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Studies on a mevaldic acid reductase of rat liver*

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SUMMARY

A TPNH-specific mevaldic reductase of rat liver has been partially purified. This enzyme fraction catalyzes the reduction of both optical isomers of mevaldic acid to isomeric mevalonic acids. Although the evidence is not unequivocal, most data are in agreement with the conclusion that a single enzyme mediates the reduction of the two isomers of mevaldic acid. The enzyme is strongly inhibited by hydroxylamine, but it is unaffected by iodoacetamide and only weakly inhibited by p-chloromercurisulfonic acid and *N*-ethyl maleimide. The enzyme exhibits no metal ion requirement and has a rather broad pH optimum (6.5 to 7.7). The biologically active isomer of mevaldic acid is reduced much more rapidly than the other isomer, and, in the reductive process, one mole of mevaldic acid is reduced and one mole of TPNH is oxidized for each mole of mevalonic acid formed. Biological activity of the enzymatic reduction product is demonstrated by its conversion to terpenol pyrophosphates and by its utilization as substrate for mevalonic kinase. Possible metabolic roles for the enzyme and its substrate are discussed. A second aldehyde-reducing activity was detected, which is DPNH specific and has activities similar to alcohol dehydrogenase. This enzyme shows significant activity in the oxidation of geraniol and farnesol in the presence of DPN.

The supposition that mevaldic acid would be a free intermediate in the conversion of HMGCoA[1] to mevalonic acid stimulated interest in this area of cholesterol biosynthesis; as a result, the conversion of mevaldic acid to mevalonic acid has been demonstrated in several laboratories. Wright *et al.* (2) demonstrated the formation of MVA from mevaldic acid by rat-liver homogenates treated with ribonuclease to prevent the synthesis of phosphorylated mevalonic acids. Lynen (3) reported upon a TPNH-specific mevaldic reductase from yeast, and Knauss and Porter (1) isolated a similar enzyme from rat liver. Studies on animal tissue mevaldic reductases that utilize DPNH more effectively than TPNH have been reported by Schlesinger and Coon (4) and Nakamura and Greenberg (5).

Studies on the reduction of HMGCoA to mevalonic acid by a yeast enzyme (6, 7) have shown that this reaction is TPNH specific. Similar studies have been made with animal preparations by Brodie and Porter (8). Efforts to isolate mevaldic acid as an intermediate in the yeast system were negative, and in the animal systems only very small amounts of mevaldic acid were demonstrable. From these reports, it appears likely that free mevaldic acid is not an intermediate in this reaction although it may have a role in cholesterol synthesis yet to be defined.

In the present paper, we report upon detailed studies of a mevaldic reductase of rat liver. These studies include the stoichiometry of the reaction, the specificity of the enzyme, the purification of the enzyme, and the biological activity of the product. During these studies, a second enzyme was detected that catalyzes the general reduction of aldehydes and is DPNH specific.

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[1] Abbreviations used are: HMGCoA, β-hydroxy, β-methyl glutaryl coenzyme A; MVA, mevalonic acid; MVALD, mevaldic acid; DPNH, TPNH, reduced di- and triphosphopyridine nucleotide; EDTA, ethylenediaminetetra acetate; PCMS, p-chloromercurisulfonate; DEAE, diethylaminoethyl.

Substrate specificity and inhibition studies indicate that this enzyme is probably alcohol dehydrogenase and that it is also active on higher terpenols but not on mevaldic acid.

EXPERIMENTAL METHODS

Chemicals. Mevaldic acid, as the dimethyl acetal of the dibenzylethylenediammonium salt, and mevalolactone were obtained from Mann Research Laboratories, Inc., New York, N.Y. DPNH, TPNH, yeast alcohol dehydrogenase, pyruvic kinase, and phosphoenol pyruvate were obtained from the Sigma Chemical Co., St. Louis, Missouri. Mevalonic kinase was prepared in our laboratory.[2] Liver alcohol dehydrogenase and Vitamin-A alcohol were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, and farnesol, geraniol, and nerolidol were obtained from Fritzsche Brothers, Inc., New York, N.Y. *n*-Butyraldehyde, isobutyraldehyde, *n*-valeraldehyde, isovaleraldehyde, and crotonaldehyde were obtained from Distillation Products Corp., Rochester, New York. *n*-Hexaldehyde, *n*-octyl aldehyde, *n*-decyl aldehyde, and *n*-hexanol were obtained from K & K Laboratories, Long Island, New York.

Cellex D, Cellex CM, and Cellex P were obtained from the Bio-Rad Laboratories, Richmond, California. Calcium phosphate gel was prepared by the method of Keilin and Hartree (10).

Butanediol succinate on Chromosorb W (the packing for the gas-liquid chromatographic separations) was obtained from Wilkens Instrument and Research, Inc., Walnut Creek, California.

Partial Purification of Mevaldic Reductase. Rat livers were excised, chilled in ice, and then homogenized in a loose fitting (0.5 mm clearance) Potter-Elvehjem homogenizer as described previously (11). The resultant homogenate was centrifuged at 500 × *g* for 15 minutes and 8500 × *g* for an additional 15 minutes to remove cell debris, nuclei, and mitochondria. Microsomes were removed from the microsomal suspension by centrifugation at 100,000 × *g* for 40 minutes. The clear supernatant solution of soluble proteins was fractionated with solid ammonium sulfate; the protein precipitated between 35% and 60% of saturation was dissolved in 0.1 M phosphate buffer, pH 7.0, and then dialyzed for 4 hours against 4 liters of 0.005 M phosphate buffer, pH 7.0, with a change of buffer at 2 hours. The protein concentration of the dialyzed solution was adjusted to 25 to 40 mg per ml with 0.005 M phosphate buffer, pH 7.0. Protein concentration was determined by the method of Gornall *et al.* (12).

The dialyzed protein solution was treated with calcium phosphate gel at a gel-protein ratio of 1.0, allowed to stand for 10 minutes, and then centrifuged. The supernatant solution was again treated with calcium phosphate gel at a gel-protein ratio of 1.0. The suspension was centrifuged, and the protein of the supernatant solution was precipitated by the addition of solid ammonium sulfate to 60% of saturation. The precipitated protein was dissolved in 5 to 10 ml of 0.1 M phosphate buffer, pH 7.0, and then dialyzed as indicated in the previous paragraph. The mevaldic reductase of this preparation was stable when stored in a dry-ice chest.

Preparation of Mevaldic Acid. The dimethyl acetal of the dibenzylethylenediammonium salt of mevaldic acid was added to an alkaline solution (KOH or NH_4OH), and the dibenzylethylenediamine was extracted with ethyl ether. The aqueous solution was then adjusted to pH 7.0 with 1 N HCl and the mevaldic acid concentration was made 0.1 M. When NH_3 was used to render the solution alkaline, the pH was adjusted by simply aerating to remove the excess NH_3. This solution was stored in the frozen state until needed. Prior to incubation, aliquots of the dimethyl acetal were hydrolyzed with 0.1 or 0.5 N HCl for 15 minutes at room temperature (13). The pH of the mevaldic acid solution was then adjusted to 7.0 with aqueous KOH. No difference was observed in enzymatic activity of the mevaldic acid liberated through each of these treatments with acid.

Properties of Mevaldic Acid. Mevaldic acid prepared in the manner described above was stable for several hours at pH 7.0, as shown by ultraviolet spectral analysis. Prolonged exposure at pH 2.0 and room temperature led to the formation of a chromophore with a light absorption maximum at 237 mμ, which corresponds to that of an α,β-unsaturated aldehyde. With crude enzyme preparations, this compound oxidized TPNH with the same efficiency as mevaldic acid.

Upon derivatization of mevaldic acid with 4-phenylsemicarbazide, a major product was formed which had an elemental analysis corresponding to the 4-phenylsemicarbazone of 3-methylcrotonaldehyde. This product would be expected if dehydration of either the reactant or derivative was followed by decarboxylation of the vinylic acid. The reaction of mevaldic acid with 2,4-dinitrophenylhydrazine under acid conditions gave from 50% to 90% anhydro product by spectrophotometric analysis.

Preparation of the Incubation Mixture. Phosphate buffer, TPNH, enzyme, and mevaldic acid were added

[2] Norgard, D. W., and Porter, J. W. Unpublished data. Mevalonic kinase was prepared by a modification of the method of Levy and Popják (9).

in the amounts stated in each table. In studies of the stoichiometry of the reaction, incubations were carried out at 38° with and without mevaldic acid, and aliquots were diluted 10- to 40-fold for absorbancy readings at 340 mμ. Other studies were performed at room temperature (22° to 23°), and the entire incubation mixture was added to the cuvette.

Assay for Quantity of Mevaldic Acid. Microcarbonyl determinations were made to determine the purity of the substrate and to determine the quantity of mevaldic acid disappearing during studies of the stoichiometry of the reaction. The method of Henick (14), as modified by Chipault *et al.* (15) and further modified in our laboratory by the substitution of ethanol for benzene and HCl for trichloroacetic acid, was used in early studies. Although this modification gave quantitative results, reproducibility was hampered by rapid deterioration of the reagents. In later work, it was found that methanol was a superior solvent and reagents were stable for periods up to 4 weeks. The components are carbonyl-free methanol (obtained by refluxing and then distilling from a solution of 2,4-dinitrophenylhydrazine and a drop of concentrated sulfuric acid), a saturated solution of 2,4-dinitrophenylhydrazine (twice recrystallized from carbonyl-free methanol), and a 10% solution of KOH in 20% aqueous methanol (16).

The sample (0.1 to 0.5 μmole in 1 ml of incubation mixture) was added to a 25-ml volumetric flask, and 1 ml of the saturated 2,4-dinitrophenylhydrazine solution was added with a drop of concentrated hydrochloric acid. The flask was stoppered and heated in a water bath at 60° for 30 minutes. At the end of this time, the sample was cooled in ice and 5 ml of the KOH solution was added with swirling. The solution was then brought to 25 ml with carbonyl-free methanol and filtered through glass wool to remove any KCl. The initial black color rapidly fades to a characteristic wine red. Readings against suitable blanks prepared in an identical manner are made at 430 and 460 mμ exactly fifteen minutes after the addition of base. A color development due to TPNH is superimposed on the normal 2,4-dinitrophenylhydrazone color and is corrected for by including TPNH in the blanks for all samples incubated with TPNH.

The equations of Chipault *et al.* (15) have been shown to be valid for this method by several criteria. The absorption maxima and extinction coefficients for pure saturated and unsaturated 2,4-dinitrophenylhydrazones in aqueous methanol (Fig. 1) correspond exactly to those published for these compounds in benzene-ethanol. Assays of known mixtures of these compounds using the equations of Chipault *et al.* gave the expected values. Further, mevaldic acid of known

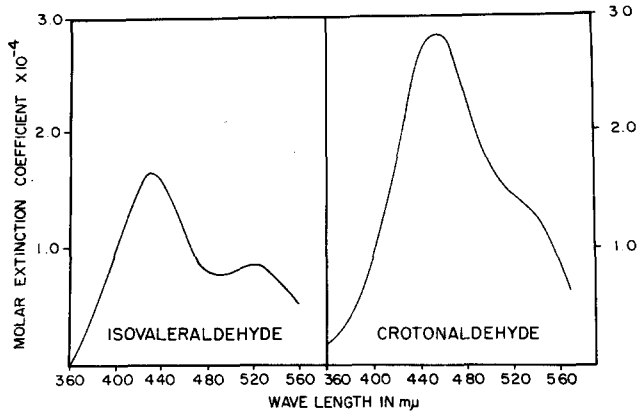

FIG. 1. Absorption spectra of pure 2,4-dinitrophenylhydrazones. Spectra were obtained on a Cary Model 14 recording spectrophotometer approximately 15 minutes after the addition of a 10% solution of KOH in 20% aqueous methanol.

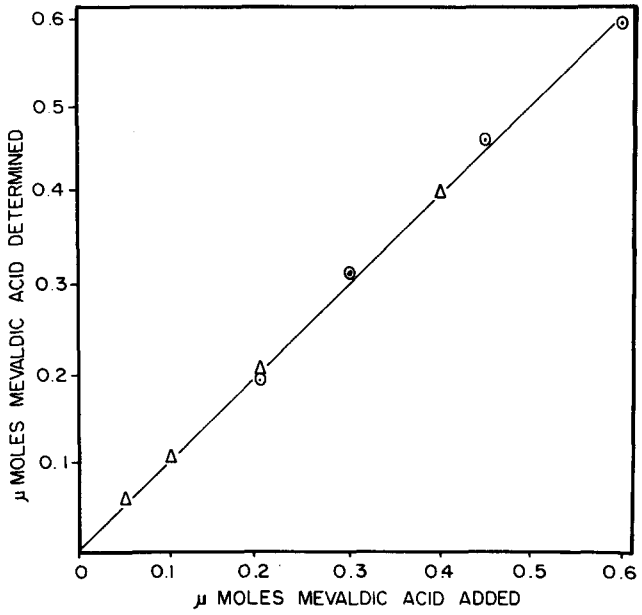

FIG. 2. Determination of mevaldic acid by the microcarbonyl procedure. Triangles represent determinations made with aqueous ethanol; circles represent results obtained with aqueous methanol. In each case, the point observed represents the summation of the 2,4-dinitrophenylhydrazone of mevaldic acid and 3-methyl crotonaldehyde as described in the text.

analysis [*N*,*N*′-dibenzylethylenediammonium-bis(3-hydroxy-3-methyl-5,5-dimethoxypentanoate); m.p. 106°; C 61.23%, H 8.17%, N 4.56%] was used as a standard to verify the accuracy of both alcohol methods (Fig. 2) and was used as the substrate in all experiments. The quantity of mevaldic acid determined spectrophotometrically varied less than 6% from the weight of mevaldic acid used over a several-fold range in concentration.

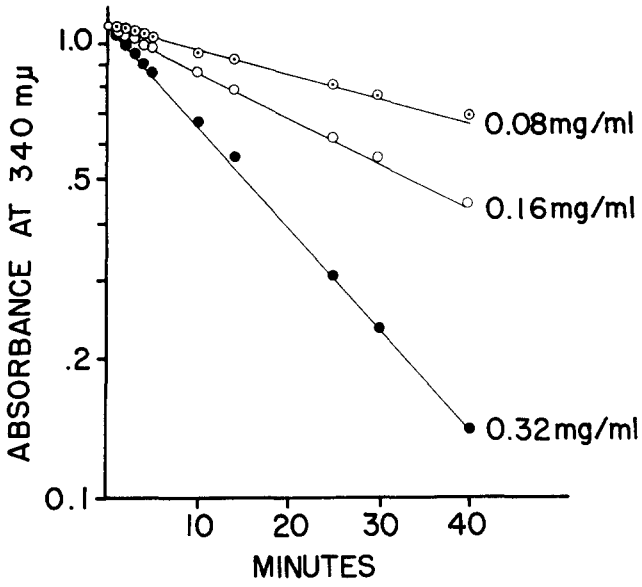

FIG. 3. Dependence of mevaldic acid reduction on protein concentration. The incubation mixture contained: phosphate buffer, pH 7.0, 240 μmoles; TPNH, 0.48 μmole; DL-mevaldic acid, 3.0 μmoles; enzyme as indicated and water to a volume of 3.0 ml.

Assay for Quantity of TPNH. The quantity of TPNH was determined spectrophotometrically on either an aliquot or on the entire incubation mixture. Substrate was omitted in control samples in all experiments, and negligible TPNH oxidation occurred in the absence of substrate.

Extraction, Identification, and Assay of Product. Extraction of the biosynthetic mevalonic acid was performed according to the method of Lynen and Grassl (17). To each milliliter of incubation mixture was added 0.1 ml of 12 N H_2SO_4. Lactonization of the mevalonic acid was achieved by incubating the solution at 38° for 10 minutes. The solution was saturated with sodium sulfate and then extracted repeatedly with ethyl ether. After evaporation of the ether, 0.5 ml of 2.5 M neutral hydroxylamine (neutralized with NaOH) was added and the solution was incubated at 38° for one hour. An aliquot was withdrawn for a quantitative hydroxamate determination, and the remainder of the sample was lyophilized. The residue was extracted six times with 0.5-ml portions of anhydrous ethanol. This solution was reduced to a volume of 0.2 to 0.3 ml and centrifuged, and 100 μl of the clear supernatant solution was chromatographed ascending on Whatman No. 1 filter paper using water-saturated butanol. Hydroxamate-positive spots were detected with a ferric chloride spray reagent (11).

Mevalohydroxamate prepared from high-purity mevalolactone was used as the reference standard for quantitative hydroxamate determinations. Spectrophotometric measurements, using the extinction coefficient of Beinert *et al.* (18), yielded a standard curve in excellent agreement with known weight of material.

In all quantitative assays for mevalonic acid, two blanks were employed; mevaldic acid was omitted in one and TPNH in the other. The summation of these blanks was subtracted from the value for the complete incubation mixture. Hydroxamate values reported in the tables thus represent only mevalonic acid formed from mevaldic acid.

Quantitative Determination of Biosynthesized Farnesyl Pyrophosphate. It appeared desirable to determine whether the mevalonic acid produced by the reduction of DL-mevaldic acid was biologically active. Therefore, the biosynthesized mevalonic acid was isolated from the incubation mixture and reintroduced into an incubation system (19) capable of converting mevalonic acid to farnesyl pyrophosphate. Aliquots of 1.0 ml were with-

TABLE 1. PARTIAL PURIFICATION OF MEVALDIC ACID REDUCTASE*

Fractionation Step	Activity†	Protein	Recovery
		mg	%
Crude solubles	0.106	7470	100
35–60 $(NH_4)_2SO_4$	0.375	2460	116
$Ca_3(PO_4)_2$ (1)		870	
$Ca_3(PO_4)_2$ (2)	1.22	434	67
0–60 $(NH_4)_2SO_4$	1.94	244	60

* The incubation systems contained: phosphate buffer, pH 7.0, 100 μmoles; TPNH, 0.5 to 0.6 μmole; DL-mevaldic acid, 5.0 μmoles; protein 0.9 to 2.05 mg; and water to a final volume of 3.0 ml. TPNH oxidation was determined spectrophotometrically and the enzyme activity was calculated from the initial rate of TPNH disappearance.

† μmoles TPNH oxidized per mg protein per hour.

TABLE 2. STOICHIOMETRY OF MEVALDIC ACID REDUCTION*

Experiment	TPNH Oxidized	Hydroxamate Formed	Carbonyl Disappearing
	μmoles	*μmoles*	*μmoles*
1	1.71	1.45	
2	1.06		0.93
3	1.05		1.09
4	0.75	0.82	0.84

* The incubation mixtures contained: phosphate buffer, 100 to 400 μmoles; TPNH, 4.8 to 12 μmoles; DL-mevaldic acid, 8 to 12 μmoles; and protein, 3.2 to 6.6 mg. A final volume of 4.0 to 6.0 ml was used. The lower phosphate concentration was employed to minimize turbidity in the microcarbonyl determinations. The course of the reaction was followed through spectrophotometric assay (340 mμ) for TPNH disappearance on 1 to 10 or 1 to 15 dilutions of aliquots of the incubation mixture.

drawn from a 5.0-ml incubation mixture at 15, 30, 60, and 120 minutes and then heat-denatured. After the protein was removed by centrifugation, the nonsaponifiable compounds were extracted directly from the incubation mixture with petroleum ether that had been purified by passing through silica gel. The aqueous residue was adjusted to pH 1.0 with 1.0 N HCl, and the solution was incubated 10 minutes at 38°. Farnesol, nerolidol, related compounds, and free higher fatty acids were extracted with purified petroleum ether. Extracts were reduced in volume to 0.10 ml and aliquots of 0.02 ml were injected into a Barber-Colman Model 10 gas-liquid chromatograph equipped with a Sr^{90} detector. A 6 ft × 0.25-inch column of butanediol succinate (25%) on Chromosorb W was operated at 180° with an argon gas flow rate of 100 ml per min at STP. Areas of peaks having the same retention time as authentic farnesol and nerolidol were determined and related to a previously obtained curve for different quantities of authentic farnesol and nerolidol. The areas of the peaks were found to be linearly related to the amount of material injected over the relatively narrow concentration range employed. Parallel experiments were performed with radioactive and nonradioactive DL-mevalonic acid. The conversion of radioactive mevalonic acid to acid-labile terpenol pyrophosphates was determined by measurement of radioactivity with a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., LaGrange, Illinois). Results were then converted to mμmoles of farnesol on the assumption that all of the radioactivity was located in this compound, since no monoterpene alcohols were detected by gas-liquid chromatography.

RESULTS

Purification of the Enzyme. Figure 3 shows the dependence of mevaldic acid reduction on enzyme concentration. Table 1 shows the results of the partial purification (about 20-fold) of mevaldic reductase. The degree of purification varied in successive experiments because of the lack of reproducibility of the activity of the initial rat liver soluble protein. However, after the first ammonium sulfate precipitation, assays of activity were reproducible. In some experiments, an apparent overall purification of as much as 40-fold was obtained.

Attempts were made to fractionate the protein remaining after the second ammonium sulfate precipitation on various cellulose resins such as DEAE cellulose, carboxymethyl cellulose, and cellulose phosphate. Although varying pH values and buffer concentrations were tried with each resin, no conditions were found that would cause either the activity or accompanying proteins to adhere to the column. Batch treatment with DEAE cellulose using the conditions of Schlesinger and Coon (4) yielded only a slight purification.

Identification of Product. A typical chromatogram of the hydroxamates of enzymatically generated mevalonic acid and the DL-compound (control) is shown in Figure 4. The coincidence in R_f values of the hydroxamates is evident. The hydroxamate spot for the enzymatically generated mevalonic acid was obtained only with the complete incubation system. When one of the essential components was eliminated (TPNH, mevaldic acid, or enzyme), no spot for the mevalonic hydroxamate was obtained on spraying with the ferric chloride reagent.

Stoichiometry. Data are presented in Table 2 on the quantity of TPNH that was oxidized, carbonyl that disappeared, and mevalonic acid that was formed. It is evident that equivalence for these quantities was obtained. Further evidence of stoichiometry, shown in Table 5, was obtained under conditions of limiting substrate.

Nucleotide Specificity. Figure 5 shows the difference in effectiveness of TPNH and DPNH. These results are in contrast to those for the mevaldic reductases obtained from pig liver by Schlesinger and Coon (4) and from rat liver by Nakamura and Greenberg (5), which utilized DPNH more effectively than TPNH.

General Properties of Mevaldic Acid Reductase. The enzyme was not inhibited by iodoacetamide in concentrations as high as 0.01 M, and it was only slightly inhibited by p-chloromercurisulfonic acid and *N*-ethyl maleimide. Hydroxylamine strongly inhibited the enzyme. These inhibitions are discussed later in the section on stereospecificity of the mevaldic reductase. No effect of Mg^{++} (0.006 M), Mn^{++} (0.006 M), Zn^{++} (0.006 M), or EDTA (0.0001 M) was observed. However, incubation of the enzyme with Cu^{++} (6×10^{-5} M) reduced the activity about 15% during the first 5 minutes. The activity of the enzyme was quite insensitive to pH changes in the 6.5 to 7.7 range, but the activity was decreased about 50% when incubation was carried out at pH 6.0 and 25% at pH 8.0.

Reversibility of the Reaction. All attempts to demonstrate reversibility of the reduction of mevaldic acid to mevalonic acid were without success, which is in accord with the results of Lynen (3) and of Schlesinger and Coon (4).

Conversion of Biosynthesized Mevalonic Acid to Farnesyl Pyrophosphate. Table 3 shows the results of an experiment on the conversion of mevalonic acid to farnesyl pyrophosphate. The biosynthesized farnesyl pyrophosphate of these experiments was rearranged and

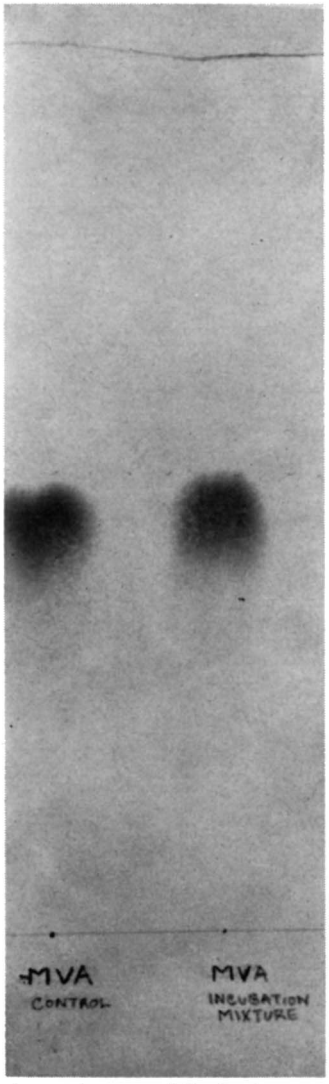

FIG. 4. Paper chromatogram of the hydroxamates of enzymatically prepared mevalonic acid and DL-mevalonic acid (control). The complete incubation system contained all of the components in the amounts listed in Table 2. Isolation procedures and the conditions of the chromatographic separation are described in the experimental section.

hydrolyzed (19) during a 10-minute incubation at pH 1.0. The resultant nerolidol (20) was extracted with petroleum ether, and the quantity formed was determined by GLC. The results show that each of the substrates was converted to farnesyl pyrophosphate.

Stereospecificity of the Enzyme. It was expected that mevaldic reductase would be active on only one isomer of the DL-mevaldic acid. Figure 6, however, demonstrates that there is a rapid enzymatic conversion of 50% of the substrate, followed by a much slower reduction until quantitative conversion is approached as measured through TPNH oxidation. This would suggest that a rapid primary phase occurs that corresponds to the reduction of one isomer, concomitant with

TABLE 3. FARNESYL PYROPHOSPHATE GENERATED FROM ENZYMATICALLY SYNTHESIZED AND DL-MEVALONIC ACID (IN MμMOLES)*

Time	Enzymatically Synthesized MVA†	DL-MVA†	2-C^{14}-DL-MVA†
min			
0			
15	4.2	5.6	
30	33.0	51.2	60.5
60	98.4	120.0	118.7
90	96.8	124.4	115.7

* The incubation mixtures contained: phosphate buffer, pH 7.0, 250 μmoles; ATP, 50 μmoles; $MgCl_2$, 30 μmoles; GSH, 30 μmoles; enzymatically synthesized mevalonic acid (sample 1), 4.07 μmoles; DL- and 2-C^{14}-mevalonic acid (samples 2 and 3), 8.2 μmoles; protein, 18.9 mg; and water to a final volume of 5.0 ml. The procedures for the isolation and the analytical determination of nerolidol are described in the experimental section.

† The values given are the quantities of farnesol, measured as nerolidol, obtained from the 1-ml aliquots removed from the incubation mixture at the indicated time.

a slow reduction of the "other" isomer. This is followed by a secondary phase in which the "other" isomer is the major substrate.

Further proof for the reduction of both isomers of mevaldic acid is found in the stoichiometry reported in Table 4, experiment 2, and Table 5. These results and those reported in the preceding paragraph unequivocally establish the enzymatic reduction of DL-mevaldic acid.

These results also gave rise to three questions: (1) Is one, or are both isomers of mevalonic acid formed; if the latter, is the biologically active isomer formed preferentially? (2) Does racemization of mevaldic acid occur? (3) Are one or two enzymes involved?

To get information on the first question, the mevaldic acid reductase was incubated in the presence of excess DL-mevaldic acid and also in the presence of limiting amounts of substrate. The biosynthesized mevalonic acid of each sample was extracted, and hydroxamate determinations were made on aliquots of the extracted material. Another aliquot was assayed in a coupled system of mevalonic kinase, pyruvic kinase, and lactic acid dehydrogenase. Such a system has been employed by several investigators for the assay of mevalonic kinase (9, 21, 22). According to Levy and Popják (9), this enzyme acts on only one isomer of mevalonic acid. Table 4 shows the results of two such experiments. It will be noted from experiment 2 that approximately 80% of the substrate was reduced when the enzyme was incubated in the presence of limiting amounts of DL-mevaldic acid. A conversion in excess

TABLE 4. UTILIZATION OF MEVALONIC ACID BY MEVALONIC KINASE*

Experiment	MVA Generation: MVALD Incubated	MVA Generation: MVA Formed	Coupled Assay: MVA Incubated	Coupled Assay: MVA Utilized	Utilized
	μmoles	*μmoles*	*μmoles*	*μmoles*	%
1	20.0		0.045	0.041	91.5
1	7.5		0.052	0.032	61.0
2	8.0	2.02	0.076	0.064	84.0
2	2.0	1.60	0.080	0.041	52.0

* The incubation mixtures for the generation of mevalonic acid in experiment 1 contained: phosphate buffer, pH 7.0, 200 μmoles; TPNH, 8.7 μmoles; mevaldic acid as indicated in the table; enzyme protein, 7.8 mg; and water to a volume of 2.6 ml. The incubation mixtures of experiment 2 consisted of a total volume of 1.2 ml but contained the same concentrations of components as in experiment 1. The incubation temperature in experiment 1 was 38°; in experiment 2, 22° to 23° (room temperature). The coupled assay mixtures contained: phosphate buffer, pH 7.5, 100 μmoles; DPNH, 0.15 to 0.18 μmoles; phosphoenolpyruvate (tricyclohexylamine salt), 1.0 μmole; $MgCl_2$, 5.0 μmoles; cysteine, 2.0 μmoles; ATP, 2.0 μmoles; KF (experiment 2), 12.0 μmoles; mevalonic acid as indicated; lactic acid dehydrogenase, 10.0 μg; pyruvic kinase, 10.0 μg; mevalonic kinase, 0.35 mg; and water to a volume of 1.0 ml. The mevalonic acid samples were treated with KOH prior to incubation to convert the lactone to the salt.

of 50% could not be shown in experiment 1 due to nonlimiting substrate. It is evident that the mevalonic acid formed in the presence of excess mevaldic acid in each experiment was considerably more active in the coupled assay than that formed in the presence of limiting amounts of substrate. These results suggest that the biologically active isomer of mevaldic acid is acted on preferentially by the enzyme to form the biologically active isomer of mevalonic acid and that the slow reduction of mevaldic acid corresponds to the formation of the biologically inactive isomer of mevalonic acid.

TABLE 5. CONVERSION OF DL-MEVALDIC ACID TO MEVALONATE*

Experiment	MVALD Incubated	Carbonyl Disappeared	Hydroxamate Formed
	μmoles	*μmoles*	*μmoles*
1	1.0	0.87	0.84
2	1.11	1.12	0.90
3	1.11	1.05	0.91

* Incubation mixtures contained: DL-mevaldic acid, 1.0 to 1.1 μmoles; TPNH, 3 μmoles; phosphate buffer, pH 7.0, 100 μmoles; protein, 4 mg; and water to a final volume of 3.0 ml. After incubation for 1 hour at 38°, 1-ml aliquots were removed for assay.

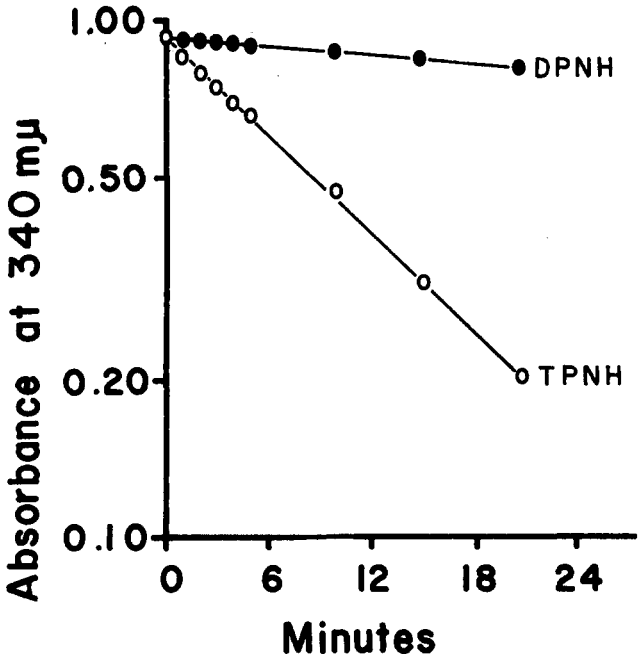

FIG. 5. Nucleotide specificity for mevaldic acid reduction. The incubation mixture contained: phosphate buffer, pH 7.0, 100 μmoles; TPNH or DPNH, 0.50 μmole; DL-mevaldic acid, 5.0 μmoles; protein, 0.92 mg; and water to a final volume of 3.0 ml.

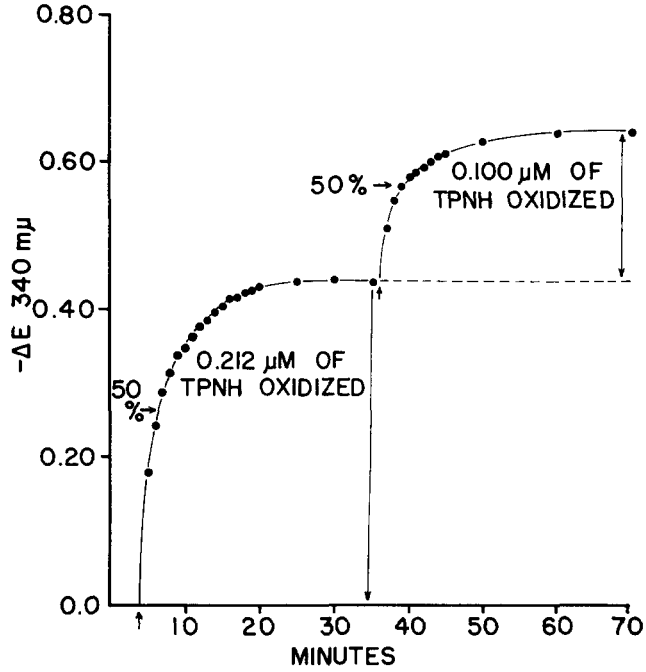

FIG. 6. Stereospecificity of mevaldic acid reductase. The incubation mixture contained: phosphate buffer, pH 7.0, 100 μmoles; TPNH, 0.50 μmole; protein, 3.0 mg; and water to a final volume of 2.95 ml. At the first vertical arrow (4 min), 0.250 μmole of DL-mevaldic acid was added; at the second vertical arrow (36 min), 0.125 μmole was added. The horizontal arrows indicate the point of 50% conversion of DL-mevaldic acid.

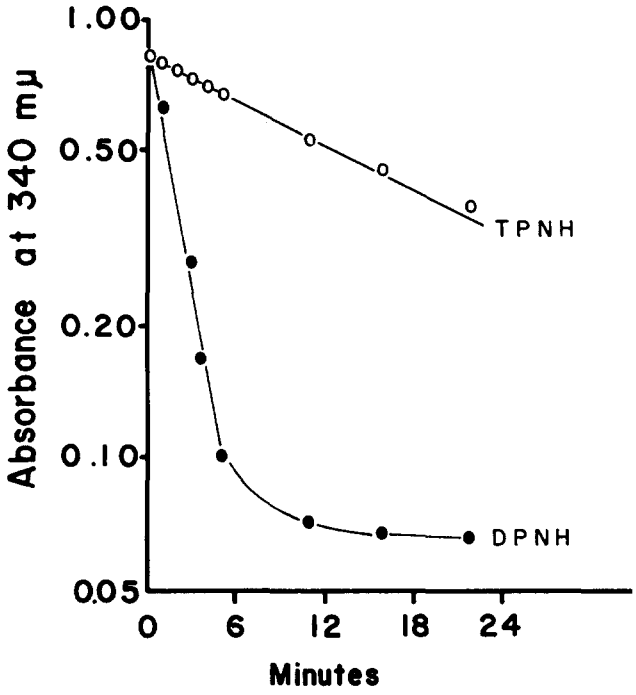

FIG. 7. Nucleotide specificity for the enzymatic reduction of *n*-butyraldehyde. The incubation mixture contained: phosphate buffer, pH 7.0, 100 μmoles; TPNH or DPNH, 0.41 μmole; *n*-butyraldehyde, 2.5 μmoles; protein (from second gel treatment, Table 1) 0.06 mg; and water to a final volume of 3.0 ml.

No evidence was obtained that would indicate racemization of mevaldic acid. In these experiments, the reaction was allowed to proceed until 50% of the substrate had been reduced and the TPNH was exhausted. The remaining mevaldic acid was incubated for 30 minutes with enzyme prior to the introduction of additional TPNH. No reappearance of the initial rapid rate of TPNH oxidation was observed. However, when DL-mevaldic acid was added with TPNH, oxidation of the latter proceeded at the initial rate. The results reported in this and previous paragraphs strongly suggest the enzymatic reduction of DL-mevaldic acid to DL-mevalonic acid.

Experiments that were conducted in order to obtain information on whether one or two enzymes are involved in the conversion of the two isomers of mevaldic acid are here summarized. The reduction of both isomers of mevaldic acid is TPNH specific, and both the primary (fast) and secondary (slow) phases persisted through all of the purification steps. Both phases were affected to the same degree when the enzyme preparation was subjected to treatment with acid and hydroxylamine (a level of 5×10^{-5} M gave an inhibition of 50%), and each remained unaffected by iodoacetamide at a concentration of 0.01 M. The relative activities of the two phases remained unchanged over the pH range of 6.0 to 8.6. Some difference was noted, however, on assaying for the activities in the presence of PCMS. The primary phase was inhibited 30% to 50% by 5×10^{-4} M PCMS while the secondary phase was inhibited 75% to 100%. There was negligible inhibition of the primary phase in the presence of 1×10^{-4} M PCMS. *N*-ethyl maleimide (5×10^{-3} M) inhibited the primary phase by only 15%.

Assay of Other Enzymes for Mevaldic Reductase Activity. Assays to determine whether crystalline liver alcohol dehydrogenase and lactic dehydrogenase reduce mevaldic acid were completely negative with both reduced pyridine nucleotides.

Aldehyde Reductase Activity Distinct from Mevaldic Acid Reductase. Substrate specificity studies revealed the presence of another enzyme in association with mevaldic reductase that is capable of reducing a number of aldehydes in the presence of DPNH. The distinctiveness of this activity compared with mevaldic reductase was demonstrated through differences in (1) nucleotide specificity, (2) sensitivity toward heat and acid, (3) ratios of the activities on purification, and (4) sensitivity toward PCMS. Figure 7 shows that DPNH was much more effective than TPNH in the reduction of *n*-butyraldehyde. Table 6 shows that the ratio of the two enzymatic activities changed by a factor of 6 to 7 on heat treatment and that the mevaldic acid reducing activity was more sensitive. An experiment demonstrating a 4-fold change in the ratio of the two activities on acid treatment is summarized in Table 7. The DPNH-specific aldehyde reductase is the more sensitive to acid treatment. Further, the ratio of the two reducing activities changed about 12-fold on enzyme purification as shown in Table 8. Treatment with 3×10^{-4} M PCMS resulted in complete inhibition of the DPNH-specific activity, whereas only 20% to 30% of the mevaldic acid-reducing activity was lost.

Substrate studies of aldehyde reduction and alcohol oxidation by the nonspecific aldehyde reductase revealed enough similarities to liver alcohol dehydrogenase (Nutritional Biochemicals Corp.) to suggest this as the identity of the enzyme associated with mevaldic reductase. We also observed that both crystalline liver alcohol dehydrogenase and the nonspecific aldehyde reductase of rat liver dehydrogenate geraniol and farnesol.

DISCUSSION

It is evident that the mevaldic acid reductase described in this paper differs in several respects from other enzymes effecting the same reduction in animal tissues. In previous reports on mevaldic reductase

TABLE 6. EFFECT OF HEAT ON ENZYME ACTIVITIES*

Treatment	Mevaldic Acid Reduction†	Butyraldehyde Reduction‡	Ratio
None	1.94	1.77	1.10
2 min, 50°	1.09	2.15	0.51
2 min, 55°	0.73	2.40	0.31
2 min, 60°	0.29	1.75	0.17

* 0.5-ml aliquot of enzyme solution (17.0 mg per ml) in 0.005 M phosphate buffer of pH 6.8 were subjected to heat treatments as indicated above, cooled, and centrifuged. Assay conditions were the same as those in Table 1.

† μmoles TPNH oxidized per mg protein per hour.

‡ μmoles DPNH oxidized per mg protein per hour.

TABLE 7. EFFECT OF ACID ON ENZYME ACTIVITIES*

pH	Mevaldic Acid Reduction†	*n*-Butyraldehyde Reduction‡	Ratio
6.8	1.77	1.77	1.00
6.0	1.64	1.56	1.05
5.5	1.67	0.84	2.00
5.0	0.58	0.13	4.50

* A 2.0-ml aliquot of enzyme solution (17.0 mg per ml) in 0.005 M phosphate buffer of pH 6.8 was progressively adjusted to the pH values indicated. Biuret determinations were made on the remaining solution when precipitation of protein occurred. Assays were then carried out as indicated in Table 1. All assays were made at pH 7.0.

† μmoles TPNH oxidized per mg protein per hour.

‡ μmoles DPNH oxidized per mg protein per hour.

(4, 5), the authors reported that DPNH was the preferred cofactor in their systems. Figure 5 shows that DPNH was ineffective with our rat liver enzyme preparation. Also, inhibition studies in our system yielded results that are consistent with an enzyme model that does not contain a sulfhydryl group at the active center; this is in marked contrast to the high sensitivity to sulfhydryl inhibitors reported in the other studies.

If only one isomer of mevalonic acid were formed in the reduction of mevaldic acid, an "all or none" effect would have been observed upon coupling with mevalonic kinase (9). However, in the conversion of limiting amounts of DL-mevaldic acid to mevalonic acid (Table 4), both optical isomers were utilized by our rat liver enzyme system, and both isomers of mevalonic acid were formed as indicated by the incomplete utilization of the biologically formed mevalonic acid in the coupling assay. In the presence of nonlimiting amounts of DL-mevaldic acid (Table 4), the biologically active isomer was reduced preferentially as shown by the almost complete utilization of the product in the coupling assay.

Whether a single enzyme is acting at different rates on the two isomers of mevaldic acid or whether two enzymes are involved is not certain, although all results in this paper may be rationalized by using a single enzyme model. Precedent for a single enzyme is found in the fact that glutamine synthetase is known to act on both isomers of its substrate under controlled conditions (23–25). The fact that both phases of the mevaldic reductase activity persisted to the same degree through all steps of enzyme purification with several individual preparations suggests that a single enzyme is acting. Further support for the single-enzyme concept is obtained from the results of studies on the effect of pH and acid treatment on the activity of mevaldic reductase. Studies with PCMS in which the secondary phase was inhibited more strongly than the primary phase may be interpreted on the basis of a progressive disruption of the tertiary structure of the enzyme and a difference in the affinity of D- and L-mevaldic acid for the enzyme.

Efforts to detect mevaldic acid as an intermediate in sterol synthesis in yeast enzyme systems when radioactive acetate or HMGCoA were used as precursors have been negative (6, 7). However, Brodie and Porter (8) detected incorporation of radioactivity into mevaldic acid by using animal systems and the same substrates, but the quantities were too low for it to be considered as a free intermediate. They proposed that mevaldic reductase has the function of metabolizing any mevaldic acid lost from the enzyme surface in the reduction of HMGCoA to mevalonic acid.

It is known that HMGCoA is formed in the microsomes (26, 27) and that the phosphorylation of mevalonic acid takes place in the soluble portion of the cell (9, 28); therefore, at some stage between HMGCoA and mevalonic phosphate, there must be a migration of a metabolite outside of the microsomes. Whether or not this metabolite is mevaldic acid, which is then reduced to mevalonic acid, is problematical; but if the conversion of HMGCoA to mevalonic acid in the rat

TABLE 8. RATIO OF MEVALDIC ACID AND BUTYRALDEHYDE REDUCTASE ACTIVITIES*

Fractionation Step	Ratio
Crude solubles	0.09
35–60 $(NH_4)_2SO_4$	0.20
$Ca_3(PO_4)_2$, two adsorptions	0.62
0–60 $(NH_4)_2SO_4$	1.10

* Assay conditions were the same as those for Table 1.

system is effected by a single enzyme, as in yeast, then free mevaldic acid will not appear outside the microsome in large quantities.

Mevaldic reductase may be operative in an alternate pathway to sterol synthesis. A suggested route would entail the synthesis of β-methylglutaconyl CoA from leucine followed by direct reduction and hydration to mevaldic acid. Such a pathway might be operative in starvation, a condition in which Bucher, *et al.* suggest that the enzyme HMGCoA reductase is either deleted or its activity seriously impaired (27).

The finding that the DPNH-specific aldehyde reducing activity of rat liver and liver alcohol dehydrogenase are active on geraniol and farnesol corroborates the report of Christophe and Popják (29) that soluble rat liver enzymes convert terpene alcohols to the corresponding carboxylic acids.

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