Simplified spectrophotometric assay for microsomal 3-hydroxy-3-methylglutaryl CoA reductase by measurement of coenzyme A

Frank H. Hulcher and Wayne H. Oleson1

Department of Biochemistry and the Arteriosclerosis Research Center, The Bowman Gray School of Medicine, Winston-Salem, North Carolina 27103

Abstract

A new assay for 3-hydroxy-3-methylglutaryl CoA reductase (mevalonate:NADP oxidoreductase [acylating CoA], EC 1.1.1.34) is based upon the measurement of released coenzyme A (SH) during the reduction of 3-hydroxy-3-methylglutaryl CoA to mevalonate. Coenzyme A was measured in the presence of dithiothreitol, required for activity, by reaction with 5,5'-dithiobis(2-nitrobenzoic acid). Sodium arsenite forms a complex with the diithiol, but not with monothiols. Thus, reduced coenzyme A reacts instantaneously with the reagent and dithiothreitol reacts slowly. The absorbance due to the coenzyme A-5,5'-dithiobis(2-nitrobenzoic acid) reaction is determined by extrapolating the linear (dithiol) absorbance-time curve to the time of addition of the reagent. After subtraction of control absorbance (deletion of NADPH), the concentration of CoA-SH is calculated from \( c_{\text{max}} = 1.36 \times 10^4 \) at 412 nm. The method of protein removal and reduction of sulfhydryl groups on the enzyme are critical. This method provides an immediate assay. Recovery of reduced coenzyme A was 98.7%. The assay is applicable for microsomes or purified enzyme and has an effective range of 0.5-50 nmoles of coenzyme A. It was applied to kinetic measurement of the pigeon liver microsomal enzyme reaction. The apparent \( K_m \) value for 3-hydroxy-3-methylglutaryl CoA was 1.75 \( \times 10^{-9} \) M, and for NADPH the value was 6.81 \( \times 10^{-4} \) M. This method was compared with the dual-label method at high and low levels of activity. The data were not statistically different.

Supplementary key words: pigeon liver · yeast microsomes · 3-hydroxy-3-methylglutaryl CoA deacylase · sulfhydryl group · arsenite-dithiol complex · enzyme kinetics

3-Hydroxy-3-methylglutaryl CoA reductase (mevalonate:NADP oxidoreductase [acylating CoA], EC 1.1.1.34) (HMG CoA reductase) is the major regulatory enzyme of hepatic cholesterol biosynthesis (1-4). The reaction catalyzed involves a two-step, NADPH-dependent reduction of 3-hydroxy-3-methylglutaryl CoA to mevalonate and formation of NADP and reduced coenzyme A (1, 4, 5). The measurement of activity of this enzyme involves a complex procedure requiring extraction, thin-layer chromatography, and double-label measurement (1, 3, 4, 6, 7-9). The direct spectrophotometric assay of NADPH oxidation is very slow and is subject to interference by other NADPH-requiring reactions. Therefore, it is not considered to be a practical assay for microsomal preparations.

In searching for an alternative method to assay the reaction immediately, we have explored the possibility of measuring reduced coenzyme A formed in the reaction. A new spectrophotometric assay based on the stoichiometric formation of coenzyme A during the reduction of 3-hydroxy-3-methylglutaryl CoA to mevalonate has been developed.

Coenzyme A is measured spectrophotometrically after the reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). DTNB is a sensitive reagent for sulfhydryl groups; the para-nitrothiophenol ion formed in the reaction has a molar extinction coefficient of 1.36 \( \times 10^4 \) at pH 8.0 (10). However, it will react with both monothiols and dithiols. A sulfhydryl reducing agent is required for HMG CoA reductase activity and for maintaining CoA in the reduced form as it is produced in the reaction. Consequently, it became necessary to be able to measure coenzyme A in the presence of a sulfhydryl reducing agent. Zahler and Cleland (11) found that arsenite forms a complex with dithiols but not with monothiols. They reported a procedure for the specific determination of dithiols in the presence of monothiols (11). Therefore, we selected dithiothreitol as the reducing agent for the reaction system. We have employed the procedure of Zahler and Cleland (11) and modified it for use as a sensitive assay for estimating coenzyme A released in the reaction.

This procedure has been used to assay microsomal 3-hydroxy-3-methylglutaryl CoA reductase. The data ob-

Abbreviations: DTT, dithiothreitol; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

1 Postdoctoral Fellow, NIH-National Heart and Lung Institute Training Grant HL-5883.
tained have been correlated with those obtained by the radioactive tracer method (6) using thin-layer chromatography.

**MATERIALS AND METHODS**

**Animals and yeast**

The livers of 3-month-old White Carneau and Show Racer pigeons fed a commercial grain diet (Purina pigeon pellets, Ralston Purina Co., St. Louis, Mo.) were used as a source of microsomes. Brewer's yeast microsomal preparations were kindly supplied by Dr. C. Madhosingh and Ian Wood, Canada Department of Agriculture, Chemistry and Biology Research Institute, Ottawa, Canada.

**Chemicals**

Triethanolamine-HCl, tris(hydroxymethyl)aminomethane, NADPH, NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and diithiothreitol were obtained from Sigma Chemical Co., St. Louis, Mo. EDTA and sodium arsenite were purchased from Fisher Scientific Co., Silver Spring, Md. Coenzyme A and 3-hydroxy-3-methylglutaryl CoA were purchased from P-L Biochemicals, Milwaukee, Wis. The 5,5'-dithiobis(2-nitrobenzoic acid), A grade, was obtained from Calbiochem, La Jolla, Calif. 3-Hydroxy-3-methyl[3-14C]glutaric acid used to prepare [3-14C]HMG CoA was supplied by New England Nuclear Corp., Boston, Mass. The reagent N,N'-dicyclohexylcarbodiimide was supplied by Schwarz/Mann, Orangeburg, N.Y.

**Preparation of microsomes**

The procedure described here is partially modified from that reported by Shapiro and Rodwell (7). The pigeons were decapitated and the livers were immediately removed and placed in cold buffer (0-4°C) at pH 7.4. The buffer contained 0.1 M triethanolamine-HCl, 0.02 M EDTA, and 2.0 mM dithiothreitol. The livers were thoroughly chilled, blotted, and weighed. The tissue was minced with scissors and homogenized with 3.5 ml of buffer/g of tissue. The homogenization was done with six strokes of a smooth-walled, glass Potter-Elvehjem homogenizer with a loose-fitting Teflon pestle (0.5 mm radial clearance). The homogenate was centrifuged for 10 min at 12,000 g to remove cell debris and mitochondria. The supernatant solution was carefully removed to prevent contamination with mitochondria and recentrifuged for 10 min at 12,000 g to ensure removal of mitochondria, it was then centrifuged at 60,000 g for 60 min. The 60,000 g microsomal pellet was then rinsed with buffer and frozen in a freezer (−20°C). Frozen microsomes were resuspended in 0.1 M triethanolamine buffer, pH 7.4, containing 0.02 M EDTA and 10 mM diithiothreitol and allowed to stand 60 min packed in ice; they were then centrifuged at 60,000 g for 45 min.

Microsomes either freshly prepared or frozen and treated with 10 mM DTT were resuspended in 2 ml of cold (0-4°C) buffer (0.1 M triethanolamine-HCl, 0.02 M EDTA, 2 mM diithiothreitol) at pH 7.4. Protein concentrations were determined with Folin phenol reagent using bovine serum albumin as a standard (12). To avoid interference by the triethanolamine buffer, the protein was precipitated with 10% trichloroacetic acid and redissolved in 0.1 M sodium hydroxide. The resuspended microsomes to be used for the assay were diluted with buffer to give a protein concentration of 5-10 mg/ml.

**Assay procedure**

For the assay of 3-hydroxy-3-methylglutaryl CoA reductase, the incubation mixtures normally contain 0.5-1.0 mg of microsomal protein, 150 nmoles of HMG CoA, and either 2 μmoles of NADPH or, preferably, 2 μmoles of NADP, 2 units of glucose-6-phosphate dehydrogenase, and 3 μmoles of glucose-6-phosphate. These components are added to 0.8 ml of 0.1 M triethanolamine-0.02 M EDTA buffer at pH 7.4 without diithiothreitol. The diithiothreitol (0.2 μmole) was added along with the microsomal preparation. The final incubation volume was 1 ml. Control tubes were as follows: (a) microsomes and buffer, (b) all components except HMG CoA, and (c) all components except NADP.

The reaction is started by the addition of microsomes, and the mixture is incubated in Corex centrifuge tubes at 37°C for 30 min. After incubation, 20 μl of 1 X 10^-2 M sodium arsenite solution is added to facilitate removal of soluble protein, and after 1 min the reaction is terminated by the addition of 0.1 ml of 2.0 M citrate buffer, pH 3.5, containing 3% sodium tungstate to give a final pH of 4.0 to precipitate the microsomal protein. After 10 min in a 37°C water bath, the tubes are centrifuged at 25,000 g for 15 min to remove protein. 1 ml of the supernate is transferred to a tube with stopper, and just before assay the pH is brought to 8.0 by addition of 0.2 ml of 2 M Tris buffer, pH 10.6, and 0.1 ml of 2 M Tris buffer, pH 8.0. The formation of the dithiol-arsenite complex is complete in 3-4 min after the addition of 50 μl of 0.4 M sodium arsenite.

The concentration of monothiol is determined by reacting DTNB with the reaction mixture in a cuvette placed in a recording spectrophotometer. 20 μl of 3 mM DTNB in 0.1 M triethanolamine-0.2 M EDTA buffer, pH 7.4, is added to 1.0 ml of the solution in a 1.0-ml cuvette, and the time is indicated on the recorder. The contents are mixed and the absorbance is measured for 4 min at 412 nm. The monothiol reacts immediately but the dithiol reacts slowly, giving a linear increase in absorbance with time. The absorbance due to monothiol is determined by extrapolating the linear portion of the curve back to the time of addition of DTNB. The concentration of monothiol is calculated using the molar extinction coefficient of 1.36 X 10^4 (10). The conversion to nanomoles of CoA formed per minute is as follows:
The value of 1.43 is the dilution factor, and $A$ is the absorbance at 412 nm. The difference in absorbance between the complete reaction and that of all components except NADP (HMG CoA deacylase control) represents the activity due to HMG CoA reductase. Controls containing only microsomes and buffer, and containing all components except HMG CoA, are used to monitor HMG CoA deacylase.

**Assay for HMG CoA reductase by the dual-label tracer method**

Microsomes, prepared as previously described, were suspended in 2.0 ml of 0.1 M triethanolamine buffer, pH 7.4, containing 0.02 M EDTA and 2 x 10^{-3} M dithiothreitol. This preparation was diluted to give a concentration of 5.0-8.0 mg of protein/ml, and 0.1 ml of this was used for the assay. The incubation mixture contained 0.5-0.8 mg of microsomal protein, 150 nmoles of [3-14C]HMG CoA, 3 μmoles of glucose-6-phosphate, 2 units of glucose-6-phosphate dehydrogenase, and 2 μmoles of NADP. The reaction was assayed according to the double-label procedure of Goldfarb and Pitot (6), using the thin-layer method reported by Shapiro, Imblum, and Rodwell (13).

**Preparation of the 14C-labeled substrate**

[3-14C]HMG CoA (R.S racemic mixture) was prepared from [3-14C]HMG anhydride (6) and coenzyme A (reduced) according to the procedure of Louw, Bekersky, and Mosbach (14) and purified by gel filtration chromatography on Sephadex G-10 (15). The lyophilized preparation was assayed after separation by paper chromatography using Whatman no. 3MM paper in a solvent system containing n-butanol-acetic acid-water 5:2:3 (v/v) according to Louw et al. (14). Two radioactive spots were identified. The compound identified as HMG CoA had an $R_F$ of 0.35 and contained 88% of the total radioactivity. The other spot was identified as 3-hydroxy-3-methylglutaric acid.

**RESULTS**

Although DTNB has been used in the determination of the sulphhydryl content of coenzyme A, it was necessary to determine if coenzyme A could be quantitatively assayed under the experimental conditions used. Data obtained when coenzyme A sulphydryl content was measured alone or with dithiothreitol according to the method of Zahler and Cleland (11) were identical. A known amount of coenzyme A was added, assayed after separation by paper chromatography using Whatman no. 3MM paper in a solvent system containing n-butanol-acetic acid-water 5:2:3 (v/v) according to Louw et al. (14). Two radioactive spots were identified. The compound identified as HMG CoA had an $R_F$ of 0.35 and contained 88% of the total radioactivity. The other spot was identified as 3-hydroxy-3-methylglutaric acid.

**Fig. 1.** Quantitative recovery of reduced coenzyme A previously added to the reaction mixture at different concentrations. The coenzyme A was measured by the assay procedure described with 5,5'-dithiobis(2-nitrobenzoic acid). The incubation mixture contained 4 μmoles of NADPH, 100 μmoles of dithiothreitol, 1 mg of microsomal protein, and coenzyme A. Control tubes were identical except for the absence of coenzyme A. The data were corrected for control tube values. The coenzyme A was 91.8% pure by the phosphotransacetylase assay from P-L Biochemicals.

**Fig. 2.** Stability of reduced coenzyme A upon exposure to air after treatment with 2 M citrate buffer at pH 4.0 containing 3% sodium tungstate. The reaction mixture and assay were those described in the legend to Fig. 1 and in the text (Assay procedure).
reagent blank. However, there was a time-dependent increase in DTNB-reacting material when HMG CoA was incubated with microsomes in the absence of the NADPH-generating system, as seen in Fig. 3. A possible source of sulfhydryl groups could be the hydrolysis of HMG CoA by HMG CoA deacylase. Microsomes isolated from livers of pigeons fed different diets elicited different amounts of deacylase activity. A control containing all components except NADPH is essential to allow correction for the activity of HMG CoA deacylase. This control was included in each HMG CoA reductase assay. The presence of NADPH in a microsomal preparation without HMG CoA gave an unexpected increase in DTNB-reacting material. This material is thought to be a soluble protein that was not precipitated in the pellet. The increase in DTNB-reacting material could be eliminated by making the incubation medium $2 \times 10^{-4}$ M with sodium arsenite just before the addition of the citrate-tungstate solution to terminate the reaction. Data are presented in Fig. 4. If the interfering protein is not removed, estimations of HMG CoA reductase activity will be high. The results shown in Fig. 5 indicate a linear increase in coenzyme A concentration with increasing amount of microsomal protein. Protein concentrations above 1.2 mg/ml no longer gave a linear increase in coenzyme A.

Kinetic properties of pigeon liver HMG CoA reductase

The time course of activity of HMG CoA reductase is shown in Fig. 6. The reaction was considered to be linear up to 30 min; increases beyond this no longer gave proportional increases in enzyme activity. The kinetic properties of the enzyme were studied to establish saturating concentrations of the substrates for the assay procedure. Fig. 7 (A) indicates the typical Michaelis-Menten kinetics.
for HMG CoA with a constant, saturating concentration of NADPH. The concentration of HMG CoA (R,S racemic mixture) in excess of 150 nmoles/ml of reaction mixture was found to be saturating. A Lineweaver-Burk plot of the data (Fig. 7, B) indicates that under these conditions the $K_m$ was $1.75 \times 10^{-5}$ M (for S form of HMG CoA) and the $V_{max}$ was 3.0 nmoles/min/mg of microsomal protein for the pigeon liver enzyme.

The effect of concentration of NADPH on the velocity of HMG CoA reductase at 150 $\mu$M concentration of HMG CoA is presented in Fig. 8A. The curve shows strong dependence on concentration of the reduced coenzyme. The estimated $K_m$ for NADPH was $6.81 \times 10^{-4}$ M with a $V_{max}$ of 0.59 $\mu$mole/min/mg of protein. A concentration of NADP of 2.4 mM was thus selected for use in the assay procedure from the double reciprocal plot in Fig. 8B.

### DISCUSSION

Several problems have been encountered with the methodology for estimating the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase in hepatic microsomes. The rate of the reaction is so slow that measurement of NADPH oxidation is impractical. Suitable methods using tracer techniques involve the incorporation of 3-hydroxy-3-methyl[3-14C]glutaryl CoA into [3-14C]mevalonate and require synthesis of the radioactive substrate. After the assay reaction is performed, the labeled mevalonate is converted to its lactone, extracted, and separated by either thin-layer chromatography or gas-liquid chromatography, and the product is measured. Although this procedure is reliable and precise, it requires a considerable amount of time and expense.

The present procedure depends upon maintaining reduced sulfhydryl groups for both 3-hydroxy-3-methylglutaryl CoA reductase and coenzyme A during the incubation and determination of the reduced coenzyme A formed in the reaction. Thus, the CoA-SH formed must be kept reduced up to the time of measurement with Ellman’s reagent, 5,5'-dithiobis(2-nitrobenzoic acid). Both of these

---

**Fig. 7.** Effect of concentration of 3-hydroxy-3-methylglutaryl CoA on the velocity of HMG CoA reductase from pigeon liver microsomes. The standard reaction mixture and assay conditions were as described in Materials and Methods, with saturating concentration of NADPH. Plot A shows the effect of different concentrations of HMG CoA upon the velocity of the reaction, and plot B is the double reciprocal plot of velocity and HMG CoA concentration.

**Fig. 8.** Effect of concentration of NADPH on the velocity of 3-hydroxy-3-methylglutaryl CoA reductase reaction in liver microsomes. The standard reaction mixture and assay conditions were as described in Materials and Methods. Plot A shows the effect of increasing the NADPH concentration upon the velocity of the reaction, and plot B is the double reciprocal plot of velocity and NADPH concentration. The reaction mixtures contained saturating concentrations of HMG CoA.
Quantitative way of doing this while maintaining reduced arsenite is specific for dithiol and does not complex with problems are overcome by the use of Cleland's reagent (dithiothreitol). The measurement of monothiol or dithiol, containing 3% sodium tungstate was the simplest, most independent of each other was accomplished by Zahler and Cleland (11) by complexing the dithiol with arsenite. Ellman's reagent, will react, producing high blank values.

Microsomal protein, if not removed before addition of CoA and dithiothreitol and without hydrolysis of HMG in Materials and Methods. The spectrophotometric assay for measuring coenzyme A was described in Materials and Methods. The incubation mixtures contained 150 nmoles of HMG CoA, 2 nmoles of NADP, 2 units of glucose-6-phosphate dehydrogenase, 3 nmoles of glucose-6-phosphate, and 1.0 mg of microsomal protein. The 14C method was performed according to Goldfarb and Pitot (6). The incubation mixture was identical with that used for the spectrophotometric method except that the substrate was 3-hydroxy-3-methyl-[3-14C]glutaryl CoA (150 nmoles) with a specific activity of 865 dpm/nmole and a purity of 88%.

Problems are overcome by the use of Cleland's reagent (dithiothreitol). The measurement of monothiol or dithiol independent of each other was accomplished by Zahler and Cleland (11) by complexing the dithiol with arsenite. Arsenite is specific for dithiol and does not complex with monothiol.

Microsomal protein, if not removed before addition of Ellman's reagent, will react, producing high blank values. It was found that removal with citrate buffer, pH 3.5, containing 3% sodium tungstate was the simplest, most quantitative way of doing this while maintaining reduced CoA and dithiothreitol and without hydrolysis of HMG CoA. This treatment showed no detrimental effect on the system. It should be noted that precipitation of protein with trichloroacetic acid produces an interaction with HMG CoA and NADPH, resulting in a product that reacts with Ellman's reagent. HMG CoA alone, when treated in the same manner, does not give this product.

The concentration of dithiothreitol in the reaction mixture must be maintained within reasonable limits. Too little will not maintain reduced enzyme and CoA-SH and too much will react more rapidly with Ellman's reagent and produce an absorbance curve that cannot be accurately extrapolated to determine the monothiol concentration. The concentration of DTT recommended for the conditions specified in the reaction mixture is 0.2 mM. This concentration was used in all experiments reported in Results and applies to enzymatic activities from 0.015 to at least 2.0 nmoles/min/mg of protein. This range of activities includes fluctuations in levels of activity due to diurnal variation and inhibition or stimulation by drugs. An excessive amount of microsomal protein (3–5 mg) in the reaction mixture will remove dithiothreitol, giving undesirable results; i.e., not enough DTT remains in the solution to protect the CoA-SH or some of the CoA-SH may be precipitated and removed with the protein.

Although we have reported here the use of commercial NADPH, the NADPH-generating system via glucose-6-phosphate dehydrogenase is considered to be preferable. This system operates as well when using an NADP-glucose-6-phosphate dehydrogenase-generating system for NADPH as with added NADPH.

The major advantages of the proposed HMG CoA reductase assay are that no synthesis or separation procedures are required, and the assays can be performed immediately and directly after incubation of the reaction mixture and results obtained rapidly.

Replicate analyses by this method are in excellent agreement. When control tubes containing the complete system are assayed with boiled microsomes or with dele-

<table>
<thead>
<tr>
<th>TABLE 1. Comparison of spectrophotometric and 14C methods for assaying activity of HMG CoA reductase in pigeon liver microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet</td>
</tr>
<tr>
<td>-----------------------------</td>
</tr>
<tr>
<td>Pigeon pellets + 0.5% cholesterol</td>
</tr>
<tr>
<td>Pigeon pellets</td>
</tr>
<tr>
<td>Pigeon pellets + 5% corn oil</td>
</tr>
</tbody>
</table>

^a Mean percentage difference, 8%; mean difference, 0.006 ± 0.004 nmoles/min/mg microsomal protein; not significantly different, P = 0.2; 95% confidence limits of the mean differences, -0.003 < 0.006 < 0.015.

^b Mean percentage difference, 1.4%; mean difference, 0.004 ± 0.002 nmoles/min/mg microsomal protein; not significantly different, P = 0.2; 95% confidence interval of the mean differences, -0.002 < 0.006.

The spectrophotometric assay was performed as described in Materials and Methods. The incubation mixtures were the same as given in Table 1. The 14C method was performed as described by Goldfarb and Pitot (6). The reaction mixture contained [3-14C]HMG CoA (150 nmoles with a specific activity of 865 dpm/nmole) with a purity of 88%. The yeast microsomal preparation was supplied by Dr. C. Madhosingh and These preparations were highly active in HMG CoA reductase.

^a Means ± SEM.

^b Not significantly different, P = 0.2.

The concentration of DTT recommended for the conditions specified in the reaction mixture is 0.2 mM. This concentration was used in all experiments reported in Results and applies to enzymatic activities from 0.015 to at least 2.0 nmoles/min/mg of protein. This range of activities includes fluctuations in levels of activity due to diurnal variation and inhibition or stimulation by drugs. An excessive amount of microsomal protein (3–5 mg) in the reaction mixture will remove dithiothreitol, giving undesirable results; i.e., not enough DTT remains in the solution to protect the CoA-SH or some of the CoA-SH may be precipitated and removed with the protein.

Although we have reported here the use of commercial NADPH, the NADPH-generating system via glucose-6-phosphate dehydrogenase is considered to be preferable. This system operates as well when using an NADP-glucose-6-phosphate dehydrogenase-generating system for NADPH as with added NADPH.

The major advantages of the proposed HMG CoA reductase assay are that no synthesis or separation procedures are required, and the assays can be performed immediately and directly after incubation of the reaction mixture and results obtained rapidly.

Replicate analyses by this method are in excellent agreement. When control tubes containing the complete system are assayed with boiled microsomes or with dele-

<table>
<thead>
<tr>
<th>TABLE 2. Comparison of 14C and spectrophotometric methods for assay of HMG CoA reductase of brewer's yeast microsomal preparations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomal Protein</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>mg</td>
</tr>
<tr>
<td>0.056</td>
</tr>
<tr>
<td>0.56</td>
</tr>
<tr>
<td>0.88</td>
</tr>
</tbody>
</table>

^a Means ± SEM.

^b Not significantly different, P = 0.2.

630 Journal of Lipid Research Volume 14, 1973
tion of NADPH, the absorbances are identical. Control analyses in which HMG CoA was deleted gave slightly less absorbance than those without NADPH. A trace amount of monothiol is present in the HMG CoA preparations. Any hydrolysis of HMG CoA by 3-hydroxy-3-methylglutaryl CoA lyase would not affect the assay system but only the effective concentration of substrate at any time. Under the conditions we use to prepare our microsomal fractions, no appreciable HMG CoA lyase activity is present because it is a mitochondrial enzyme. Any contribution to the coenzyme A by action of HMG CoA deacylase is corrected for by the control tube without NADPH.

The apparent $K_m$ value for pigeon liver microsomal HMG CoA reductase of $1.75 \times 10^{-5}$ M determined by this procedure is comparable with the values for the rat liver enzyme of $0.625 \times 10^{-5}$ M reported by Shefer et al. (8) and $0.6 \times 10^{-5}$ M reported by Kawachi and Rudney (16) for the $S$ enantiomer. However, the apparent $K_m$ for NADPH was high ($6.81 \times 10^{-4}$ M) for the microsomal enzyme, since Kawachi and Rudney reported a value of $8.7 \times 10^{-5}$ M for the solubilized enzyme. We attribute this high $K_m$ value for NADPH with the microsomal enzyme to the bound form of the enzyme or to species difference.

The present spectrophotometric assay for HMG CoA reductase was compared with the radioactive tracer method in several experiments. For the liver microsomal experiments, the percentage differences for data obtained by the two methods were 8% and 1.4% for low and high levels of activity, respectively. Nearly all of the data from both methods were within 95% confidence limits at three activity levels of the enzyme. Further comparisons using highly active brewer’s yeast microsomal HMG CoA reductase yielded data in which the percentage of difference from the means was 2.5–7.7% when five replicates at three levels of activity were measured. Student’s $t$ test was applied to these data and the probability that the means were not different was greater than 0.2.

Although there are inherent problems in both methods due to this complex enzymatic reaction, the spectrophotometric method in our hands provides a rapid means of estimating the activity of HMG CoA reductase and correlates statistically with the tracer method. The spectrophotometric method, however, is not as sensitive as the tracer method and requires approximately 0.5 nmole of CoA-SH to obtain meaningful spectral differences between reactions and controls.

We wish to express our gratitude to Drs. Thomas B. Clarkson, Jr., and Noel Lehner for supplying the pigeons used in this work. We wish to thank Mr. Tracy Watson for his careful and diligent technical assistance.

This work was supported by the U.S. Public Health Service, National Heart and Lung Institute, grant HL-14164-01-SCOR.

References