LOCALIZATION OF HYDROGEN ACTIVATING ENZYMES IN PSEUDOMONAS SACCHAROPHILA*

D. H. Bone

Department of Microbiology, Yale University,
New Haven, Conn.

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Bacteria which derive their energy from the oxidation of hydrogen have been found to contain several kinds of hydrogen activating systems, collectively known as "hydrogenases". Atkinson and McFadden (1954) found in Hydrogenomonas facilis a particle-bound hydrogenase which in their experiments could only use redox dyes as electron acceptors. Extracts of H. eutropha contain a FMN-requiring enzyme which reduces DPN with hydrogen (Wittenberg and Repaske, 1958; Repaske and Seward, 1960). H. ruhlandii contains yet another enzyme which reduces DPN (Packer and Vishniac, 1955). Purification of the DPN reducing hydrogenase of H. ruhlandii has shown it to be a single enzyme with the properties of a dehydrogenase, and subsequently named hydrogen dehydrogenase (Bernstein, 1960). The purpose of the present communication is to describe the distribution of two hydrogen activating enzymes in extracts of Pseudomonas saccharophila.

P. saccharophila kindly supplied by Dr. M. Doudoroff was grown on a salts medium containing trace elements. Twenty-two liters of a gas mixture (7% N_2, 15% CO_2, 20% O_2, and 58% H_2) were recirculated through two liters of medium at a rate of about 10 liters per minute. Exponential phase cells were harvested after 20 hrs of growth at 30°C, (4 g wet weight of cells) washed twice with distilled water, and resuspended in 10 ml of .05 M potassium phosphate buffer, pH 7.8. The cells were disrupted either by

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being passed twice through a French pressure cell or by oscillation in a 10 kc. Raytheon oscillator for 5 minutes. Extracts were centrifuged at 10,000 Xg for 10 min. to remove whole cells. The supernatant E was then centrifuged at 20,000 Xg for 30 min. to give a sediment of large particles LP. The second supernatant was centrifuged at 105,000 Xg for 90 min. to yield particle fraction SP. The upper half of the resulting third supernatant was carefully removed by pipette (fraction S). LP and SP were resuspended in 10 ml of .05 M potassium phosphate buffer, pH 7.8, and recovered by centrifugation at 105,000 Xg for 90 min. to give WLP and WSP particle fractions and washing fractions W_1 and W_2. WSP were washed again giving fraction WWSP and washings W_3.

Hydrogenase in the fractions was assayed manometrically at 30°C. The reaction mixture contained 150 µmoles of Tris buffer, pH 7.8; 16 µmoles of methylene blue, pH 7.8; hydrogen atmosphere; and sufficient enzyme to give about 10 µl of H_2 uptake in 1 min. Rate of H_2 uptake was linear for 10 minutes. Reduction of pyridine nucleotides was followed at 340 mµ in a Cary Model 11 spectrophotometer at 25°C. The cuvette contained 150 µmoles of Tris buffer, pH 7.8; 5 mg of bovine serum albumin; and sufficient enzyme to give an O.D. change of up to 0.20 per min. Hydrogen was bubbled through the cuvette for 4 min. and the cuvette was then sealed. The reaction was started by adding 2 µmoles of pyridine nucleotide. Protein was determined by the method of Lowry *et al.* (1951).

Hydrogen reduces DPN but not TPN in fraction E. Methylene blue is also reduced but the electron acceptors benzyl viologen, safranin O, and ferricyanide have only slight activity. On fractionation of E, DPN reduction is separated from methylene blue reduction (table). DPN reduction is clearly contained in the soluble fraction and is only present as a contaminant of WSP. Removal of DPN reducing activity requires two washings. It is of interest that the first washing, W_2, removes 5.2 mg of protein and only a small amount of DPN reducing power while the second washing removes only 1.2 mg of protein but an increased amount of DPN reducing activity, suggesting

some loose binding between DPN reducing activity and the SP fraction.

Fractionation of French Pressure Cell Extracts of P. saccharophila

Fraction	Hydrogenase		Reduction of DPN	
	Specific activity μmoles/mg protein/min	Total activity μmoles/min	Specific activity μmoles/mg protein/min	Total activity μmoles/min
E	0.71	203.8	0.19	54.5
S	0.81	117.4	0.23	33.3
WLP	0.12	2.6	0	0
WSP	1.49	145.3	0.03	3.0
WWSP	1.82	123.7	0	0
W_1	--	--	0.01	0.2
W_2	--	--	0.01	0.5
W_3	--	--	0.2	2.4

Hydrogenase activity, though not so sharply limited to one fraction is evidently concentrated in WWSP. As fraction S contains a diaphorase, the reduction of methylene blue could involve the coupling of the DPN reducing activity with diaphorase. A reconstituted fraction E, prepared from WLP, WSP, and S, showed 95% of DPN reducing activity and 108% of hydrogenase normally expected in fraction E. This indicates that the two reducing activities are not denatured during fractionation and can exist as separate entities. Attempts to convert the hydrogenase activity of WWSP and WLP into DPN reducing activity by sonication, solubilization with detergents, and hydrolytic enzymes did not prove successful.

The kinetics of release of the DPN reducing system and hydrogenase from whole cells of P. saccharophila during sonication was investigated using the methods of Marr and Cota-Robles (1957). In this case, hydrogenase was measured in the presence of a saturated solution of dicumarol to inhibit the diaphorase activity but not the hydrogenase. It was found that the turbidity, weight of unbroken cells, hydrogenase, and DPN reducing activity followed first order kinetics; and that the four components measured had

the same first order velocity constant, 0.6 $min.^{-1}$ This result suggests that sonication causes an instantaneous complete breakage of the cell into LP, SP, and S, and provides no information on the distribution of reducing activities.

The DPN reducing activity has been partially purified using protamine sulfate precipitation, ammonium sulfate fractionation, and DEAE column chromatography. This purification indicates that the enzyme responsible for the DPN reducing activity can be called hydrogen dehydrogenase. Further characterization of hydrogen dehydrogenase and hydrogenase is in progress.

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