CONTROL OF ETHANOL DEHYDROGENASE LEVELS IN *AEROBACTER AEROGENES*

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Received for publication September 26, 1960

Aerobacter aerogenes strain 1033 grows on a number of polyhydric alcohols under both aerobic and anaerobic conditions. Among these compounds are glycerol, D-arabitol, D-sorbitol, and D-mannitol. Under anaerobic conditions the utilization of glycerol and D-arabitol seemed to depend on two diphosphopyridine nucleotide (DPN)-linked enzymes, glycerol dehydrogenase and D-arabitol dehydrogenase. The levels of these two enzymes could be greatly reduced in cells growing on their respective substrates if the cultures were vigorously aerated (Lin, Levin, and Magasanik, 1960; Lin, 1961). In the case of glycerol it was possible to show that the reduction in glycerol dehydrogenase level was compensated by an increase in the level of glycerol kinase which mediated a separate pathway for the dissimilation of this polyhydric alcohol. The control of the levels of glycerol and D-arabitol dehydrogenases by oxygen tension in the growth media stimulated our interest in the possible regulatory role played by oxygen on another DPN-linked enzyme, ethanol dehydrogenase. It has been previously reported that anaerobic dissimilation of glycerol produced equimolar quantities of ethanol and formate (Magasanik, Brooke, and Karibian, 1953). This observation suggests that ethanol dehydrogenase serves as a mediator for the terminal electron transfer during fermentation in this organism.

The present studies showed that the level of ethanol dehydrogenase increased severalfold when aerobically grown cultures were adapted to anaerobiosis on a number of polyhydric alcohols. Data were also obtained which indicate that the level of this dehydrogenase was not repressed by molecular oxygen itself, but was regulated by hydrogen acceptors within the cells.

MATERIALS AND METHODS

Bacteria. A. aerogenes strain P14 is a guanine auxotroph derived from strain 1033 (Brooke and Magasanik, 1954). The basal culture medium was of the following composition: guanine, 40 μg/ml; KH_2PO_4, 1.26%; K_2HPO_4, 0.54%; $(NH_4)_2SO_4$, 0.20%; $MgSO_4 \cdot 7H_2O$, 0.02%; and $CaCl_2$, 0.001%. The final pH was adjusted to 6.8 by addition of NaOH. Starting cultures were usually primed with 0.01% glucose in addition to the main source of carbon and energy which was usually present at a level of 0.2% unless otherwise specified. Cells from guanine-enriched agar slants were suspended and transferred aseptically into the growth medium (2-liter Erlenmeyer flasks each containing 1 liter of medium). Cultures were incubated on a rotary action shaker operated at about 200 cycles per min in a room maintained at 37 C. Anaerobic cultures were incubated in the same manner under 95% N_2 and 5% CO_2.

Preparation of enzyme. The cells were collected by centrifugation at 0 C, washed once with distilled water, and then suspended in 0.05 M glycylglycine at pH 8.0 (10 to 15 ml of buffer for every liter of culture). The suspension was then sonically disrupted by treatment in a 10 kc, 250 Watt sonic oscillator at 0 C for 4 min. This treatment did not inactivate the ethanol dehydrogenase. The sonically treated preparations were centrifuged at approximately 20,000 $\times$ *g* and the supernatant fraction was assayed for enzyme activity. This supernatant fraction was found to be as active as the crude sonic material in ethanol dehydrogenase activity. The enzyme was found to be more stable in glycylglycine than in phosphate, presumably because the former acted also as a mild metal complexing agent and protected the enzyme from traces of certain heavy metals.

Growth rates of bacteria were determined in 50-ml cultures in 300-ml Bellco Nephelo-culture flasks. The flasks were incubated on a gyrotory shaker operated at 240 cycles per min. Growth was monitored by reading the optical density in the side arm in a Klett colorimeter with a no. 42 filter.

Chemicals. Commercial ethanol was redistilled

before use. Other chemicals were used without further purification. These were: glycerol from Mallinckrodt Chemical Works, D-arabitol from Pfanstiehl Laboratories, D-sorbitol from H. M. Chemical Company, D-mannitol from Eastman Organic Chemicals, fumaric acid from California Corporation for Biochemical Research, and crystalline pyridine nucleotides from Pabst Laboratories.

Enzyme assay. The activity of ethanol dehydrogenase was measured by following the reduction of DPN at 340 mμ in a spectrophotometer. The temperature of the cuvette compartment was maintained at 25 ± 0.2 C. The enzyme was first mixed with 0.2 ml of neutralized 0.01 M DPN. This was followed by the addition of 2.0 ml of 0.5 M sodium carbonate buffer at pH 9.0 and sufficient water to give a final volume of 3.0 ml. The reaction was initiated by the addition of 0.4 ml of 12 M ethanol. The reaction blank contained all components except the substrate. The slope during the first 2 min was used to calculate the enzyme activity which was expressed as μmoles of DPN reduced per min per mg protein. The enzyme exhibited maximal ethanol dehydrogenating activity at pH 9. Under the assay conditions the rate of DPNH disappearance was negligible. High ethanol concentrations are required to saturate the enzyme. No inactivation of the enzyme was noted during the period of assay as judged by the kinetics. The protein was determined by the use of the Folin-Ciocalteu phenol reagent according the procedure described by Lowry and co-workers (1951).

The dehydrogenase was specific for DPN and gave no measurable activity with triphosphopyridine nucleotide (TPN). The reaction with DPN was readily reversible by the addition of acetaldehyde. Addition of reduced glutathione to the crude extract did not activate the enzyme. Unlike the glycerol dehydrogenase in this organism (Lin and Magasanik, 1960) the ethanol dehydrogenase did not seem to be activated by ammonium or potassium ions.

Analytical procedures. For determination of glycerol and fumarate in the growth media, samples of cultures were first centrifuged to remove the cells. Samples of the clear supernatant fluids were acidified with equal volumes of 0.25 N H_2SO_4. Glycerol was estimated by the periodic acid-chromotropic acid method (Korn, 1955).

TABLE 1

Variation of ethanol dehydrogenase levels in cells grown under different conditions

Source of Energy and Carbon	Aerobic		Anaerobic	
	Doubling time	Enzyme	Doubling time	Enzyme
	min	*units/mg protein*	*min*	*units/mg protein*
Glycerol	40	0.023	85	0.368
D-Arabitol	35	0.039	107	0.227
D-Sorbitol	40	0.030	97	0.152
D-Mannitol	32	0.024	48	0.105
Ethanol	160	0.035		

To minimize the variation in enzyme levels with growth phase, all cultures were harvested at approximately the same population density.

Fumarate was measured by absorption at 220 mμ in a spectrophotometer (Racker, 1950).

RESULTS

Effect of oxygenation on levels of ethanol dehydrogenase. Cells grown under well aerated conditions exhibited low activities of ethanol dehydrogenase irrespective of the source of carbon and energy (Table 1). The slight variations in the levels of this enzyme among these aerobically grown cells were perhaps not significant in view of the low activities which reduced precision. The low activities of ethanol dehydrogenase observed are probably not attributable to instability of the enzyme, since cells with high levels of this enzyme could be incubated aerobically in the absence of an energy source for many hours without appreciable loss of the enzyme activity. It might also be noted that the level of ethanol dehydrogenase was low in cells growing aerobically on ethanol as the sole sorce of carbon and energy.

The fact that rates of growth were slower under anaerobic than under aerobic conditions might at first suggest that the increase in the ethanol dehydrogenase during anaerobiosis reflected a release from the glucose effect. Compounds such as glucose, which supports rapid growth, are generally found to repress induced enzyme formation (Neidhardt and Magasanik, 1957). However, further inspection of the data will reveal that there is a lack of correlation between the levels of the enzyme and the anaerobic growth rates. If slow growth led to release of the enzyme then D-arabitol and not glycerol-grown cells

should give the highest activity. As may be seen, the data showed otherwise.

The observation that fluctuations in the level of this enzyme may be obtained in cells grown anaerobically allows us to conclude that molecular oxygen was not necessary for the formation of repressors of ethanol dehydrogenase.

A third possibility that acetate or acetaldehyde was the inducer of the enzyme and that anaerobiosis caused their accumulation was rendered unlikely by the observation that the presence of 0.2% of acetate or of acetaldehyde in cultures growing aerobically on glycerol did not increase the enzyme.

There is yet the possibility that the formation of ethanol dehydrogenase depended upon the balance of hydrogen donors and acceptors inside the cell. More specifically, reduced DPN (DPNH) accumulation might have caused induction or DPN accumulation might have caused repression of the enzyme. In this connection it should be noted again that the level of the enzyme obtained from anaerobically grown cells varied with the polyhydric alcohol presented to the organism. Thus, fermentation of glycerol, the most reduced of the series summarized in Table 1 (more hydrogen atoms per unit mass), gave the highest level of enzyme. The level of the enzyme was progressively lower as the chain length of the polyhydric alcohol increased.

The above data are consistent with the results of another experiment in which the growth rates of cells were limited by two different factors under anaerobic conditions. In one culture growth was limited by guanine which was dripped into a medium containing 0.2% glucose as the source of carbon and energy. In a parallel culture, guanine was provided in excess (40 μg per ml) and growth was limited by slow delivery of glucose. The specific activity of ethanol dehydrogenase was found to be 0.086 in the cells whose growth was limited by guanine and 0.048 in cells limited by glucose.[1] Thus it would appear that the level of dehydrogenase depended on the rate at which a fermentable sugar was fed to the cell as well as on the oxidation state of the compound. Both of these parameters are expected to influence the balance of reduced to oxidized pyridine nucleotides or their metabolic equivalents in the cells. The importance of such a balance in the regulation of ethanol dehydrogenase formation is further supported by the effect of an externally added hydrogen acceptor on the level of the enzyme during anaerobiosis.

Effect of addition of a hydrogen acceptor to cells fermenting glycerol. Previous studies indicate that fumerate could be used as a hydrogen acceptor by cells fermenting glycerol. Addition of fumarate under these conditions resulted in increased growth and in the formation of acetylmethylcarbinol presumably at the expense of the production of ethanol and formate (Magasanik et al., 1953). This observation suggests that fumarate was able to shunt the hydrogen from the acetaldehyde-ethanol pathway during anaerobiosis. To test whether the formation of ethanol dehydrogenase was affected when hydrogen was thus diverted, levels of this enzyme were measured in cells grown on glycerol in the presence and absence of fumarate.

The result of a typical experiment is summarized in Table 2 which shows that fumarate was able to repress the level of the enzyme to one-fifth of the control in cells growing on glycerol under 95% nitrogen and 5% carbon dioxide. Therefore, it would appear that the level of the enzyme was not regulated actually by the particular polyhydric alcohol which was utilized, but must depend on the disposition of the hydrogen. That fumarate did not repress the enzyme by its suppression of ethanol production is indicated by the fact that the addition of ethanol to cultures growing anaerobically on glycerol in the presence of fumarate had no further effect on the ethanol dehydrogenase level.

TABLE 2

Effect of fumarate on the level of ethanol dehydrogenase in cells fermenting glycerol

Compounds in Media	Ethanol Dehydrogenase
	units/mg protein
Glycerol, 0.2%	0.45
Glycerol, 0.2%, + fumarate, 0.1%	0.11
Glycerol, 0.2%, + fumarate, 0.2%	0.088

[1] Glucose was used as the source of energy and carbon in this experiment because it was difficult to obtain satisfactory growth rates with the polyhydric alcohols under these conditions. Although the effects of limiting glucose or guanine were found to be similar from experiment to experiment, the absolute activities attained in the cells seemed to depend on the state of the starting culture used for inoculation.

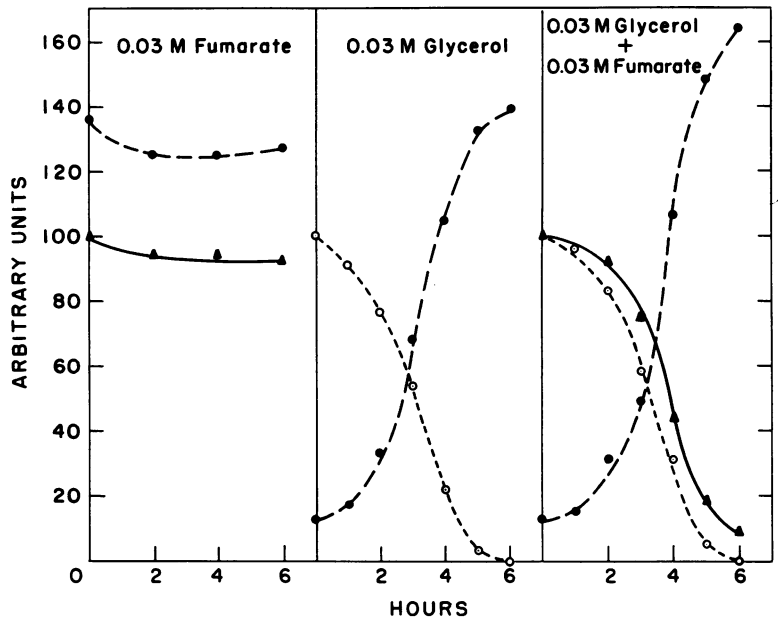

Fig. 1. Utilization of fumarate in the presence of glycerol. Cells previously grown anaerobically on glycerol were inoculated into fresh media and again incubated under 95% nitrogen and 5% carbon dioxide. Sources of carbon are given at the top of each set of curves. Cell density is denoted by ●-----●, glycerol concentration by O-----O, and fumarate concentration by ▲——▲.

In confirmation of the previous report cited above, fumarate increased the final growth in the culture by about 20% (Fig. 1). It also had the effect of accelerating the growth rate by about 10% during the logarithmic phase. It is of interest that fumarate was utilizable by the cells if it was presented with glycerol but not when offered alone. This would indicate that the main function served by the unsaturated dicarboxylic acid under anaerobic conditions was that of a hydrogen acceptor. An enzyme system which coupled the reduction of fumarate with the oxidation of DPNH has been reported in *Streptococcus faecalis* (Jacobs and VanDemark, 1960). We have now found a similar fumarate reductase system in *Aerobacter aerogenes*. The level of this system can be increased either by anaerobiosis or by the addition of fumarate to the growth medium. Preliminary experimental results indicate that there is a summation of these two effects.

DISCUSSION

Substrate induction and end product repression have been recognized as two important mechanisms of regulation which permit a cell to minimize redundant protein biosynthesis. Control of enzyme formation by induction is more frequently encountered in degradative pathways, and that by repression is more often seen in biosynthetic pathways although these two controls are not exclusive and may be mechanistically related (Pollock, 1959; Pardee, 1959). So far comparatively little attention has been focussed on the control of enzymes which catalyze freely reversible reactions, the directions of which may vary with the particular organism possessing the enzyme system and perhaps also with environmental conditions.

Such an enzyme is represented by ethanol dehydrogenase which catalyzes a coupled reaction involving four components. There is no reason to doubt that in certain cells, for example those in the liver, the enzyme primarily mediates the oxidation of ethanol, whereas in other cells, as in some microorganisms, the role of the enzyme is primarily for the production of ethanol. (Whether the enzymes are homologous or not is not pertinent to the present consideration.) Clearly the cellular function of such an enzyme must be determined either by the concentrations of the component substrates, by the type of controls existing in the cell, or both.

In the case of *A. aerogenes* we have an organism which is capable of both producing and utilizing ethanol, depending on the conditions of growth. That the ethanol dehydrogenase plays an essential role during fermentation is indicated by the increased formation of this enzyme during anaerobiosis. Whether, under aerobic conditions, the enzyme reverses its metabolic function, by catalyzing the first step of dissimilation of ethanol, cannot be unequivocally answered at present. Attempts to demonstrate a separate pathway for the aerobic utilization of ethanol by this organism failed to give any positive evidence. Thus, when the capacity to reduce triphenyltetrazolium chloride was tested with suspensions of intact cells (Neidhardt and Magasanik, 1956) in the presence of ethanol, it was found that cells grown aerobically on ethanol were not able to reduce the dye faster than those grown aerobically on glycerol. In contrast, cells grown anaerobically on glycerol reduced the dye about 10 times faster than cells grown aerobically either on glycerol or on ethanol. Cells grown first on ethanol also failed to utilize oxygen in the presence of ethanol at a significantly faster rate than cells grown first on glycerol when incubated in Warburg respirometers. On the other hand, it should not be ignored that growth on ethanol was exceedingly slow. Therefore, the low level of ethanol dehydrogenase in these cells might be quite sufficient to account for the aerobic utilization of the alcohol. A definitive answer to the question of the potential role of this enzyme may have to be obtained from mutants which lack the dehydrogenase.

It is not yet clear what caused the formation of the enzyme to be increased under anaerobic conditions, although it seems that acetate, acetaldehyde, and ethanol were not controlling agents. The possibility that molecular oxygen was the repressor of the enzyme was excluded by the observation that the level of the enzyme could be repressed anaerobically by providing a hydrogen acceptor system such as fumarate. That fumarate exerted its effect by virtue of its capacity to accept hydrogen and not simply by being a member of the tricarboxylic acid cycle was indicated by the fact that succinate was totally ineffective in reducing the level of ethanol dehydrogenase in cells fermenting glycerol. (Aerobically, the cells were demonstrated to grow on succinate, a finding which eliminates the question of succinate entering the cell.) Therefore, the control of ethanol dehydrogenase formation is unlike that of the isocitratase whose biosynthesis appeared to be repressed by members of the tricarboxylic acid cycle (Kornberg, Gotto, and Lund, 1958). There remains the possibility that the production of ethanol dehydrogenase is controlled by intracellular redox potentials. Such a control might be specifically mediated by DPNH as an inducer or by DPN as a repressor. Unfortunately both the strength and weakness of this hypothesis lie in the fact that at present there is no known way of varying the DPNH to DPN ratio independently of the redox potentials inside the cell.

ACKNOWLEDGMENTS

We wish to thank Boris Magasanik for helpful discussions during the course of this work.

This investigation was supported by research grants E3096 of the National Institutes of Health and G7479 of the National Science Foundation.

SUMMARY

Formation of ethanol dehydrogenase in *Aerobacter aerogenes* was increased during anaerobiosis. When the cells were grown anaerobically on polyhydric alcohols as sole sources of carbon and energy, it was found that the most reduced compound gave the highest ethanol dehydrogenase level. The formation of ethanol dehydrogenase in cells growing anaerobically on glycerol was found to be repressed severalfold if a hydrogen acceptor was provided in the growth media. Formation of the enzyme was found not to be appreciably influenced by ethanol, acetaldehyde, or acetate. These data permit us to conclude that the formation of the enzyme was regulated by the availability of hydrogen acceptors and not by molecular oxygen itself.

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