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HYDROXYMETHYLGLUTARYL-COENZYME A REDUCTASE

PURIFICATION AND PROPERTIES OF THE ENZYME FROM *FUSARIUM OXYSPORUM* *

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Summary

The hydroxymethylglutaryl-coenzyme A reductase (mevalonate:$NADP^+$ oxidoreductase, EC 1.1.1.34) system in *Fusarium oxysporum*, a soil inhabiting plant pathogen, has been examined. Two forms of the enzyme catalyzing the conversion of hydroxymethylglutaryl-coenzyme A were obtained in the supernatant after precipitation at 75% $(NH_4)_2SO_4$ saturation of the soluble culture extract which was previously separated from cell wall, mitochondria and microsomes. The two forms of the enzyme were separated electrophoretically.

A third form, contained in the precipitate obtained at 35—75% $(NH_4)_2SO_4$ saturation of the same extract, was further purified by Sephadex G-50 column chromatography. This purified form moved as a single band in sodium dodecyl sulphate electrophoresis and in immunological tests and has a molecular weight of 11 000. The apparent Michaelis constant for the substrate hydroxymethylglutaryl-coenzyme A is 21 μM at 2 μM NADP. NADPH is a more efficient reductant on a molar basis than NADH for the deacylation of the hydroxymethylglutaryl-coenzyme A substrate. Optimum activity of the enzyme was obtained at pH 7.4 and 37°C. The enzyme demonstrated no cold sensitivity but rather was more stable at 4°C than at 25°C. The protection with dithiothreitol, though minimal compared to other systems, was more effective at the higher temperature.

Introduction

Hydroxymethylglutaryl-coenzyme A reductase (mevalonate:$NADP^+$ oxidoreductase, EC 1.1.1.34), the enzyme which catalyzes the conversion of hydro-

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xymethylglutaryl-coenzyme A to mevalonate, has been examined extensively from different sources during recent years. The enzyme, which has a wide distribution [1], appears also to be variable in its properties. The main differences relate to temperature stability [2,3], coenzyme requirement [4—7], subunit size [9,10], and to its association with mitochondria or microsomes [5,11]. This latter property has led to extensive efforts to solubilize the enzyme [1].

The species, *Fusarium oxysporum*, constitutes a number of widely distributed strains which are responsible for a number of economically significant plant diseases [12]. Endemic yield losses range from 5 to 20% in affected crops. Although the organisms contain an abundance of sterols [13,14], the hydroxymethylglutaryl-coenzyme A reductase system has not been examined previously. Sterols, which have been implicated in the tolerance of temperature extremes by several fungi and other microorganisms [15—17], and which form an integral component of the cell membranes, presumably also play a role in the perennial survival of these soil organisms under diverse environmental conditions. The present study examines the hydroxymethylglutaryl-coenzyme A reductase system in *F. oxysporum*. One enzyme component is purified and characterized.

Materials and Methods

Extraction and purification of hydroxymethylglutaryl-CoA reductase

F. oxysporum Schl. em Sny. et Hans., obtained from the Commonwealth Mycological Institute, Kew, England, was grown in a modified Fries medium at 15°C for 30 h with continuous rotary agitation [18]. The filtered and washed cultures were extracted immediately.

All extraction and purification procedures were performed at 0—4°C. The cultures (100 g) were disrupted in 150 ml cold 0.1 M triethanolamine · HCl buffer, pH 7.4, containing 0.02 M EDTA and 2 mM dithiothreitol with 200 g small glass beads in an Omnimixer (Sorvall) at high speed in four 2-min periods while maintaining the homogenate temperature at 4°C. The homogenate was centrifugated at 30 $\times g$ for 1 min to remove the glass beads then for 10 min at 17 000 $\times g$. The residue was washed four times. The combined washings and the supernatant fluid were again centrifuged at 100 000 $\times g$ for 75 min. The supernatant fluids combined with four washings of the residue, constituted the crude extract.

$(NH_4)_2SO_4$ precipitation. Crystalline $(NH_4)_2SO_4$ (enzyme grade) was added slowly to 35%, then 75% saturation of the crude extract. After constant slow stirring for 2 h the mixture was centrifuged for 10 min at 27 000 $\times g$ and the precipitates were separated from the supernatants at each salt concentration. The final supernatant fluid (B) was concentrated by freeze-drying, washed on Amicon PM10 ultrafilter (Amicon, Lexington, Mass., U.S.A.) and subjected to electrophoresis. The precipitate (A) obtained at 75% $(NH_4)_2SO_4$ saturation was purified further by column chromatography. Protein was determined by the method of Lowry et al. [19].

Chromatography. Sephadex G-50 (fine) was allowed to swell in a boiling water bath for 1 h, then packed in a glass column (2.5 × 100 cm). 1 l of 0.1 M triethanolamine · HCl buffer, pH 7.4, was passed through the column to stabi-

lize the bed. The homogeneity of the packing was checked by monitoring the passage through the column of 1 ml (2 mg) of Blue Dextran 2000.

Assay for hydroxymethylglutaryl-coenzyme A reductase. The activity of the enzyme was determined by the amount of coenzyme A released from the substrate hydroxymethylglutaryl-coenzyme A and was measured by the method of Hulcher and Oleson [20].

One unit of enzyme is that amount catalyzing the turnover of 1 nmol of substrate per min at 37° C. Since 1 mol of hydroxymethylglutaryl-coenzyme A produces 1 mol of coenzyme A in the catalytic reaction, the units of enzyme were based on the coenzyme A equivalence.

Radioassay for hydroxymethylglutaryl-coenzyme A reductase activity. The microassay technique reported by Shapiro et al. [21] was employed to verify the data obtained by the colorimetric method described by Hulcher and Oleson [20] which was used routinely in these studies. The commercial radioactive hydroxymethylglutaryl-coenzyme A substrate (New England Nuclear) was chromatographed on thin-layer chromatography [21]. The substrate was isolated by elution from the appropriate location on the gel and used in the assays. After the reaction mixture was incubated for 15 min at 37°C and the proteins were removed by precipitation, the supernatant was chromatographed on the same thin-layer chromatography system to separate the substrate from the mevalonate product. Each compound was eluted and their radioactivities were measured on an Ansitron liquid scintillation counter. The radioactivity (cpm) per mol of the product was the same for the substrate. Controls without the enzyme preparation or the substrate did not show mevalonate on thin-layer chromatography. Identical samples of the enzyme preparation produced 0.885 and 0.893 nmol mevalonate per min by the radioactive assay [21] and the colorimetric method [20], respectively.

Electrophoresis. (a) Analytical: The technique of polyacrylamide gel electrophoresis described by Davis [22] was employed using Tris/glycine buffer, pH 8.3, at 4°C and 10.5% acrylamide monomer concentration. Samples were separated on gels in 75 × 5 mm glass tubes at 3 mA per gel with bromphenol blue as the tracking dye. The separated proteins in the gels were then fixed with 12.5% trichloroacetic acid for 30 min at 65°C, stained with Coomassie Brilliant Blue reagent (0.2% Coomassie Brilliant Blue R in 45% ethanol/10% acetic acid (v/v) for 30 min at 65°C, then destained with ethanol/acetic acid (25 : 10, v/v, in 100 ml water) during two 30-min intervals at 65°C. The gels were rinsed in several changes of 10% acetic acid then maintained in this solution [23].

(b) Enzyme activity: The hydroxymethylglutaryl-coenzyme A reductase activity on the gels were detected by the method of Bensch and Rodwell [7]. Directly after electrophoresis as described above, the gels were incubated for 20—30 min at room temperature in the dark in a mixture containing 60 ml 0.1 M Tris · HCl buffer, pH 7.1, 27 mg DL-mevalonic acid, 139 mg NAD^+, 49 mg nitroblue tetrazolium, 60 mg phenazine methosulfate and 34 mg coenzyme A. The hydroxymethylglutaryl-coenzyme A reductase activity was indicated by a purple band on a light violet background. The gel was then maintained in 10% acetic acid solution.

Molecular weight determination. (a) Electrophoresis: The molecular weight of the one enzyme component (precipitate A) was estimated by electrophoresis

in sodium dodecyl sulfate (SDS)-acrylamide gel electrophoresis as described by Weber and Osborn [24]. The enzyme samples and protein standards were individually incubated at 65°C for 10 min in 0.01 M sodium phosphate buffer, pH 7.0, containing 1% SDS, 2.5% mercaptoethanol, 2 M urea (ultra pure). Electrophoresis was performed at room temperature at 8 mA per gel and stained with Coomassie Brilliant Blue R as described previously. Standards consisted of 5 μg of the following proteins: ovalbumin, chymotrypsin A, myoglobin and cytochrome *c*.

(b) Chromatography: The molecular weight of the enzyme was also estimated by chromatography in a calibrated Sephadex G-50 (fine) column previously equilibrated with 0.1 M Tris buffer as described previously.

Kinetic experiments. Double reciprocal plots according to Lineweaver and Burk [29] were used for calculating the apparent kinetic constants. K_m and V were determined by varying the concentration of hydroxymethylglutaryl-coenzyme A as substrate while maintaining the other components at concentrations which did not limit the enzyme activity.

Immunochemistry. Immune serum was developed in rabbits as described previously [25] by intravenous injections of the purified form of the enzyme. The titre of the immune serum was determined by the microprecipitin method [26] and the specificity of the reactants was determined by the Ouchterlony double diffusion in agar technique [25,27] and by immunoelectrophoresis [28].

Results

Table I summarizes the purification of hydroxymethylglutaryl-coenzyme A reductase from the crude extract by ammonium sulfate precipitation and Sephadex G-50 chromatography. Whereas there is no significant increase in the specific activity of the precipitate (A) obtained at 75% $(NH_4)_2SO_4$ saturation, the specific activity of the supernatant fluid (B) was more than eleven times greater than that of the crude extract.

Neither the precipitate obtained at 35% saturation, nor the mitochondrial nor microsomal fractions obtained by differential high speed centrifugation, demonstrated any reductase activity.

Chromatographic purification (Fig. 1) of the precipitate (A) increased the specific activity of the enzyme fraction about 200 times over that of the crude

TABLE I

PURIFICATION OF HYDROXYMETHYLGLUTARYL-CoA REDUCTASE FROM *FUSARIUM OXYSPORUM*

Fraction *	Protein (mg)	Enzyme (units)	Units/mg protein
(1) Crude extract (100 00 × *g* supernatant)	1350	998	0.74
(2) Precipitate A, 35—75% $(NH_4)_2SO_4$ saturation of (1)	460	352	0.75
(3) Supernatant B, 75% $(NH_2)_2SO_4$ saturation of (1)	47	398	8.50
(4) Active fractions **, Sephadex G-50 chromatography of (2)	0.175	26	145

* The precipitates obtained after 17 000 × *g* and 100 000 × *g* centrifugation and 35% $(NH_4)_2SO_4$ saturation of the crude extract showed negligible enzyme activities.

** The active fractions 74—77 (Fig. 2) were selected for this enzyme and protein analysis.

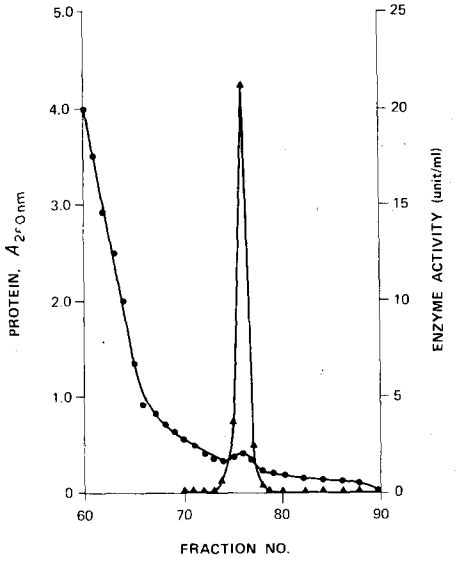

Fig. 1. Sephadex G-50 (fine) chromatography of precipitate A obtained at 35—75% $(NH_4)_2SO_4$ saturation of the 100 000 × *g* supernatant fluid from the crude extract. Column, 2.5 × 100 cm; buffer, 0.1 M triethanolamine · HCl buffer, pH 7.4. Temperature, 4°C; rate, 0.2 ml/min; fractions, 3.5 ml. Sample: 184 mg precipitate (141 enzyme units).

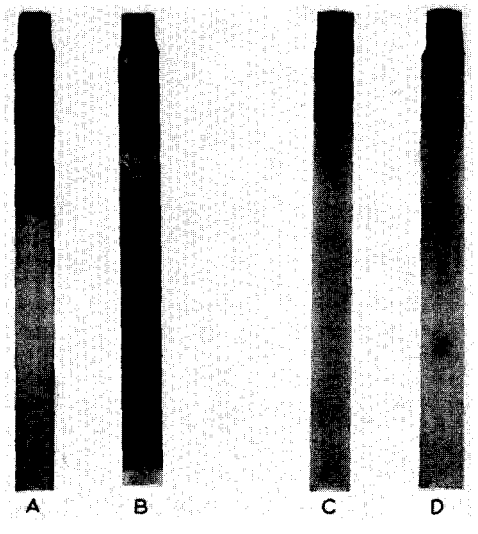

Fig. 2. Polyacrylamide gel electrophoresis after Davis [22] of precipitate A (230 μg) and supernatant B (60 μg), obtained at 35—75% $(NH_4)_2SO_4$ saturation of the 100 000 × *g* supernatant from the crude extract. Both samples were washed on molecular filters before electrophoresis. C and D are identical enzyme fractions, each containing 40 μg protein, from Sephadex G-50 chromatography. C, protein stained with Coomassie Brilliant Blue R. A, B and D, enzyme reaction according to Bensch and Rodwell [7]. Separating gel, 10.5% acrylamide. Duration, 2 h, 3 mA/gel.

extract. The active enzyme fractions emerged well behind the bulk of proteins which eluted immediately after the void volume.

Polyacrylamide gel electrophoresis revealed three oxidoreductases (Fig. 2). The 35—75% saturation ammonium sulfate precipitate (A) had the slowest moving enzyme entity (Fig. 1A). The hydroxymethylglutaryl-coenzyme A reductase-active fraction from the Sephadex gel chromatography had the same electrophoretic mobility and reactivity as did the precipitate (A) enzyme.

Acrylamide gel electrophoresis of the four most active chromatographed fractions combined, demonstrated a single protein with enzyme activity on the gel (Fig. 2).

The molecular weight of this protein fraction was 11 000 determined by SDS-acrylamide gel electrophoresis (Fig. 3) and also by Sephadex G-50 gel filtration. Only one protein band was obtained in the SDS gel.

The effect of time and protein concentration on the enzyme activity. Fig. 4 illustrates that the optimum activity of the enzyme, under the conditions of the assay system, occurred between 20 and 30 min after initiation and maintained a linear rate at protein concentration ranging from 4 to 40 mg/ml (Fig. 5) after 30 min incubation.

Kinetic studies. The K_m value obtained from the Lineweaver-Burk plot (Fig. 6) was $2.11 \cdot 10^{-5}$ M and was determined with the purified enzyme and the sub-

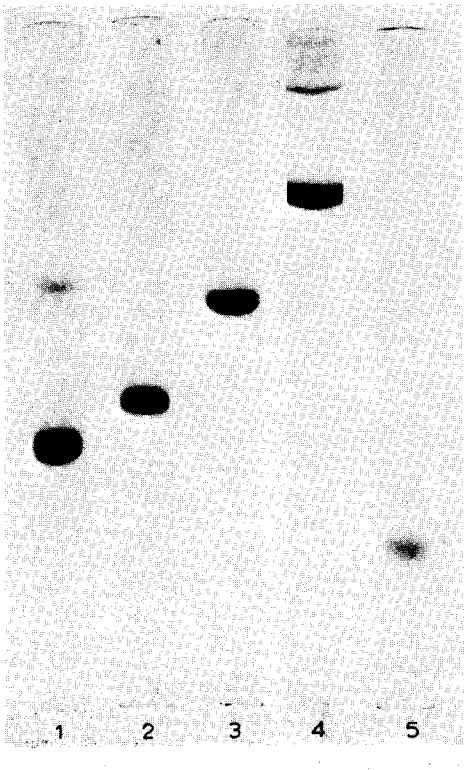

Fig. 3. SDS gel electrophoresis according to Weber and Osborn [24]. Standards, (1) cytochrome *c* (12 000), (2) myoglobin (17 800), (3) chymotrypsinogen A (25 000), (4) ovalbumin (45 000), (5) sample 1-μg active fractions 74—77, Sephadex G-50 chromatography (Fig. 2). Duration, 5 h, 8 mA/gel.

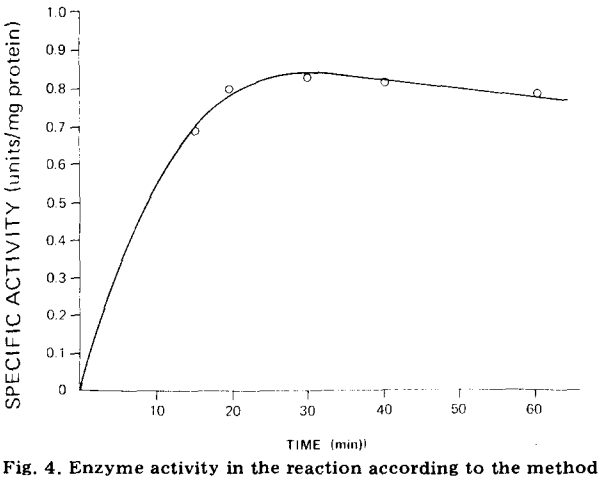

Fig. 4. Enzyme activity in the reaction according to the method of Hulcher and Oleson [20].

strate hydroxymethylglutaryl-coenzyme A. The V was 33.3 nmol/min.

The effects of temperature and dithiothreitol hydroxymethylglutaryl-coenzyme A on reductase stability. The data illustrated in Fig. 7 show the effects of 0°C, 25°C and dithiothreitol on the stability of the purified enzyme during a 24 h period. The enzyme was more stable at 0°C than at 25°C. Without dithiothreitol the enzyme lost 75% of its activity at 25°C and 25% at 0°C after 24 h. During the initial 4 h period at 25°C, the enzyme lost 30% activity, however, the rate of inactivation decreased progressively. Dithiothreitol exerted only a minimal stabilizing effect generally on the enzyme. This was more pronounced at the higher temperature and during the later stages of incubation.

Reductants for hydroxymethylglutaryl-coenzyme A reductase activity. NADPH and NADH demonstrated differential capacities to reduce the CoA released from the substrate. Whereas the V are similar for both reductants, 11.0 and 10 nmol/min, respectively, the K_m for NADH ($1.96 \cdot 10^{-5}$ M) is almost twice as much as that for NADPH ($1 \cdot 10^{-5}$ M) (Figs. 8b and 8c). The regenerating system with NADP in the incubation mixture proved to be more efficient

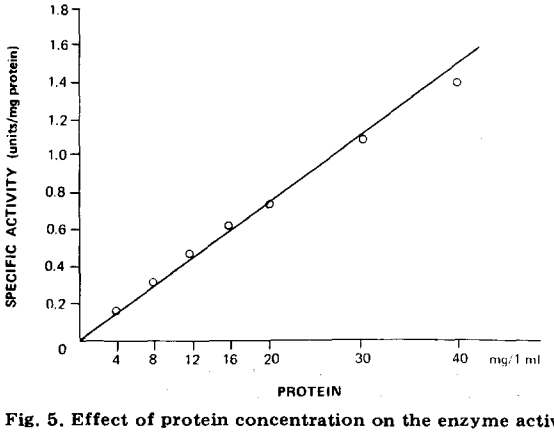

Fig. 5. Effect of protein concentration on the enzyme activity.

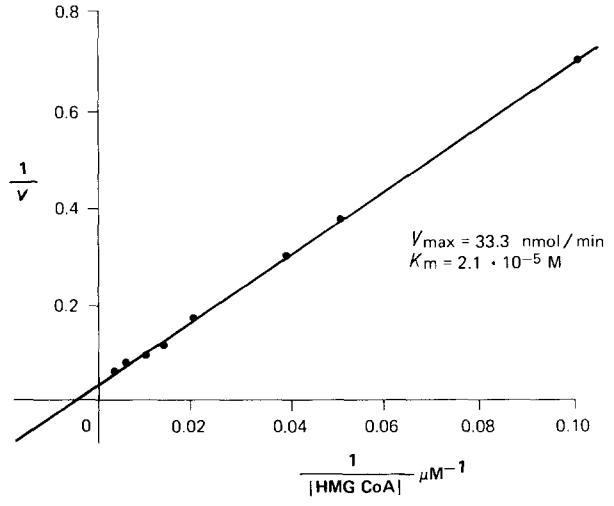

Fig. 6. Double reciprocal plots of the enzyme activity with the substrate hydroxymethylglutaryl-CoA. Incubation as described in Materials and Methods.

than either reduced coenzyme. The V was 20 nmol/min and the K_m was $5.6 \cdot 10^{-5}$ M (Fig. 8a).

The effect of pH on hydroxymethylglutaryl-coenzyme A reductase activity. The activity of the enzyme was examined in the range pH 6—pH 9 and optimum activity was obtained at pH 7.4 (Fig. 9).

The effect of temperature on the activity of hydroxymethylglutaryl-coenzyme A reductase. Examination of the activity of the enzyme incubated at temperatures in the range 15—50°C indicate an activity optimum at 37°C (Fig. 10).

The effect of freezing on the extractability of hydroxymethylglutaryl-coen-

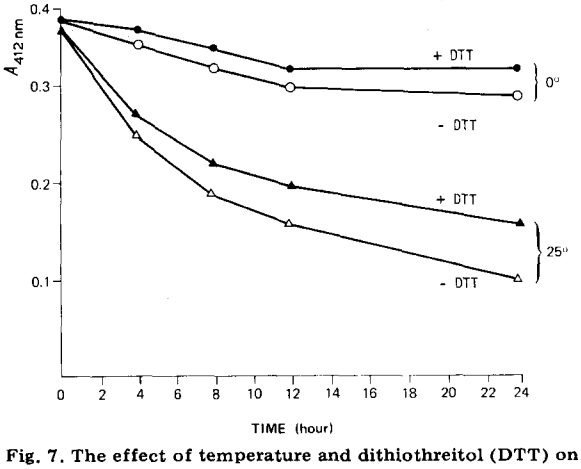

Fig. 7. The effect of temperature and dithiothreitol (DTT) on the stability of hydroxymethylglutaryl-CoA reductase. The enzyme preparations were maintained at 0 and 25°C without and with dithiothreitol (0.4 μmol). Samples were assayed as described in Materials and Methods.

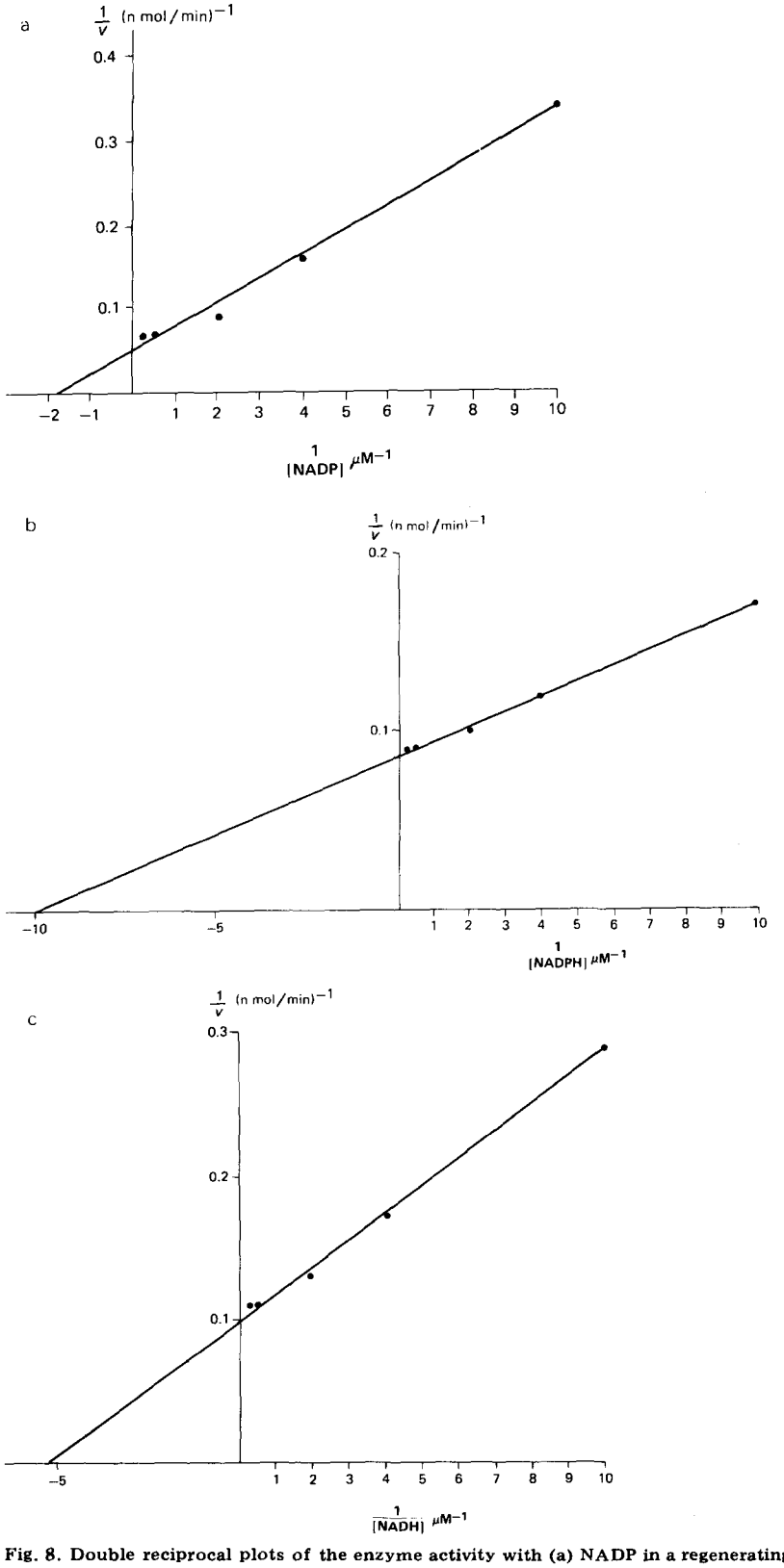

Fig. 8. Double reciprocal plots of the enzyme activity with (a) NADP in a regenerating incubation system (hydroxymethylglutaryl-CoA, 150 nmol; glucose 6-phosphate, 3 μmol; dithiothreitol, 0.2 μmol; glucose 6-phosphate dehydrogenase, 2 units; protein (enzyme), 4.5 mg; (b) NADPH and (c) NADH.

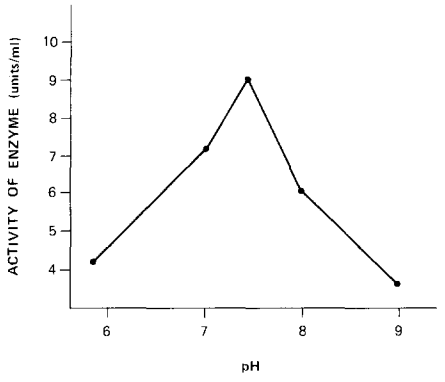

Fig. 9. The effect of pH on hydroxymethylglutaryl-CoA reductase activity.

zyme A reductase. Initially cultures were frozen at −20°C before extraction of the enzyme. However, the relative ease in obtaining the soluble enzyme directly compared to the solubilization difficulties obtained with the particle-bound enzyme from other organisms [1] suggested the extraction of fresh non-frozen cultures. The enzyme obtained from the extracts of these cultures had a higher activity (0.51 unit/mg protein) than that obtained from the frozen cultures (0.29 unit/mg protein).

Immunochemistry. The titre of the immune serum was 1/2048. The absorbed immune serum was specific for the precipitate A (35—75% $(NH_4)_2SO_4$ saturation) enzyme protein as indicated by the single precipitin reaction line in the agar gel in double diffusion and immunoelectrophoresis experiments (Figs. 11a and 11b). There were no reactions with the other fractions from the purification procedure. The precipitin obtained did not react with the substrate "stain" used previously to identify the enzyme in acrylamide gels [7]. Higgins et al. [10] also observed inactivation of the enzyme when it forms a precipitin. The precipitin in the double diffusion experiment was slow in forming and became apparent only after 6 days incubation and diffusion at 4°C.

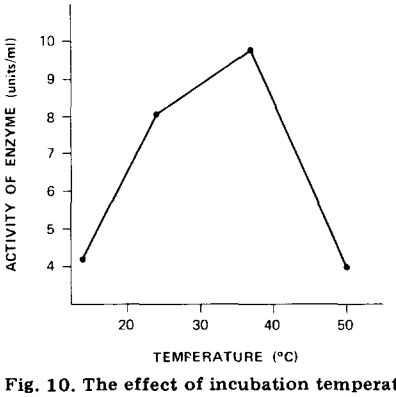

Fig. 10. The effect of incubation temperature on hydroxymethylglutaryl-CoA reductase activity.

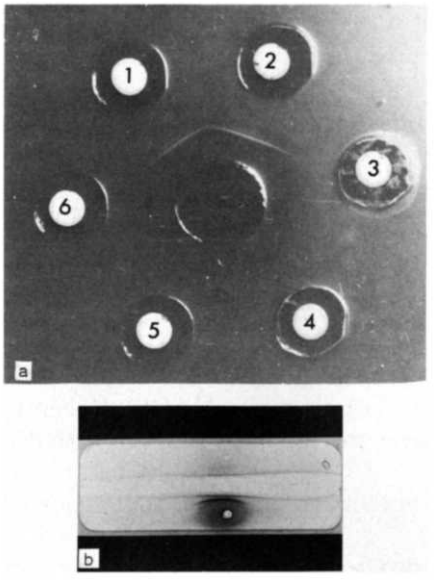

Fig. 11. Immunochemical reactions with purified hydroxymethylglutaryl-CoA reductase. (a) Double-diffusion in agar indicating a single reaction producing one precipitin band. Reactants are as follows: central well contained adsorbed immune serum prepared for the purified enzyme. The periferal wells 1,2 contained the purified enzyme preparation (A) and wells, 3, 4 contained the supernatant after 75% saturation with $(NH_4)_2SO_4$ of enzyme extract (B); wells 5, 6 contained physiological saline. (b) Immunoelectrophoresis of enzyme precipitate (A) and enzyme supernatant (B). Adsorbed immune serum was placed in the trough after electrophoresis (see ref. 28).

Discussion

Examination of the hydroxymethylglutaryl-coenzyme A reductase system in the fungus *F. oxysporum* indicate characteristics of the enzyme additional to those already reported. The unique ecological and relative taxonomic position of the organism should be considered in assessing these parameters.

The rat liver enzyme examined by Heller and Gould [31] was obtained in the precipitate at 35—50% $(NH_4)_2SO_4$ saturation whereas in *F. oxysporum* two forms of the enzyme remained in solution even at 75% $(NH_4)_2SO_4$ saturation.

For the first time, multiple forms of the enzyme have been demonstrated electrophoretically. Lynen [32] reported two forms in yeast but the subsequent study by Durr and Rudney [4] could not substantiate that claim. The temperature reactions of the enzyme led Heller and Gould [2] to postulate a multiple enzyme system. Recently Steel and Hulcher [33] obtained three catalytic forms of hydroxymethylglutaryl-coenzyme A reductase from pigeon liver and Ness et al. [34] reported two forms of the enzyme from rat liver microsomes.

The molecular weight (11 000) of the enzyme obtained from *F. oxysporum* in this study is low compared to those which range in size from $1.5 \cdot 10^5$—$2.8 \cdot 10^5$ obtained from rat liver and other microorganisms [1]. Subunits with molecular weights of 47 000 [9] and 65 000 [10] have been reported for this enzyme. Steel and Hulcher [33] have recently obtained a multimeric form (M_r

= 22 000) from pigeon liver. Since the SDS treatment did not alter the electrophoretic pattern of protein, a single peptide chain is indicated for the *F. oxysporum* enzyme.

The K_m = 10.5 μM value obtained for DL-hydroxymethylglutaryl-coenzyme A reductase compares well with the K_m = 12 μM value obtained under similar conditions with the yeast enzyme [35]. Values of K_m = 6 μM and K_m = 7.5 μM were obtained for the purified rat liver enzyme [8,36]. The K_m = 25 μM value was obtained for the *Pseudomonas* enzyme by Bensch and Rodwell [7].

The *F. oxysporum* enzyme utilized both NADPH and NADH as reductants indicating a requirement not as specific as the enzymes of yeast [4], *Neurospora crassa* [5] or the pea seedling [6] all of which require NADPH specifically. However, the *Pseudomonas* enzyme requires NADH [7]. The NADPH, on a molar basis, was a more active reductant of CoA than the NADH. Recently Ness et al. [34] described two forms of rat liver microsomal enzyme with different requirements for NADPH.

The pH optimum of 37°C for the enzyme activity is similar to that reported for the enzyme for other sources [1].

Reports on the temperature stability of hydroxymethylglutaryl-coenzyme A reductase have been variable depending partly on the source and the method of preparation of the enzyme [1]. Heller and Gould [2] reported the loss of 80% activity at 0°C within 30 min by the enzyme from rat liver solubilized bu a slow freeze-thaw procedure. They also obtained complete restoration of activity by heating the enzyme preparation at 37°C for 20 min and suggest that the enzyme possibly exists in two forms. Ackerman et al. [3] however, were unable to obtain a similar cold sensitivity with their rat liver preparation. The *F. oxysporum* enzyme examined here demonstrated no cold sensitivity and is actually more stable at 4°C than at 25°C. Also, at these temperatures, the rate of loss of activity is much less than that observed for the rat liver enzyme [37].

Thiol compounds have been added to preparations of this enzyme to maintain activity [8]. The stabilizing effect on the enzyme by dithiothreitol suggest the protection of sulfhydryl groups. The effect on the fungus enzyme however, was not as substantial as reported previously [8] for the rat liver enzyme.

The enzyme was obtained in the soluble form without recourse to any of the variety of solubilization procedures employed previously in the purification of this enzyme [1]. A soluble enzyme was also obtained from the bacterium *Pseudomonas* [7]. In *N. crassa*, an organism taxonomically closer to *Fusarium*, however the enzyme is associated with the microsomal membranes [5] and in yeast the enzyme is bound to the mitochondria [11]. Studies in yeast by Boll et al. [30] suggest that the hydroxymethylglutaryl-coenzyme A reductase is synthesized in the cytoplasm and becomes particulate by subsequent transfer and association with the mitochondria. The relative ease in obtaining the soluble enzyme suggests the non-particulate distribution in the cytoplasm of *F. oxysporum*. Freeze-thaw techniques have been used in other studies [1] to solubilize the enzyme from its association frozen at −20°C for at least 12 h and thawed slowly before extraction. However, when the total enzyme activity was found only in the soluble fraction after 70 000 $\times g$ centrifugation, the enzyme was extracted from fresh unfrozen cultures. The total activity per unit tissue almost doubled by this procedure suggesting further that the enzyme existed

freely in the cytosol at the time of extraction.

Steel and Hulcher [33] have reported recently that cytosolic hydroxymethylglutaryl reductase accounted for over 30% of the total activity in the pigeon liver. There is, however, the possibility of proteolytic activity within the cell and particularly during the extraction from the fungus although the rapid processing at low temperatures would minimize proteolysis. In any case, the particle-free enzyme is obtainable more easily from this organism than any previously reported source.

In consideration of Boll's [30] observation that in the yeast *Saccharomyces cerevisiae*, the enzyme is synthesized in the cytoplasm and subsequently associates with the mitochondria, it is conceivable that similar events occur in this fungus also. Therefore, the observation that all the hydroxymethylglutaryl-coenzyme A reductase activity appeared to originate in the cytosol at the time of extraction under the cultural conditions of these experiments does not preclude the particulate association of the enzyme in this organism. Steel and Hulcher [33] have observed that in the pigeon liver the cytosolic enzyme occurred prior to the microsomal forms and Hulcher suggests the possible cytoplasmic synthesis of the enzymes (Hulcher, F.H., unpublished data).

The recent observations of multiple forms of the enzyme introduce another parameter in the study of this enzyme system. In our experiments the specific absorbed antiserum for the precipitate A form of the enzyme did not react with the supernatant containing the two other forms of the enzyme. This suggests possible significant structural differences between the forms.

These two recent developments involving the multiplicity of forms and the possible cytoplasmic origin and soluble state of the enzyme in vivo will undoubtedly alter the experimental approaches regarding the regulation of the synthesis and activity of the hydroxymethylglutaryl-coenzyme A reductase system and the control of sterol synthesis generally.

The other forms of the enzyme which have been shown electrophoretically in these experiments are being purified and characterized presently. Their structural and immunochemical relationships and their origins and distribution will also be investigated.

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