLD at about 3.7% of the rate at which it reduced acetaldehyde, and oxidize glycerol at about 0.3% of the rate at which it oxidized ethanol (Table 4). This led us to conclude that GDH1 is in fact ADH. In spite of the extensive studies which have been carried out on ADH, particularly the enzymes from horse liver and yeast ('yeast' in this context meaning *Saccharomyces*), there are few reports of it possessing glycerol dehydrogenase activity. Winer (1958) reported that the horse liver enzyme could reduce GLD at about one-fifteenth the rate for acetaldehyde, while Feraudi & Schmoltz (1976) considered ADH to be the main enzyme responsible for the reduction of D-glyceraldehyde, L-glyceraldehyde and DHA in rat liver, but neither reported activity in the reverse direction. The yeast enzyme was reported to oxidize glycerol at 0.4% (Barron & Levine, 1952) or 0.7% (van Eys & Kaplan, 1957) of the rate found for ethanol but neither DHA nor GLD was tested in the reverse reaction. When we tested commercial yeast ADH (Boehringer) for its ability to catalyse oxidation of glycerol or reduction of DHA or GLD, no activity was detected. Thus the ADH from *S. pombe* appears to be more active against glycerol or GLD than the *Saccharomyces* enzyme. Since *S. pombe* has high ADH activity which does not vary with changes in cultural conditions, a property previously noted by Schlenderer & Dellweg (1974), the low activity with glycerol or GLD may still make a significant contribution to the overall cellular metabolism of glycerol. To decide whether it does so requires further investigation.

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