**References and Notes**

1. D. Keilin and E. F. Hartree, *Biochem. J.* **50**, 331 (1952).
2. G. B. Levy and E. S. Cook, *ibid.* **57**, 55 (1954).
3. A. S. Keston, *Science* **120**, 355 (1954).
4. Activity of the enzyme is essentially independent of *p*H in the 6.0 to 7.5 range; it is stable in the 4.0 to 8.5 range.
5. J. M. Bailey and P. G. Pentchev, *Proc. Soc. Exp. Biol. Med.* **115**, 796 (1964).
6. Plant tissues were the commonly available forms obtained at local grocery stores (fruits and vegetables) or seed merchants (plant bulbs, and whole chrysanthemum plant). We are indebted to Dr. Kittie F. Parker, Smithsonian Institution, Washington, D.C., for assistance in classifying the species used.
7. J. M. Bailey and P. G. Pentchev, *Biophys. Biochem. Acta* **94**, 124 (1965).
8. A. S. Keston, *J. Biol. Chem.* **239**, 3241 (1964).
9. Column was 26 × 2 cm and was eluted successively with 125-ml portions of 0.05, 0.1, 0.15, 0.2, 0.3, and 0.5*M* phosphate buffers, *p*H 6.4.
10. J. M. Bailey and P. G. Pentchev, *Amer. J. Physiol.* **208**, 385 (1965).
11. ———, unpublished data.
12. ———, *Biochem. Biophys. Res. Commun.* **14**, 161 (1964).
13. R. K. Crane, *Physiol. Rev.* **40**, 789 (1960).
14. W. H. Arisz, *Ann. Rev. Plant Physiol.* **3**, 109 (1952).


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## D(—)-Lactate Dehydrogenase in Lower Fungi


Abstract. *A lactate dehydrogenase, specific for nicotinamide-adenine dinucleotide and D(−)-lactate, has been detected in extracts from two fungi of the order Leptomitales (Oomycetes). Several fungi of this order carry out a lactic acid fermentation under conditions of reduced oxygen tension.*


Although the fungi are generally an aerobic group of organisms, many of them grow readily under microaerobic or, as in the case of yeasts, anaerobic conditions. The energy required for anaerobic growth is obtained by fermentation of carbohydrates, with the production of lactic acid or ethyl alcohol. Although alcoholic fermentation has been studied extensively in yeasts, only a few investigators (*1*) have dealt with lactic fermentation in fungi. If the process of lactic acid formation is like that in animals, one would expect to find in fungi a lactate dehydrogenase whose coenzyme is nicotinamide-adenine dinucleotide (NAD) rather than a flavin (*2*).

Only about a dozen genera produce appreciable quantities of lactic acid during fermentation, but these are distributed randomly throughout the major groups of fungi. A NAD-dependent lactate dehydrogenase has been found in two of these genera, *Blastocladiella* (*3*) and *Rhizopus* (*1*).

In this report, we show that NAD-dependent lactate dehydrogenase (*4*), specific for the D(−) isomer of lactic acid, is present in high concentration in *Sapromyces elongatus* (strain 54-1) and *Mindeniella spinospora* (strain 57-14), two lactic acid–producing fungi belonging to the order Leptomitales (Oomycetes). Both were isolated in pure culture from decaying fruit by one of us (R.E.).

The *Sapromyces* mycelium was grown in a liquid medium consisting of peptone, yeast extract, and glucose (Cantino's PYG Broth, Difco); the organisms were harvested, washed on a Buchner funnel, and then stored in a freezer at −10°C. A cell-free extract was readily prepared by grinding 1 g (fresh weight) of mycelium in a glass homogenizer with 2 ml of cold 0.25*M* sucrose solution. The extract was clarified by centrifugation at 12,000*g* and then stored at −10°C. Samples of the extract were thawed, further diluted with 0.25*M* sucrose, and assayed spectrophotometrically for lactate dehydrogenase activity at 25°C. The reaction mixture contained sodium pyruvate ($3.3 \times 10^{-4}M$), the reduced form of nicotinamide-adenine dinucleotide (NADH) ($1.4 \times 10^{-4}M$), potassium phosphate buffer (0.1*M*, *p*H 7.5), and diluted extract in a final volume of 3.0 ml.

Oxidation of NADH, as measured by loss of absorbance at 340 mμ, proceeded linearly with respect to time and amount of extract added. No NADH was oxidized when pyruvate was omitted from the reaction mixture or when the extract had been boiled. The extract lost about one-half of its activity when incubated at 52°C for 20 minutes. The reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH) was not a satisfactory replacement for NADH. Proteins in the extract were precipitated with cold 5-percent trichloroacetic acid, and their amount was estimated by the Lowry method (*5*) with bovine serum albumin as a standard. The specific activity of the lactate dehydrogenase present in the *Sapromyces* extract was 18 μmole of NADH oxidized per minute per milligram of protein. An extract of chicken breast muscle, prepared and assayed in the same way, had a specific lactate dehydrogenase activity of 10. The reaction rate was maximum at a pyruvate concentration of about $2 \times 10^{-3}M$; higher concentrations slightly inhibited the enzyme. The Michaelis constant ($K_m$), as determined from a Lineweaver-Burk plot, was $4 \times 10^{-4}M$.

The reverse reaction, namely the conversion of lactate to pyruvate, was studied at 25°C in a 3-ml reaction mixture containing lithium lactate (0.1*M*), NAD ($2 \times 10^{-4}M$), tris hydrochloride buffer (0.1*M*, *p*H 9.0), and extract. As shown in Fig. 1, NAD reduction was detected when the D(−) isomer of lithium lactate was used but not when L(+)-lactate was substituted for D-lactate. The $K_m$ for D-lactate was found to be $5 \times 10^{-2}M$.

Samples of the *Sapromyces* extract were subjected to electrophoresis in starch gel at *p*H 7.0 (citrate-phosphate buffer) for 16 hours, with a voltage gradient of about 10 volt/cm, according to the procedure described by Fine and Costello (*6*). After electrophoresis, a slice of the gel was incubated for 30 minutes with a reaction mixture containing nitro-blue tetrazolium, phenazine methosulfate, NAD, lithium lactate, and tris buffer (*p*H 9.0) in order to find out where lactate dehydrogenase activity was located (*6*). A single, cathodic band of lactate dehydrogenase activity was revealed 2.3 cm from the application slit when D-lactate was present in the reaction mixture. No band appeared when another slice of the gel was incubated with a reaction mixture

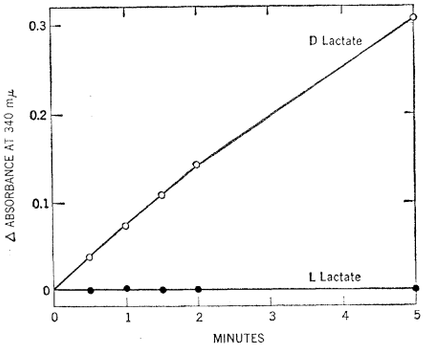


Fig. 1. *Sapromyces* lactate dehydrogenase assayed in the lactate to pyruvate direction. At 0 minutes, 0.02 ml of extract was added to the reaction mixture (see text for composition) which contained either lithium D(−)-lactate or lithium L(+)-lactate as substrate. Absorbance at 340 mμ was measured with a Zeiss spectrophotometer.

containing L-lactate instead of D-lactate.

A lactate dehydrogenase specific for NAD and D-lactate was also found in *Mindeniella*. Extracts from both organisms had similar activity in the pyruvate assay, and in both cases high pyruvate concentrations were slightly inhibitory. However, the reverse reaction, with NAD and D-lactate, proceeded very slowly in the presence of the *Mindeniella* enzyme. A twofold faster reaction was obtained when the 3-acetylpyridine analog of NAD was used in place of the natural coenzyme. In the case of the *Sapromyces* enzyme, the analog was no more effective than NAD. A third difference between the enzymes from *Sapromyces* and *Mindeniella* was evident. When the two extracts were run in the same starch gel, that from *Mindeniella* showed a single anodic band of lactate dehydrogenase activity 2.7 cm from the application slit, whereas that from *Sapromyces* showed a cathodic band.

Further characterization of the *Mindeniella* enzyme is in progress. In addition, preliminary studies indicate that representatives of two other genera in the order Leptomitales, *Rhipidium* and *Aqualinderella*, also contain NAD-dependent D-lactate dehydrogenase.

The D-lactate dehydrogenase has been regarded as uncommon. L-Lactate dehydrogenase (*7*), on the other hand, is widely distributed and is probably responsible for lactate production by all animal tissues, many bacteria (*8, 9*), and some vascular plants (*10*). The D-lactate dehydrogenase is probably responsible for lactate production in certain lactic acid bacteria (*8*), in some species of *Chlorella* (*11*), and throughout the order Leptomitales. NAD-dependent D-lactate dehydrogenase has also been detected in a very few other microorganisms, for example, the hyphomycete *Piricularia* (*12*), the cellular slime mold *Polysphondylium* (*13*), and the photosynthetic flagellate *Euglena* (*14*), but in these cases the biological function of the enzyme has not been determined.

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**References and Notes**

1. J. W. Foster, *Chemical Activities of Fungi* (Academic Press, New York, 1949), pp. 282–295; E. C. Cantino, *Am. J. Botany* **36**, 95 (1949); J. M. Crasemann, *ibid.* **41**, 302 (1954); C. G. Golueke, *J. Bacteriol.* **74**, 337 (1957); M. Margulies and W. Vishniac, *ibid.* **81**, 1 (1961).
2. The flavoprotein lactate dehydrogenases comprise a class of enzymes entirely distinct from the NAD-dependent lactate dehydrogenases. These flavo-enzymes are widely distributed in microorganisms. Their biological function may be to permit the utilization of D- or L-lactic acid as a carbon and energy source, usually during aerobic growth. They do not seem to be involved in lactate production in vivo.
3. E. C. Cantino and J. S. Lovett, *Physiol. Plantarum* **13**, 450 (1960).
4. Number 1.1.1.28 in *Enzyme Nomenclature, Recommendations of the International Union of Biochemistry* (Elsevier, New York, 1965).
5. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
6. I. H. Fine and L. A. Costello, in *Methods in Enzymology*, S. P. Colowick and N. O. Kaplan, Eds. (Academic Press, New York, 1963), vol. 6, pp. 958–972.
7. Number 1.1.1.27 in *Enzyme Nomenclature* (see *4*).
8. D. Dennis and N. O. Kaplan, *J. Biol. Chem.* **235**, 810 (1960).
9. A. Yoshida, *Biochim. Biophys. Acta* **99**, 66 (1965); E. M. Tarmy and N. O. Kaplan, *Biochem. Biophys. Res. Commun.* **21**, 379 (1965).
10. F. A. Loewus and H. A. Stafford, *J. Biol. Chem.* **235**, 3317 (1960).
11. O. Warburg, H. Klotsch, G. Krippahl, *Z. Naturforsch.* **12b**, 722 (1957); R. A. Lewin, Ed., *Physiology and Biochemistry of Algae* (Academic Press, New York, 1962).
12. K. Yamada, H. Yamada, Y. Takesue, S. Tanaka, *J. Biochem. Tokyo.* **50**, 72 (1961).
13. R. Garland and N. O. Kaplan, personal communication.
14. C. A. Price, *Biochem. J.* **82**, 61 (1961).
15 Supported in part by a grant to A.C.W. from NSF.

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## Mutagenicity of Cycasin Aglycone (Methylazoxymethanol), a Naturally Occurring Carcinogen

Abstract. *Methylazoxymethanol, a carcinogenic and hepatotoxic methylating agent prepared from cycad plants, has been found to be a good mutagen in* Salmonella typhimurium.

Cycasin (methylazoxymethanol-β-D-glucoside) is a carcinogenic and hepatotoxic compound occurring in plants of the family Cycadaceae. The frequent ingestion of various parts of these plants by people and domesticated animals in tropical parts of the world makes the compound of special interest (*1*). In its naturally occurring form cycasin is a β-glucoside, but there is mounting evidence that it is the aglycone methylazoxymethanol that is actually toxic and carcinogenic, and that deglucosylation must be performed by intestinal microorganisms before the toxicity can be manifested (*2*). Matsumoto and Higa (*3*) have demonstrated that methylazoxymethanol is a methylating agent, with 7-methlyguanine being formed by its reaction with DNA or RNA. The genetic damage resulting from such methylation should be re-

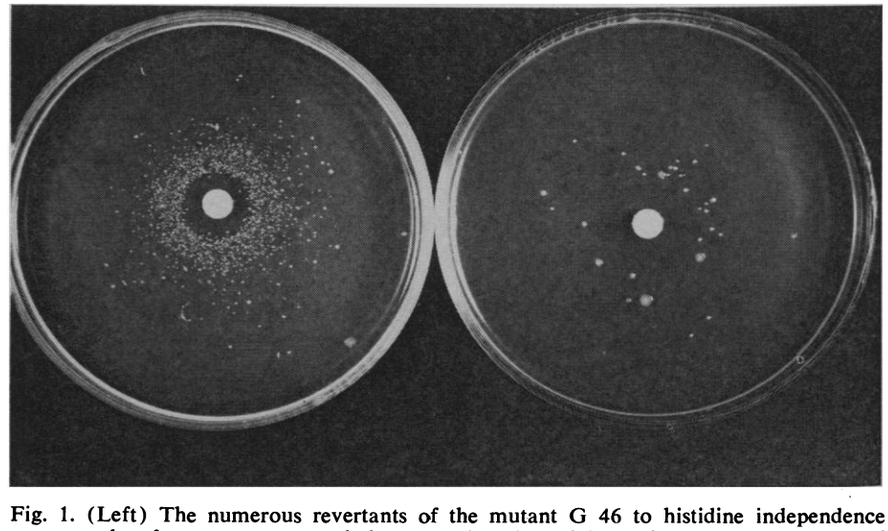

Fig. 1. (Left) The numerous revertants of the mutant G 46 to histidine independence as a result of exposure to methylazoxymethanol. (Right) The smaller number of revertants seen when less sensitive histidine-requiring mutants (C 496 in example shown) are placed under the same condition. The localization of mutants around the disc containing methylazoxymethanol is typical.