Fahrney, D. E., and Gold, A. M. (1963), *J. Am. Chem. Soc. 85,* 997.
Greenstein, J. P., and Winitz, M. (1961), Chemistry of the Amino Acids, Vol. 2, New York, N.Y., Wiley, p 926.
Hersch, L. B., and Jencks, W. P. (1967), *J. Biol. Chem. 242,* 3481.
Jaenicke, L., and Lynen, F. (1960), *Enzymes, 2nd Ed. 3,* 3.
Kitz, R., and Wilson, I. B. (1962), *J. Biol. Chem. 237,* 3245.
Lane, R. S., Manning, J. M., and Snell, E. E. (1971), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 30,* 1212.
Lane, R. S., and Snell, E. E. (1976), *Biochemistry 15,* preceding paper in this issue.
Mardashev, S. R., and Gonchar, N. A. (1968), in Symposium on Pyridoxal Enzymes, Yamada, K., Katunuma, N., and Wada, H., Ed., Tokyo, Maruzen, p 77.
Mardashev, S. R., Semina, L. A., Dabagov, N. S., and Gonchar, N. A. (1968), in Pyridoxal Catalysis: Enzymes and Model Systems, Snell, E. E., Braunstein, A. E., Severin, E. S., and Torchinski, Yu. M., Ed., Interscience, New York, N.Y., p 451.
Meloche, H. P. (1967), *Biochemistry 6,* 2273.
Recsei, P. A., and Snell, E. E. (1970), *Biochemistry 9,* 1492.
Riley, W. D., and Snell, E. E. (1968), *Biochemistry 7,* 3520.
Riley, W. D., and Snell, E. E. (1970), *Biochemistry 9,* 1485.
Rosenthaler, J., Guirard, B. M., Chang, G. W., and Snell, E. E. (1965), *Proc. Natl. Acad. Sci. U.S.A. 54,* 152.
Solomon, F., and Jencks, W. P. (1969), *J. Biol. Chem. 244,* 1079.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30,* 1190.

Purification of β-Hydroxy-β-methylglutaryl-coenzyme A Reductase from Yeast†

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ABSTRACT: β-Hydroxy-β-methylglutaryl-coenzyme A reductase of yeast has been solubilized by two different methods and then purified approximately 5000-fold. The purified enzyme shows a single precipitin band on immunodiffusion, and it moves as a single band of protein and enzyme activity on gel filtration and diethylaminoethylcellulose column chromatography. It also shows one major band on polyacrylamide gel electrophoresis. The specific activity of the pure enzyme is 18 000 to 22 000 nmol of reduced nicotinamide adenine dinucleotide phosphate oxidized per min per mg of protein. The molecular weights of the enzyme, estimated by gel filtration, and the subunits, determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis, are 2.6×10^5 and 6.0×10^4, respectively.

β-Hydroxy-β-methylglutaryl-coenzyme A reductase (mevalonate:$NADP^+$ oxidoreductase (acetylating coenzyme A), EC 1.1.1.34) has generated a great deal of interest, since it is the rate-limiting enzyme of hepatic cholesterol biosynthesis. This enzyme catalyzes the reduction of D-HMG-CoA[1] by NADPH according to the following equation:

$$\text{HMG-CoA} + 2\ \text{NADPH} + 2\text{H}^+ \rightarrow \text{MVA} + 2\ \text{NADP}^+ + \text{CoASH}$$

Mammalia hepatic HMG-CoA reductase is localized in the endoplasmic reticulum, whereas in yeast this enzyme is in the mitochondria (Shimizu et al., 1973). Yeast reductase was solubilized in earlier studies (Durr and Rudney, 1960) by prolonged autolysis and then purified (Kirtley and Rudney, 1967) approximately 200-fold to a specific activity of 1400 nmol of NADPH oxidized per min per mg of protein. This partially purified enzyme was very unstable.

Rat liver microsomal reductase has also been solubilized and partially purified. The various methods used for the solubilization and partial purification of the rat liver enzyme have been reviewed by Dugan and Porter (1976). The activities reported for this enzyme are much less than the activity reported by Kirtley and Rudney (1967) for the yeast enzyme.

HMG-CoA reductase has been induced in *Pseudomonas* by growing the organism on mevalonic acid (Bensch and Rodwell, 1970). The induced bacterial enzyme differs from the naturally occurring reductases of yeast and mammalian systems in that it utilizes NADH instead of NADPH as the reducing agent. The *Pseudomonas* enzyme was purified to a specific activity of 56 000 nmol of NADH oxidized per min per mg of protein.

In the present investigation, we have used two different methods of solubilization of the yeast HMG-CoA reductase. These are autolysis of dry yeast and sonication of mitochondria obtained through disruption of cells of frozen cakes of fresh yeast. The enzyme in each of these solubilized preparations has been purified to homogeneity, or near homogeneity, by classical techniques and affinity chromatography. This is the first

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[1] Abbreviations used are: HMG-CoA, β-hydroxy-β-methylglutaryl-coenzyme A; MVA, mevalonic acid; NADH, reduced nicotinamide adenine dinucleotide; NADPH, NADH phosphate; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid.

demonstration of the preparation of a homogeneous HMG-CoA reductase from yeast cells.

Materials and Methods

Chemicals. Chemicals were obtained from the following sources: β-hydroxy-β-methylglutaric acid from K&K Laboratories; CoASH, as the lithium salt, and NADPH from Sigma;DEAE-cellulose from Schwarz-Mann; Agarose–hexane–CoA affinity gel, type V, from P-L Biochemicals. Fleischmann's active dry yeast for bakers and cakes of fresh yeast were purchased locally. All other reagents were of analytical grade. DEAE-cellulose was acid and base washed (Peterson and Sober, 1962) and calcium phosphate gel was prepared as described by Tsuboi and Hudson (1957).

HMG-CoA was prepared by reacting coenzyme A with HMG anhydride. The latter compound was prepared as described by Goldfarb and Pitot (1971). HMG-CoA was then purified by paper chromatography (Brodie and Porter, 1960).

Spectrophotometric Assay of Enzyme Activity. Spectrophotometric assays were carried out with a Gilford recording spectrophotometer, Model 2400-S, equipped with a thermostated 1-cm cuvette compartment and a recorder with an adjustable zero and a multispeed chart drive. The temperature of each cell compartment was maintained at 30 °C by circulating water from an external Haake water bath. A unit of enzyme activity is defined as the amount of enzyme required to catalyze the oxidation of 1 nmol of NADPH per min per ml of incubation mixture. The oxidation of NADPH was followed spectrophotometrically at 340 nm. Specific activity of the enzyme is expressed as units of enzyme activity per mg of protein. HMG-CoA reductase was assayed in a solution containing potassium phosphate buffer, pH 7.0, 100 mM, dithiothreitol, 5 mM, NADPH, 0.16 mM, and HMG-CoA, 300 μM. The amount of enzyme used was variable depending on the stage of purification.

Radiochemical Assay of Enzyme Activity. The assay system was the same as described by Nepokroeff et al. (1974).

Assay for Protein. Protein assays were carried out by the biuret method and by the method developed by Lowry et al. (1951) after the protein solution was dialyzed against water to remove dithiothreitol.

Solubilization of Enzyme. HMG-CoA reductase was solubilized from Fleischmann's active dry yeast for bakers by a modification of the autolysis procedure of Kirtley and Rudney (1967). The liquid nitrogen freezing step prior to autolysis was omitted and dried yeast was substituted for yeast cakes. One pound of dry yeast was suspended in 1 l. of dibasic phosphate, 0.3 M, containing dithiothreitol, 1 mM, and EDTA, 1 mM. The suspended yeast was stirred 12 h at 4 °C. The suspension was centrifuged 15 min at 20 000*g* and the supernatant was discarded. The gummy precipitate was resuspended in 750 ml of the same buffer and stirred for another 48 h at 4 °C. The suspension was centrifuged as before, and the precipitate was resuspended in 750 ml of the same buffer and stirred for 40 h. The latter step resulted in the solubilization of a considerable portion of the enzyme (method 1). The supernatant solution was decanted and retained.

In order to determine whether the prolonged autolysis had caused any adverse effects on the enzyme, HMG-CoA reductase was solubilized by another procedure. Tzagoloff's (1969) procedure for the large-scale preparation of yeast mitochondria was used. Fleischmann's cakes of fresh yeast (1 lb) were crumbled into 1 l. of liquid nitrogen in a Dewar flask. This mixture was then transferred to a gallon-sized stainless steel Waring blender and the frozen pellets were homogenized at medium speed for 1 min. This procedure was repeated three times. The frozen powder was transferred to 1 l. of 0.05 M Tris-Cl buffer, pH 8.2, containing sucrose, 0.4 M, and EDTA, 1 mM. After thawing, the pH was adjusted to 7.8 with KOH and the suspension was homogenized for 30 s in the Waring blender. The homogenate was centrifuged at 2500*g* for 15 min to remove unbroken cells and debris. The supernatant solution was then centrifuged at 20 000*g* for 15 min. The mitochondrial pellet was suspended in 0.1 M potassium phosphate buffer, pH 7.0, containing dithiothreitol, 1 mM, and EDTA, 1 mM, and centrifuged again. In order to solubilize HMG-CoA reductase, the mitochondria were sonicated in a Branson sonicator generating 72 W for 1 min. This procedure was repeated three times. The supernatant solution, after centrifugation at 105 000*g* for 60 min, was retained for further purification of the enzyme (method 2).

Heat Treatment and Ammonium Sulfate Precipitation. The crude supernatant solutions (obtained by each of the above procedures) were heated in a water bath until a bath temperature of 65 °C was reached, which took approximately 30 min, and then for an additional 5 min. This mixture was immediately cooled on ice and centrifuged at 20 000*g* for 20 min.

The supernatant solution was brought to 60% saturation with solid ammonium sulfate. The protein precipitate was dissolved in a minimal volume of 0.1 M potassium phosphate buffer, pH 7, containing dithiothreitol, 1 mM, and EDTA, 1 mM. (The latter components were present in all buffers used in subsequent steps.) The enzyme was dialyzed against the same buffer for 3 h, with a change of buffer after 1.5 h.

Calcium Phosphate Gel Adsorption and Ammonium Sulfate Precipitation. The dialyzed protein was diluted with water containing dithiothreitol, 1 mM, and EDTA, 1 mM, to a final concentration of 0.025 M potassium phosphate and a protein concentration of approximately 7 mg/ml. The solution was then mixed with calcium phosphate gel so that the weight ratio of protein to gel was 1:1. After centrifugation for 5 min at 8000*g*, the calcium phosphate gel was washed twice with one-tenth of the original volume of 0.1 M potassium phosphate buffer. HMG-CoA reductase was removed from the gel by washing three or four times with 0.4 M potassium phosphate buffer. Each wash was assayed for reductase activity. The active fractions (0.4 M buffer eluates) were combined and enzyme protein was precipitated between 0 and 65% of saturation with solid ammonium sulfate. The precipitate was dissolved in a minimum volume of 0.1 M potassium phosphate buffer and then dialyzed against the same buffer for 3 h with a change of buffer at 1.5 h.

DEAE-Cellulose Column Chromatography. The dialyzed protein was diluted to a concentration of 0.01 M potassium phosphate buffer and then put through a DEAE-cellulose column, previously equilibrated by washing overnight with 0.01 M monobasic potassium phosphate, followed by 0.01 M potassium phosphate buffer, pH 7.0. Protein was eluted from the column with 450 ml of a linear concentration gradient, 0.01–0.50 M potassium phosphate buffer, pH 7.0. Ten-milliliter fractions were collected. The enzymatically-active fractions were pooled and concentrated by ultrafiltration in an Amicon cell with a PM-10 membrane.

Affinity Chromatography. Agarose–hexane–CoA (type V) gel[2] (0.5–1.5 ml) was suspended in 0.025 M potassium phos-

[2] The use of CoA-bound gel for the partial purification of rat liver HMG-CoA reductase was first developed by Beg and Gibson (unpublished) who then kindly suggested this method to us.

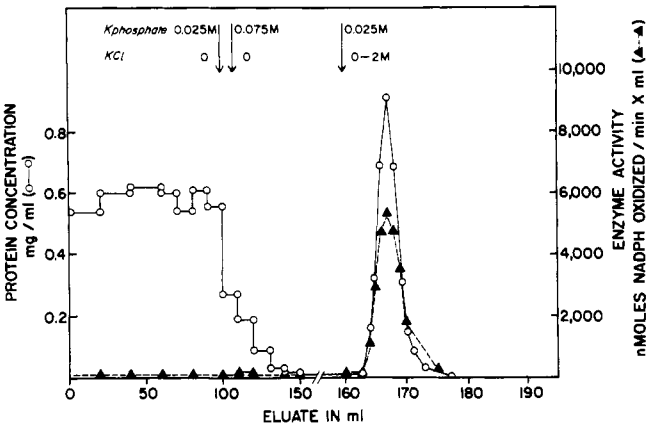

FIGURE 1: Affinity chromatography of the DEAE-cellulose-purified HMG-CoA reductase from autolyzed yeast. DEAE-cellulose purified enzyme (150 000 units and 72 mg of protein in 20 ml of 0.125 M potassium phosphate buffer) was diluted fivefold with water containing EDTA, 1 mM, and dithiothreitol, 5 mM, and then applied to a 0.75 × 3.4 cm column (1.5 ml of gel) of Agarose-hexane-CoA at 4 °C. The column was previously equilibrated with 0.025 M potassium phosphate buffer, pH 7.0, containing EDTA, 1 mM, and dithiothreitol, 5 mM. Nonadsorbed protein was washed from the column at 22 °C with 10 ml of the same buffer and then with 50 ml of 0.075 M potassium phosphate buffer containing 5 mM dithiothreitol. The enzyme was eluted with a KCl gradient (0–2 M, 14 ml) in 0.025 M potassium phosphate buffer, pH 7.0. This buffer also contained 5 mM dithiothreitol. Reductase activity (▲- - -▲) and protein (O—O) were assayed in the effluent. The rate of elution was 1 ml/min. One-milliliter eluate fractions were collected after the start of the gradient; before that, 10-ml fractions were collected.

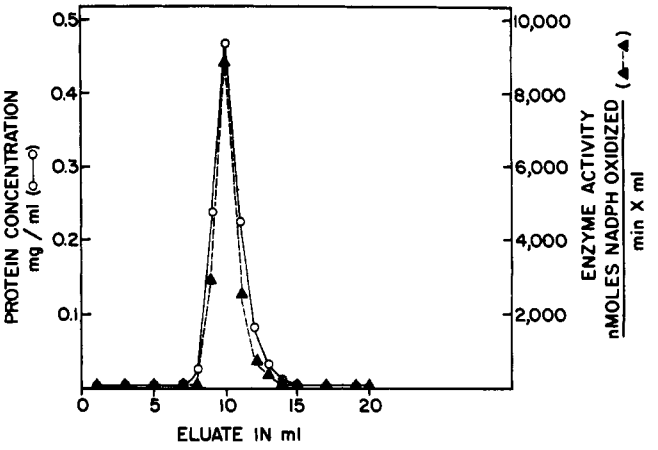

FIGURE 2: Bio-Gel filtration of HMG-CoA reductase solubilized by the autolysis procedure and purified by affinity chromatography. A column (1 × 14.5 cm) of Bio-Gel A-1.5m, 200–400 mesh, was equilibrated with 0.1 M potassium phosphate buffer, pH 7.0, containing EDTA, 1 mM, and dithiothreitol, 5 mM. Enzyme, 16 000 units and 0.8 mg of protein in 0.5 ml of the above buffer, was purified as reported in Table I and applied to the Bio-Gel column. Aliquots of 1.0 ml were collected from the column and assayed for enzyme activity (▲- - -▲) and protein (O—O).

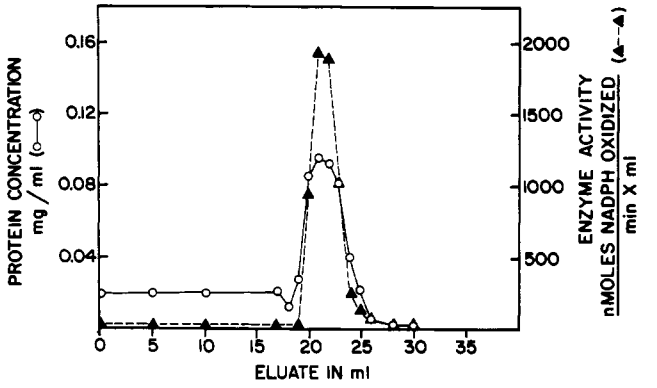

FIGURE 3: DEAE-cellulose chromatography of HMG-CoA reductase. Enzyme was solubilized by the autolysis procedure and then purified as reported in Table I. Protein, 0.85 mg, purified through affinity chromatography, was diluted to a concentration of 0.01 M potassium phosphate and then applied to a DEAE-cellulose column (0.5 × 7.5 cm) previously equilibrated overnight with the same buffer. Protein was eluted from the column with 10 ml of a linear concentration gradient, 0.01–0.5 M potassium phosphate buffer, pH 7.0, and 1-ml fractions were assayed for enzyme activity (▲- - -▲) and protein (O—O).

phate buffer, pH 7.0, containing dithiothreitol, 5 mM, and EDTA, 1 mM. The gel was then washed with 50 volumes of the same buffer. The procedure for affinity chromatography of HMG-CoA reductase on this gel is presented in the legend to Figure 1.

Gel Filtration. The procedure for this separation is presented in the legend to Figure 2.

Second DEAE-Cellulose Column Chromatography. The procedure for this step is given in the legend to Figure 3.

Preparation of Antiserum. HMG-CoA reductase was purified through the DEAE-cellulose chromatographic step (Table I). Enzyme protein was then mixed with an equal volume of complete Freund's adjuvant and 2.5 mg of protein was injected subcutaneously into a rabbit. Subsequent injections were given at 1-week intervals with 2–3 mg of protein mixed with an equal volume of complete Freund's adjuvant. The rabbit was bled 5 weeks after the first injection. The blood was coagulated for 30 min at 37 °C, and the globulin fraction was isolated as described by Levy and Sober (1960). Sera were frozen at −10 °C until used.

Immunological Procedure. Ouchterlony micro-double diffusion was carried out on microscope slides in 0.5% Agarose containing 1.0% (w/v) sodium chloride (Craig et al., 1972).

Gel Electrophoresis. Disc gel electrophoresis was carried out according to the method of Davis (1964). Gels were made 5% in polyacrylamide and 30% in glycerol (Brown et al., 1974). Protein, 50–100 μg, was subjected to a current of 3 mA/gel for approximately 3–4 h. The gels were stained with Amido-Schwarz, and then destained with 7% acetic acid. A duplicate of each gel was also run. However, instead of staining, each was sliced. The number of segments and the length of each, starting from the origin, were as follows: 1 of 1, 1 of 0.5, 9 of 0.25, 2 of 0.5, and 2 of 1 cm, respectively. Each slice was macerated and then incubated for 30 min in order to determine enzyme activity. The radioassay method previously described was used.

Sodium Dodecyl Sulfate Gel Electrophoresis. The gels (5% polyacrylamide with sodium dodecyl sulfate) were run according to the procedure of Weber and Osborn (1969). The enzyme purified by affinity chromatography was dialyzed for 1 h, in a collodion tube against 0.01 M sodium phosphate buffer, pH 7.0, containing 1% sodium dodecyl sulfate and 1% mercaptoethanol, and then incubated at 37 °C for 3 h in the same buffer. The protein concentration was normally 0.2–0.5 mg/ml. Proteins of known molecular weights were treated in a similar manner. About 50–75 μl of the samples were applied onto the gels. A current of 8 mA/gel was applied for 4 h (Weber and Osborn, 1969). After electrophoresis, the sodium dodecyl sulfate was leached out, and the proteins were precipitated by soaking the gels for 24 h in 20% sulfosalicylic acid (Dunker and Rueckert, 1969). The proteins were stained with 0.275% Coomassie brilliant blue, in a mixture of 50% methanol and 10% glacial acetic acid. The gels, after rinsing with distilled

TABLE I: Purification of HMG-CoA Reductase.

Fraction	Vol (ml)	Protein (mg/ml)	Total Units ($\times 10^{-4}$)	Sp Act.[a]	Recovery (%)	Purification (Fold)
Crude	7130	23.2	66	4	100	1
Heated	6620	19.7	65	5	98	1.25
1st $(NH_4)_2SO_4$ ppt	345	47.2	42	26	63	6.5
$Ca_3(PO_4)_2$ gel	2000	0.5	39	386	58	96
2nd $(NH_4)_2SO_4$ ppt	82	11.2	32	345	47	86
DEAE-cellulose	270	0.46	23	1 860	35	465
Affinity chromatography	3	1.16	6.9	19 800	10	4950

[a] nmol of NADPH oxidized min^{-1} mg of $protein^{-1}$.

water, were destained for 48 h in an aqueous solution containing 7.5% acetic acid and 5% methanol.

Molecular Weight of HMG-CoA Reductase. For molecular weight determinations, a Bio-Gel A-1.5m (200–400 mesh) column (1.7 × 44 cm) was used. Equilibration and elution were carried out as described above. Eluate fractions of 1 ml were collected at the rate of 1 ml/3.5 min. Standard proteins of known molecular weights were passed through the column under the same conditions.

Results

The purification of HMG-CoA reductase from yeast autolysate outlined in Table I is an extension of the method described by Dugan and Porter (1971) for the partial purification of this enzyme. The final activity reported in this table, a specific activity of 19 800 nmol of NADPH oxidized per min per mg of protein, represents a purification of nearly 5000-fold.[3]

The purification of enzyme solubilized by sonication of mitochondria prepared from several 20-lb lots of fresh yeast was also carried out by the steps outlined in Table I. The specific activity of the crude extract varied from 5.0 to 6.7 nmol of NADPH oxidized per min per mg of protein. That of the protein obtained after affinity chromatography was 17 000 to 20 000 nmol of NADPH oxidized per min per mg of protein. Thus, a purification of the enzyme from fresh yeast of over 2500-fold was obtained.

Enzyme solubilized by either autolysis (method 1) or by sonication of mitochondria (method 2) and then purified through the DEAE-cellulose column chromatographic step was very stable after concentration in the presence of 1 mM dithiothreitol. Very little loss of activity occurred in 3–4 months of storage at 4 °C.

In the final step of enzyme purification (affinity chromatography) most of the protein passed directly through the column without binding. The bound HMG-CoA reductase was then removed by gradient elution with KCl. A single peak of reductase activity was obtained, Figure 1. Results similar to that shown on this figure were obtained with enzyme preparations purified from sonicated mitochondria of fresh yeast.

The enzyme purified by affinity column chromatography moved as a single component on Bio-Gel filtration (Figure 2). This enzyme retained 80% of its activity after a 2-day period of storage at 4 °C. The enzyme also moved as a single component when chromatographed on a DEAE-cellulose column (Figure 3) and when electrophoresed on 5% acrylamide gel (Figures 4 and 5). On sodium dodecyl sulfate polyacrylamide gel electrophoresis a major and one or two trace bands were observed (Figure 6). The latter might be due to a trace of contaminating protein or to incomplete dissociation of the enzyme, or both.

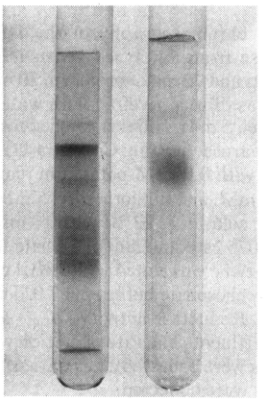

FIGURE 4: Disc gel electrophoresis of HMG-CoA reductase from autolyzed yeast. The experimental details of the procedure used in this separation are reported in the text. The amount of protein used was as follows: left, DEAE-cellulose purified enzyme, 70 μg; right, affinity gel purified enzyme, 75 μg. The specific activity of the latter preparation was 20 000 nmol of NADPH oxidized min^{-1} mg of $protein^{-1}$.

The purity of HMG-CoA reductase obtained after affinity chromatography was also determined by Ouchterlony micro-double diffusion analysis. Antiserum was prepared by injecting a rabbit with an enzyme preparation (from autolyzed yeast) purified through DEAE-cellulose chromatography (see Table I). Ouchterlony double-diffusion patterns were then obtained by testing antisera against preparations of HMG-CoA reductase purified through DEAE-cellulose and affinity chromatography (Figure 7). When the antiserum was tested against DEAE-cellulose-purified HMG-CoA reductase, three or four precipitin bands were observed. When the antisera were tested against the affinity-purified enzyme, only one precipitin band was visible.

An estimation of the molecular weight of the reductase was obtained from plots of its elution volume from a Bio-Gel column relative to those of proteins of known molecular weight. This method gave a molecular weight of 2.6 to 2.7 × 10^5.

An estimation of the molecular weights of the subunits of the reductase was obtained from a plot of their relative mobilities on sodium dodecyl sulfate gel electrophoresis. Proteins of known molecular weights were used as standards. This method gave a subunit molecular weight of approximately 60 000 for the only subunit species of the yeast enzyme that was detected, which indicates that yeast HMG-CoA reductase consists of four subunits of the same molecular weight.

[3] A range in specific activity of 18 000 to 22 000 nmol of NADPH oxidized per min per mg of protein was obtained in several purifications of enzyme.

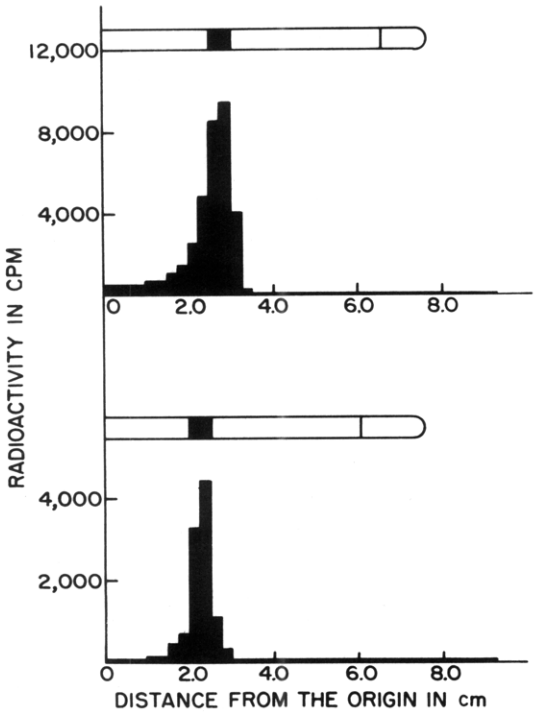

FIGURE 5: Enzyme activity profile of affinity gel purified HMG-CoA reductase on 5% acrylamide gel. Top, enzyme protein from fresh yeast; bottom, from autolyzed yeast. Duplicate gels were run. One was stained with Amido-Schwarz, while the other was sliced into several sections. Each slice was macerated and assayed radiochemically for enzyme activity as described under Methods.

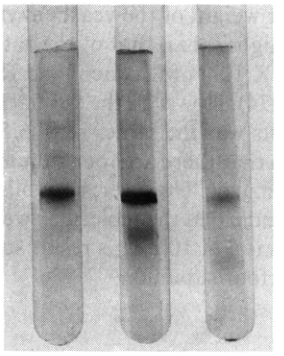

FIGURE 6: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of HMG-CoA reductase. The amount of protein used was: left, affinity gel-purified HMG-CoA reductase (from autolyzed yeast), 10 μg; center, catalase, 20 μg; right, affinity gel-purified reductase (from fresh yeast), 7 μg. Further experimental details are reported under Methods.

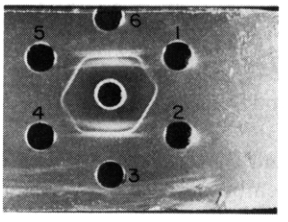

FIGURE 7: Ouchterlony double-diffusion analysis. The antibody used in this assay was prepared against DEAE-cellulose purified HMG-CoA reductase. The antigens were preparations of HMG-CoA reductase that had been purified partially or completely from autolyzed yeast. Agarose gels, 0.5% (w/v) in 1.0% sodium chloride (w/v), were prepared on microscope slides and then developed for 18–22 h at 24 °C before photographs were taken. The wells contained the following: center well, antiserum prepared against DEAE-cellulose purified HMG-CoA reductase; wells 1 and 4, HMG-CoA reductase purified through the affinity chromatographic step and then subjected to Bio-Gel filtration; wells 2 and 5, HMG-CoA reductase purified through the affinity gel chromatographic step and then subjected to DEAE-cellulose column chromatography; wells 3 and 6, HMG-CoA reductase purified through the DEAE-cellulose chromatographic step. (See Table I for the steps in the HMG-CoA purification procedure.)

Discussion

HMG-CoA reductase in each of the crude preparations obtained by the two different solubilization techniques used in this study was purified to homogeneity. Enzyme purified by either technique migrated the same on gel filtration, immunodiffusion, and 5% polyacrylamide gel electrophoresis. However, enzyme purified by each technique moved as a diffuse band on polyacrylamide gel electrophoresis with R_f's of 0.37 and 0.4 for purified reductase solubilized by autolysis of dried yeast or by sonication of mitochondria from fresh yeast, respectively. It is evident, therefore, that the diffuse band on polyacrylamide gel electrophoresis is not the result of hydrolytic action on peptide bonds during autolysis. It is evident also that the species of HMG-CoA reductase obtained by the two procedures are identical by all of the assays we carried out.

The purification of HMG-CoA reductase presented several difficulties. These included (a) a low percentage of enzyme in the crude solubilized protein fraction, (b) instability of the enzyme at low ionic strength and in the absence of a strongly reducing sulfhydryl compound, and (c) variable results in the recovery of enzyme activity after affinity chromatography.

Precautions were taken to overcome the above difficulties. All operations were carried out with a minimum exposure of enzyme to low ionic strength. For example, in the purification steps utilizing $Ca_3(PO_4)_2$ gel adsorption and DEAE-cellulose chromatography, the solutions containing enzyme were diluted to a low ionic strength just prior to the operation, and separations were carried out as quickly as possible. Dithiothreitol was present in the media at all times, including during autolysis, and in each purification step carried out after the solubilization of the reductase.

One of the most critical steps in the purification procedure was affinity chromatography. The recovery of enzyme was usually about 40–45%, but varied from 20 to 70%. In order to obtain a good yield, careful regulation of the ratio of gel to protein was necessary. The column was almost saturated with enzyme in order to achieve maximum recovery of active enzyme. After elution, the protein concentration was very low and the enzyme lost activity quickly unless it was concentrated immediately. Dialysis with collodion tubes was used to concentrate the enzyme and to remove salt from the protein.

The yeast enzyme exhibited properties similar in some respects to the NADH-requiring HMG-CoA reductase induced in *Pseudomonas* by growing the organism on mevalonic acid (Bensch and Rodwell, 1970). The specific activities, molecular weights, and kinetic patterns of the two enzymes were similar. The molecular weight obtained for the yeast enzyme was 260 000 to 270 000 as compared to 260 000 to 280 000 for the enzyme induced by *Pseudomonas*. However, the specific activity obtained for the *Pseudomonas* enzyme was 56 000 nmol of NADH oxidized per min per mg protein, which is 2.5 times that obtained for the yeast enzyme. A sequential pattern for the addition of substrates to the enzyme was obtained by kinetic analysis for the *Pseudomonas* and for the yeast enzyme purified in this study. The kinetics of yeast HMG-CoA reductase is discussed at length in the accompanying paper (Qureshi et al., 1976).

The molecular weight of the yeast enzyme, $2.6-2.7 \times 10^5$, is significantly higher than that of the rat liver enzyme (approximately 2.0×10^5) of Kawachi and Rudney (1970) and Higgins et al. (1974). However, the estimation of the molecular weight of subunits was the same—$6.0-6.5 \times 10^4$. This leads to the conclusion that there are four subunits in the yeast and three in the rat enzyme. However, unpublished data from this laboratory[4] has indicated the molecular weight of the rat liver enzyme subunits is 5×10^4. This result suggests that the rat enzyme also has four subunits.

References

Bensch, W. R., and Rodwell, V. W. (1970), *J. Biol. Chem. 245,* 3755.

Brodie, J., and Porter, J. W. (1960), *Biochem. Biophys. Res. Commun. 3,* 173.

Brown, M. S., Dana, S. E., and Siperstein, M. D. (1974), *J. Biol. Chem. 249,* 6585.

Craig, M. C., Nepokroeff, C. M., Lakshmaran, M. R., and Porter, J. W. (1972), *Arch. Biochem. Biophys. 152,* 619.

Davis, B. J. (1964), *Ann. N.Y. Acad. Sci. 121,* 404.

Dugan, R. E., and Porter, J. W. (1971), *J. Biol. Chem. 246,* 5361.

Dugan, R. E., and Porter, J. W. (1976), in The Enzymes of Biological Membranes, Vol. 2, Martonosi, A., Ed., New York, N.Y., Plenum Press, p 161.

Dunker, A. K., and Rueckert, R. R. (1969), *J. Biol. Chem. 244,* 5074.

Durr, I. F., and Rudney, H. (1960), *J. Biol. Chem. 235,* 2572.

Goldfarb, S., and Pitot, H. C. (1971), *J. Lipid Res. 12,* 512.

Higgins, M. J. P., Brady, D., and Rudney, H. (1974), *Arch. Biochem. Biophys. 163,* 271.

Kawachi, T., and Rudney, H. (1970), *Biochemistry 9,* 1700.

Kirtley, M. E., and Rudney, H. (1967), *Biochemistry 6,* 230.

Levy, H. B., and Sober, H. A. (1960), *Proc. Soc. Exp. Biol. Med. 103,* 250.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. T. (1951), *J. Biol. Chem. 193,* 265.

Nepokroeff, C. M., Lakshmanan, M. R., Ness, G. C., Dugan, R. E., and Porter, J. W. (1974), *Arch. Biochem. Biophys. 160,* 387.

Peterson, E. A., and Sober, H. A. (1962), *Methods Enzymol. 5,* 6.

Qureshi, N., Dugan, R. E., Cleland, W. W., and Porter, J. W. (1976), *Biochemistry 15* (following paper in this issue).

Qureshi, N., Dugan, R. E., and Nimmannit, S. (1975), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 567.

Shimizu, I., Magai, J., Hatanaka, H., and Katsuki, H. (1973), *Biochim. Biophys. Acta 296,* 310.

Tsuboi, K. K., and Hudson, P. B. (1957), *J. Biol. Chem. 224,* 879.

Tzagoloff, A. (1969), *J. Biol. Chem. 244,* 5020.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.

[4] Kleinsek, D. A., Ranganathan, S., Winkelman, D., and Porter, J. W., unpublished results.