THE METABOLISM OF 2-HYDROXYMUCONIC SEMIALDEHYDE BY AZOTOBACTER SPECIES

J.M. SALA-TREPAT and W.C. EVANS
Department of Biochemistry and Soil Science,
University College of North Wales, Bangor, Great Britain.

Received February 20, 1971

SUMMARY. Azotobacter species grown on benzoate have been found to metabolize 2-HMS, the meta cleavage product of catechol, to γ-oxalocrotonate by the action of a NAD^+-dependent 2-HMS dehydrogenase. The presence of very low levels of 2-HMS hydrolase activity has also been found in crude extracts from Azotobacter cells. However, the fact that these very low levels are not induced by growth on benzoate indicates that the hydrolase activity is not of any physiological significance for the metabolism of 2-HMS.

Azotobacter species were previously found to oxidise benzoate via catechol, which was further metabolized by meta cleavage (1). No attempts were made to detect any subsequent enzymes of the pathway. In Pseudomonas species two different catechol meta cleavage pathways have been reported (2,3). Nishizuka et al (2) reported a NAD^+-dependent enzymatic conversion of 2-hydroxymuconic semialdehyde (2-HMS) to γ-oxalocrotonate, whereas Dagley and Gibson (3) showed that a hydrolytic fission of the ring cleavage product occurs, yielding formate and 4-hydroxy 2-oxovalerate. Cain and Farr (4) with a Pseudomonas strain grown on benzene-sulphonate found that γ-oxalocrotonate was formed in NAD^+-supplemented enzymic reaction mixtures using 2-HMS as substrate. However, to account for other results, they precluded the participation of γ-oxalocrotonate as a direct metabolite of catechol, and supported the general scheme proposed by Dagley and Gibson (3). This scheme does not account for the observation made in different laboratories (4,5,6) that added NAD^+ increases the rate of conversion of 2-HMS to non-absorbing products, nor does it explain the role of γ-oxalocrotonate in catechol metabolism. The volume of data reported (3,4,7,8) leaves, however, the

existence of a hydrolytic fission of 2-HMS in no doubt. In a study of the metabolism of 2-HMS by Azotobacter species we re-investigated this apparent discrepancy using a new approach. The results of this study are reported here.

MATERIALS AND METHODS: The origin, growth media and culture conditions of the Azotobacter strains used in this work were described previously (1). Cell-free extracts were made up in 100 mM phosphate buffer, pH 7.5, supplemented with 10% acetone as previously reported (1). The activity of the 2-HMS metabolizing enzymes in cell-free extracts was determined by measuring the rate of decrease in absorbance at 375 nm in a Unicam SP 800 spectrophotometer. In a similar way the activities of the enzymes degrading 2-hydroxy 5-methylmuconic semialdehyde (2-hydroxy-5-MMS) and 2-hydroxy-6-oxohepta-2,4-dienoic acid were measured by observing the rate of decrease in A_{382} and A_{388} respectively. Assay cuvettes contained in a total volume of 3 ml : phosphate buffer, pH 7.5, (250 μmoles), substrate (0.1 μmoles) and extract(0.3-3 mg protein). NAD^+ (0.25 μmole) was added where indicated. Substrates for these assays were prepared by adding heat-treated extracts (55^o for 10 min) from benzoate-grown cells to 3 μmoles of the corresponding catechol (catechol, 4-methylcatechol or 3-methylcatechol). The volume was made up to 3ml with 100 mM phosphate buffer, pH 7.5. The mixtures were incubated at 30^o until the increase in absorbance at 375 nm (382 nm or 388 nm) ceased. The solutions were afterwards kept in ice and used the same day.

NADase (NAD glycohydrolase EC 3.2.2.6) was prepared from a 10 l culture of Pseudomonas fluorescens KB1 grown on the medium described elsewhere (9). Extracts were made up in 100 mM phosphate buffer, pH 7.5, and activated by heating for 3 min at 100^o as reported by Mather and Knight (10). The activity of these heat-treated preparations was 0.38 μmoles NAD^+ destroyed/min/mg protein, as tested by the KCN method (11). Protein concentrations were measured by the biuret procedure (12). Formate was determined after deproteination of the reaction mixtures and reduction to formaldehyde (13)

Table 1. Specific activities* of 2-HMS, 2-hydroxy-5MMS, and 2-hydroxy-6-oxohepta-2,4-dienoic acid metabolizing enzymes in cell-free extracts of Azotobacter Vinelandii, Strain 206.

Growth substrate	Assay substrate	No NAD added	NAD added to the reaction mixture	Ratio of activities $\frac{\text{NAD}}{\text{Non NAD}}$	NADase treated cell-free extracts**	Remaining activity after NADase treatment %
Benzoate 15mM	2-HMS	40	350	8.7	0.08	0.2
	2-Hydroxy-5MMS	38	362	9.5	0.092	0.24
	2-Hydroxy-6-Oxohepta-2,4-Dienoate	0.16	0.16	1.0	0.16	100
Succinate 20mM	2-HMS	0.17	0.56	3.2	0.068	40
	2-Hydroxy-5MMS	0.19	0.61	3.2	0.108	56
	2-Hydroxy-6-Oxohepta-2,4-Dienoate	0.18	0.18	1.0	0.18	100

* Expressed as nmoles/min/mg. protein.

** 0.25 ml of NADase preparation was incubated in 100 mM sodium-potassium phosphate buffer, pH 7.5, at room temperature with varying amounts of crude cell-free extracts in a total volume of 0.8 ml. After 10 min. incubation 2.1 ml of 100 mM sodium-potassium phosphate buffer, pH 7.5, and 0.1 ml of the corresponding assay substrate were added. The decrease in A_{375} (A_{382} or A_{388}) was followed spectrophotometrically.

by the acetylacetone method of Nash (14). γ-Oxalocrotonate was synthesised according to the method of Lapworth (15).

RESULTS AND DISCUSSION: Cell-free extracts from benzoate-grown Azotobacter vinelandii, strain 206, were found to metabolize 2-HMS much faster in the presence of added NAD^+ (Table 1). As can be seen these extracts could also metabolize the ring fission products of 4-methylcatechol and 3-methylcatechol. 2-Hydroxy-5-MMS was metabolized at about the same rate as 2-HMS and the addition of NAD^+ to the reaction mixture elicited a similar increase in activity. 2-Hydroxy-6-oxohepta-2,4-dienoic acid, the ring fission product of 3-methylcatechol, where the terminal aldehyde group is absent, was metabolized much more slowly and the activity was not affected by added NAD^+. These results were interpreted in terms of two different and non-specific enzymes, one NAD^+-dependent and the other non NAD^+-dependent, being involved in the metabolism of 2-HMS. In an attempt to purify these two enzymes the non NAD^+-dependent activity was easily lost. The kinetics of thermal inactivation and stability to pH changes, as well as the pH activity curves, were found to be identical for both activities (unpublished results). These findings suggested the possible existence of just one, the NAD^+-dependent enzyme. The activity found in the absence of added NAD^+ in the crude extracts could be ascribed to the endogenous NAD^+ and NADH oxidase present in these extracts. To test this hypothesis crude extracts were treated with NADase preparations. The results shown in Table 1 concerning the activities found in these NADase treated extracts indicate that more than 99% of the 2-HMS and 2-hydroxy-5MMS supposed to be non NAD^+-dependent activities are attributable to the endogenous NAD^+. Accordingly the NAD^+-dependent activity seems to be the one operating in cell-free extracts for the metabolism of 2-HMS in this strain. Nevertheless, the constant levels of 2-hydroxy-6-oxohepta-2,4-dienoate activity, not affected by added NAD^+ or NADase treatment, and the measurable, although low, levels of 2-HMS and 2-

hydroxy-5-MMS activities remaining after the NADase treatment, points to the presence of a non NAD^+-dependent enzyme in these extracts as well. To ascertain the physiological role of this very low non NAD^+-dependent activity, similar experiments were carried out in extracts from succinate-grown cells. As shown in Table 1 the non NAD^+-dependent activity proves to be non-inducible. The NAD^+-dependent activity, however, is increased about 620-fold by growth on benzoate.

The reaction products of the NAD^+-dependent activity were found to

Table 2. The stoicheiometry of γ-oxalocrotonate formation from 2-HMS by cell-free extracts of Azotobacter vinelandii, strain 206, grown on benzoate.

2-HMS metabolized nmoles	γ-Oxalocrotonate formed nmoles	Formate formed nmoles
100	98	N.D.
150	155	N.D.
200	208	N.D.

N.D. Not detectable.

The reaction mixture contained in a total volume of 3 ml: phosphate buffer, pH 7.5, (250 μmoles), Na_2-EDTA (50 μmoles), 0.1 ml of cell-free extract (1.5 mg of protein) and 2-HMS (0.1, 0.15 or 0.2 μmole). The disappearance of 2-HMS was followed spectrophotometrically at 375nm. When the reaction stopped the γ-oxalocrotonate formed was determined by the absorbance at 350 nm after adding 1 drop of 10 M NaOH (to pH 12.0) to the reaction mixture. A standard curve was plotted by taking varying amounts of synthetic γ-oxalocrotonic acid through the same procedure. The absorbance was proportional to the amount of γ-oxalocrotonic acid at least up to 0.2 μmole. Formate was assayed as indicated in Materials and Methods.

Table 3. The stoicheiometry of NADH formation from 2-HMS in NAD-supplemented reaction mixtures by cell-free extracs of benzoate grown Azotobacter vinelandii, strain 206

2-HMS metabolized nmoles	NADH formed nmoles	Formate formed nmoles
100	110	N.D.
200	200	N.D.
400	380	N.D.

N.D. Not detectable.

The reaction mixture contained in a total volume of 3 ml: phosphate buffer, pH 7.5, (250 μmoles), amytal (60 μmoles), NAD (2 μmoles), cell-free extract (300 μg of protein) and 2-HMS (0.1, 0.2 or 0.4 μmole). The amount of NADH formed was determined by the A_{340} when all the 2-HMS had disappeared.

be γ-oxalocrotonate and NADH. The stoicheiometry of the reaction is shown in Table 2 and 3. EDTA was added to the reaction mixture when γ-oxalocrotonate was to be estimated in order to inhibit the γ-oxalocrotonate decarboxylase activity found to be present at high levels in cell-free extracts from benzoate-grown cells (16). Amytal was added in a similar way when estimating NADH, as it was found to inhibit the NADH oxidase activity. Formate was never detected in these reaction mixtures and was not metabolized by the extracts. The NAD^+-dependent activity would be responsible for reaction 1 (fig. 1). The enzyme is a 2-HMS dehydrogenase (2-HMS : NAD oxidoreductase). The partial purification and properties of this enzyme will be published elsewhere.

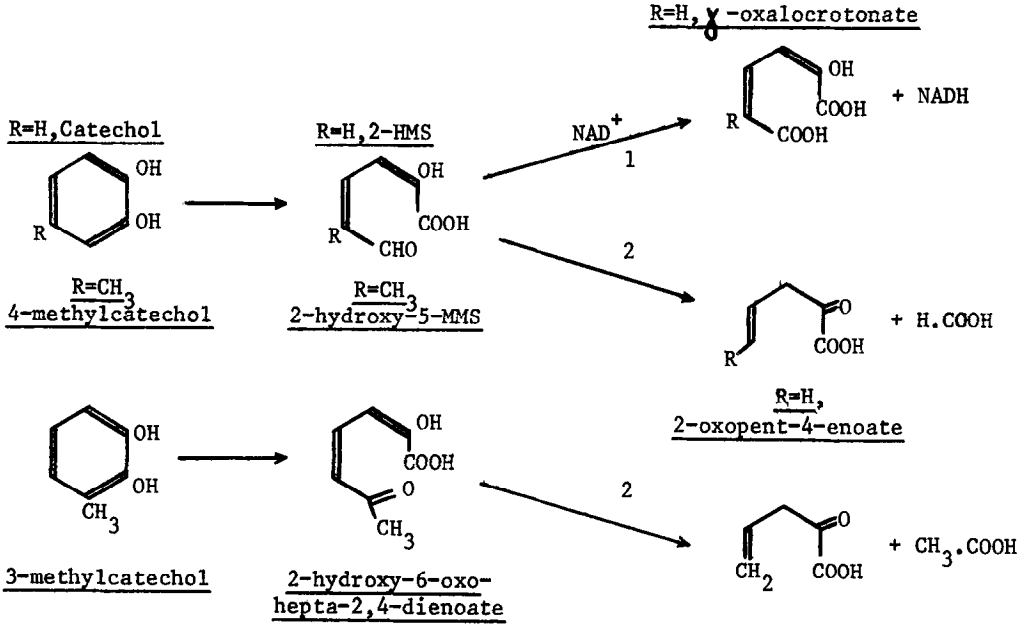

Fig.1.

The results presented in Table 4 indicate that the non NAD^+-dependent activity corresponds to the hydrolytic fission of 2-HMS reported by Dagley and Gibson (3). Formate was formed from 2-HMS when NADase-treated cell-free extracts were used.

Similar results to the ones reported were found using two other

Table 4. Formate production from 2-HMS by NADase treated extracts of benzoate grown Azotobacter vinelandii, strain 206.

2-HMS metabolized nmoles	Formate formed nmoles
100	80
200	178

0.3 ml of NADase preparation was incubated with 0.5 ml of crude cell-free extract for 30 min. 0.1 (0.2) ml of 2-HMS solution was added and the volume was made up to 3 ml with 100mM phpsphate buffer, pH 7.5. The reaction mixture was incubated overnight at 30° to allow for the total disappearance of the 2-HMS. Formate was determined after deproteination of the reaction mixture as described in Materials and Methods.

Azotobacter strains: Azotobacter chroococcum, strain 203 and Azotobacter beijerinckii, strain V. Our studies show that the metabolic pathway used by Azotobacter species for the degradation of 2-HMS involves an inducible, NAD^+-dependent dehydrogenase converting this product to γ-oxalocrotonate. The low, non-induced levels of 2-HMS hydrolase activity (reactions 2, fig.1) found in cell-free extracts could be due to the non-specific action of a hydrolase accomplishing another function in the cell.

REFERENCES

(1) C. Hardisson, J.M. Sala-Trepat and R.Y. Stanier, J. Gen. Microbiol. 59, 1 (1969)
(2) Y. Nishizuka, A. Ichiyama, S. Nakamura and O. Hayaishi, J. Biochem. 237, PC 268 (1962).
(3) S. Dagley and D.T. Gibson, Biochem. J. 95, 466 (1965).
(4) R.B. Cain and D.R. Farr, Biochem. J. 106, 859 (1968).
(5) W. Zubair, Ph.D. Thesis, University of Leeds (1963).
(6) C.F. Feist and G.D. Hegeman, J. Bacteriol. 100, 869 (1969).
(7) R.C. Bayly, S. Dagley and D.T. Gibson, Biochem. J. 101, 293 (1966).
(8) R.C. Bayly and S. Dagley, Biochem. J. 111, 303 (1969).
(9) J. London and M. Knight, J. Gen. Microbiol. 44, 241 (1966).
(10) I. Mather and M. Knight, J. Gen. Microbiol. 56, i (1969)
(11) N.O. Kaplan, in: Methods in Enzymology, vol. 2, eds. S.P. Collowick and N.O. Kaplan (Academic Press, New York, 1955) p.660.
(12) A.G. Gornall, C.J. Bardawill and M.M. David, J. Biol. Chem. 177, 751 (1949).
(13) W.M. Grant, Analyt. Chem. 20, 267 (1948).
(14) T. Nash, Biochem. J. 55, 416 (1953).
(15) A. Lapworth, J. Chem. Soc. 79, 1265 (1901).
(16) J.M. Sala-Trepat and W.C. Evans, in preparation.