Stimulation of Hydrogenation of Linoleate in *Treponema (Borrelia)* sp. Strain B₂5 by Reduced Methyl Viologen and by Reduced Benzyl Viologen

By M. T. YOKOYAMA* and C. L. DAVIS

Department of Dairy Science, University of Illinois, Urbana, Ill. 61801, U.S.A.

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A number of studies have been conducted to elucidate the properties and mechanism of the hydrogenation of C₁₈ unsaturated fatty acids by rumen micro-organisms. Progress in this area has been reviewed by Dawson & Kemp (1970). During screening of a number of pure cultures of rumen bacteria, several species have been shown to hydrogenate linoleate to *trans*-octadecenoate (Polan, McNeill & Tove, 1964; Kemp & White, 1968; Sachan & Davis, 1969; Mills, Scott, Russell & Smith, 1970). A rumen bacillus has been reported to effect the complete hydrogenation of linoleate to stearate (White, Kemp & Dawson, 1970).

Treponema (Borrelia) sp. strain B₂5, a rumen spirochaete, has been shown to hydrogenate linoleate to octadec-*trans*-11-enoate (Sachan & Davis, 1969). In further studies with the spirochaete we demonstrated that the hydrogenation of linoleate occurred as a two-step process, involving an initial isomerization to a conjugated fatty acid, octadeca-*cis*-9,*trans*-11-dienoate, and the subsequent reduction of this intermediate to octadec-*trans*-11-enoate (Yokoyama & Davis, 1971). A similar sequence of reactions was previously shown to occur in *Butyrivibrio fibrisolvens* strain A38 (Kepler, Hirons, McNeill & Tove, 1966).

During our studies it was observed that disruption of the spirochaete by a number of methods (sonication, Braun shaker and French press) results in the inability of the cell-free extract obtained to effect the second reaction in the hydrogenation process, the reduction of octadeca-*cis*-9,*trans*-11-dienoate to octadec-*trans*-11-enoate. This phenomenon has also been observed after the disruption of mixed rumen micro-organisms (Wilde & Dawson, 1966). Since a biological electron carrier, such as ferredoxin, has been suggested as being involved in hydrogenation (Kepler *et al.* 1966; Viviani, Borgatti & Crisetig, 1967; Dawson & Kemp, 1970), we investigated the effects of two artificial electron carriers, methyl viologen and benzyl viologen, on the conversion of linoleate into octadec-*trans*-11-enoate by a cell-free extract of the spirochaete.

Materials and methods. The spirochaete was

cultured in 20 litres of medium (Bryant & Robinson, 1961) containing clarified rumen fluid (20%, v/v) and glucose as the energy source. After 18-20 h of growth, cells were harvested by Sharples centrifugation and washed once with 200 ml of 50 mM-potassium phosphate buffer, pH 7.0, containing 2-mercaptoethanol (0.01%) and resuspended in the same buffer. Cells were ruptured with a French pressure cell as described by Yokoyama & Davis (1971) and centrifuged at 20 000g for 30 min at 5°C to remove large debris. The crude extract was passed through a column (20 cm × 3 cm) of DEAE-cellulose (Mortenson, Valentine & Carnahan, 1962). The DEAE-cellulose-treated extract was kept in an ice bath until used.

The complete incubation system contained 2 μCi of [1-¹⁴C]linoleic acid (56 mCi/mmol; Amersham-Searle Corp., Arlington Heights, Ill., U.S.A.) emulsified in 1 ml of water, 2 μmol of either methyl viologen or benzyl viologen, 20 μmol of sodium dithionite and 1 ml of the DEAE-cellulose-treated extract (29.9 mg of protein/ml) in 50 mM-potassium phosphate buffer, pH 7.0, containing 2-mercaptoethanol (0.01%). Methyl viologen and benzyl viologen dyes (Mann Research Laboratories Inc., New York, N.Y., U.S.A.) were chemically reduced with an excess of sodium dithionite (Peck & Gest, 1956). Incubations were conducted in test tubes (18 mm × 150 mm) flushed with either H₂ or N₂ and placed in a 37°C water bath for 2 h.

At the end of the incubation fatty acids were extracted from the incubation media with chloroform-methanol (2:1, v/v) and methylated with 2% (v/v) sulphuric acid in methanol, and the labelled fatty acids were examined by radio-g.l.c. and t.l.c. on 25%-(w/v)-silver nitrate-impregnated silica gel G as described by Yokoyama & Davis (1971).

Results and discussion. The results are shown in Table 1. The complete system in which the viologen dyes had been reduced with sodium dithionite (tube 1) showed the greatest effect on the hydrogenation of linoleate to *trans*-monoenoate by the cell-free extract. Under either H₂ or N₂ atmosphere the reduced viologen dyes resulted in 79-84% of the recovered radioactivity being associated with octadec-*trans*-11-enoate. Sodium dithionite also stimulated the reduction to octadec-*trans*-11-enoate

* Present address: Department of Animal Sciences, Washington State University, Pullman, Wash. 99163, U.S.A.

Table 1. *Effects of viologen dyes and sodium dithionite on the hydrogenation of linoleate to octadec-trans-11-enoate*

The complete system (tube 1) contained 1.0 ml of DEAE-cellulose-treated extract (29.9 mg of protein/ml) in 50 mM-potassium phosphate buffer, pH 7.0, containing 2-mercaptoethanol (0.01%), 2 μ Ci of [$1-^{14}$ C]linoleic acid (56 mCi/mmol) emulsified in 1 ml of water, 2 μ mol of either methyl viologen or benzyl viologen and 20 μ mol of Na₂S₂O₄. Omissions from tubes 2-5 were respectively viologen dye, Na₂S₂O₄, viologen dye + Na₂S₂O₄ and DEAE-cellulose-treated extract. Incubations were carried out under either H₂ or N₂ for 2 h at 37°C.

Experiment		% of recovered radioactivity						
		In linoleate		In conjugated dienoate		In octadec- <i>trans</i> -11-enoate		
		H ₂	N ₂	H ₂	N ₂	H ₂	N ₂	
Methyl viologen	Gas phase	...						
Tube 1			2.2	2.3	1.5	8.1	84.1	78.8
Tube 2			11.4	12.4	34.6	41.4	21.4	15.7
Tube 3			2.5	2.7	37.8	48.1	42.2	32.5
Tube 4			15.7	5.1	45.0	72.3	0.2	6.9
Tube 5			83.9	81.4	1.4	1.6	0.2	0.3
Benzyl viologen								
Tube 1			6.9	3.5	3.3	3.4	72.7	83.2
Tube 2			8.5	4.3	48.5	61.2	16.8	21.8
Tube 3			2.8	3.5	46.6	41.7	38.5	42.0
Tube 4			3.6	3.9	74.4	73.0	5.5	8.1
Tube 5			87.5	85.9	1.5	1.2	0.3	0.2

(tube 2); however, the response (16–22%) over all treatments was not as great as that observed for the viologen dyes. The addition of either oxidized methyl viologen or oxidized benzyl viologen under either H₂ or N₂ atmosphere (tube 3) partially restored the ability of the cell-free extract to effect the reduction to octadec-trans-11-enoate. The viologen dyes appeared to be equally effective in their response, with 32–42% of the recovered radioactivity being associated with the *trans*-mono-enoate. Linoleate was readily isomerized to octadeca-cis-9,trans-11-dienoate by the addition of the treated cell-free extract in the absence of viologen dyes and sodium dithionite (tube 4). On the basis of the recovered radioactivity, 45–74% of the radioactivity present in the respective treatments was associated with the conjugated fatty acid intermediate. The cell-free extract was, however, unable to effect the reductive step to form octadec-trans-11-enoate. The negative controls (tube 5) for the respective treatments showed a small, but consistent, isomerization of linoleate to a conjugated dienoate as an artifact of methylation. Radioactivity associated with the *trans*-monoenoate was negligible.

The stimulatory effect of the viologen dyes and sodium dithionite strongly suggest the involvement of a biological electron carrier, such as ferredoxin, in the hydrogenation reaction. The results also imply that the loss in hydrogenation that occurs when the spirochaete is ruptured is probably due to the inactivation of the electron-carrier system.

An amber-coloured protein, with an absorption maximum at 400 nm, has been isolated from extracts of the spirochaete by DEAE-cellulose column chromatography; however, its role (if any) in the hydrogenation reaction has not been established.

It is noteworthy that, in this study, the hydrogenation reaction occurred to about the same extent under N₂ and H₂ gas phases. This suggests that, although anaerobic conditions may be required, H₂ gas (via a hydrogenase reaction) is not directly involved in the reduction. Analysis of the fermentation products of glucose indicate that H₂ gas is not produced by the spirochaete.

The electron donor for the hydrogenation reaction is at present not known. Attempts to stimulate hydrogenation by the addition of potential electron donors such as pyruvate, formate, 2-oxoglutarate and succinate have not been successful. Additions of clarified rumen fluid and the supernatant from extracts of *Butyrivibrio fibrisolvens* strain A38 have been shown also to be without effect in stimulating hydrogenation of linoleate by ruptured cells of the spirochaete.

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