Improved methods for the solubilization and assay of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase

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Abstract A method for solubilizing HMG-CoA reductase is described that reproducibly yielded approximately 190% of the activity assayed in rat liver microsomes. Optimal solubilization occurred when microsomal membranes were frozen at a fixed concentration, thawed, homogenized in a buffer containing 50% glycerol, and incubated at 37°C for 60 minutes. A rapid spectrophotometric assay of the reductase has been developed and the optimal conditions defined. Using this assay, the kinetics were determined for HMG-CoA reductase purified to a specific activity of 17,400 nmol NADPH oxidized per minute per mg protein.

Supplementary key words HMG-CoA reductase kinetics.

The rate-limiting enzyme for cholesterol biosynthesis from acetate is considered to be 3-hydroxy-3-methylglutaryl coenzyme A reductase (1, 2). A number of investigators have reported purification of the rat liver microsomal enzyme although the specific activities are reported to vary between 3.0 and 10,000 nmol mevalonate formed per min per mg protein (3–6). The reason for these large variations in enzyme specific activity has yet to be established.

Solubilization of the reductase has been performed by slowly freezing and thawing the microsomes (4–7) or by exposing the membranes to buffers containing deoxycholate (3), 1.0 M KCl (8), glycerol and 1.0 M KCl (8), or phospholipase A (8). However, none of these methods reproducibly resulted in solubilization of all the membrane-bound enzyme. The slow freeze–thaw technique of Heller and Gould (7) has been used by most investigators and has resulted in solubilization of 30–114% of the enzyme activity originally found in the microsomal fraction. We report here a rapid method of solubilizing approximately 190% of the reductase activity originally assayed in rat liver microsomes.

The radioassay for HMG-CoA reductase that is currently used by most investigators is tedious; it involves the enzymic conversion of [3-^{14}C]HMG-CoA to [3-^{14}C]mevalonolactone and subsequent isolation of [3-^{14}C]mevalonolactone by thin-layer chromatography (9) or ion exchange chromatography (10).

We have developed a spectrophotometric assay that can be used to assay the activity of soluble HMG-CoA reductase and report here the optimal conditions and kinetic data for the purified enzyme. During the course of this work, Kleinsek, Ranganathan, and Porter (6) reported the use of a spectrophotometric assay to measure reductase activity with conditions significantly different from those reported here.

MATERIALS AND METHODS

Chemicals

Chemicals were obtained from the sources indicated: AG1-X8 formate from Bio-Rad; glycerol from Mallinckrodt; Coomassie brilliant blue G-250 from Serva; Triton X-100 from Baker; lithium diiodosalicylate from Eastman was a gift from Dr. C. F. Fox; deoxycholate and NADPH from Sigma. The deoxycholate was recrystallized three times from ethanol. Questran (cholestyramine) was from Mead Johnson. The sources of all other chemicals have been previously reported (11).

Animals

Male Sprague-Dawley rats (200–300 g body weight) were housed one per cage in a room in which the lights were off from 4:00 AM to 4:00 PM. Food and water were available ad libitum. For 3 days before

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; DTT, dithiothreitol; DOC, deoxycholate; LIS, lithium diiododsalicylate.

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killing, the rats were fed powdered rat chow containing 5% cholestyramine. Animals were killed at 10:00 AM, at the peak of the HMG-CoA reductase cycle (11).

Each rat liver was homogenized at 4°C in 25 ml of buffer A (0.1 M sucrose, 0.05 M KCl, 0.04 M potassium phosphate and 0.03 M potassium EDTA; pH 7.2) with a motor-driven, tight-fitting, glass–Teflon Potter-Elvejhem homogenizer and the microsomes were prepared as previously described (12). Unless otherwise stated, the microsomes were resuspended in buffer A to a concentration of approximately 82 mg protein/ml, solid DTT was added to a final concentration of 10 mM and the microsomes were homogenized with a hand-driven, all-glass Potter-Elvehjem homogenizer (Kontes; clearance 0.004″ to 0.006″). Three-ml aliquots of the microsome suspension were frozen in glass tubes at a rate of 6–8°C per min as described by Heller and Gould (7) unless otherwise stated, and stored at −20°C for up to 2 months.

For optimal solubilization of the reductase the frozen microsomes were allowed to thaw either at room temperature or at 37°C before addition of an equal volume of 50% glycerol in buffer B (buffer A plus 10 mM DTT) preheated to 37°C. The suspension was rehomogenized with 10 downward passes of a hand-driven, all-glass Potter-Elvehjem homogenizer and then incubated at 37°C for 60 min. The suspension was diluted threefold with buffer B preheated to 37°C to a final glycerol concentration of 8.3%, rehomogenized with 10 downward passes of the glass homogenizer pestle, and centrifuged at 100,000 *g* for 60 min at 25°C. The supernatant containing solubilized HMG-CoA reductase was removed and used immediately for enzyme purification. Various modifications of the above procedure are given in the text.

Assay of HMG-CoA reductase

Microsomal reductase. The microsomal enzyme was assayed in 0.5 ml of buffer B containing 3.5 μmol of NaCl, 5 μmol of D-glucose-6-phosphate, 1 μmol of NADP, 2.2 units of glucose-6-phosphate dehydrogenase, 150 nmol of *RS*-[3-^{14}C]HMG-CoA (0.34 Ci/mol), and 0.4–1.0 mg of microsomal protein. The pH of the incubation mixture was 7.2. The [3-^{14}C]-mevalonate synthesized during the assay was isolated by a minor modification of the method of Avigan, Bhalthena, and Schreiner (10). Briefly, the reaction was stopped with 50 μl of 33% KOH and [5-^{3}H]-mevalonate (189,000 dpm; 2.5 μg) was added as the internal standard. After 30 min, 25 μl of bromophenol blue (0.05%) was added followed by 5 N HCl (105–110 μl) until the indicator changed to yellow. After 30 min at the acid pH, the precipitated protein was removed by centrifugation and the supernatant was applied to an AG1-X8 formate column (0.7 × 10 cm) made up in water. The sample was eluted with water, the first 1.8 ml was discarded, and the mevalonolactone was collected in the next 5-ml fraction. Four ml of this fraction was mixed with 15 ml of Aquasol and the ^{14}C and ^{3}H content were determined in a Beckman LS100 scintillation counter. Recovery of tritium in the 5-ml fraction ranged from 75 to 95%. However, the variation of recovery within each experiment was generally within 1 or 2%. Assays performed in the absence of microsomal protein gave background values which were subtracted from experimental values. The duplicate backgrounds for each experiment were in close agreement and gave values approximating those obtained previously by thin-layer chromatography. The ^{14}C radioactivity in the blanks (150 dpm) was significantly lower than in experimental assays (2000–9000 dpm).

Low background values were only obtained when the above procedure was used. When assays were stopped with acid as described by Avigan et al. (10) and the thioester was not hydrolyzed before column chromatography, the background values were more than twofold higher.[3]

Solubilized reductase. The activity of the solubilized HMG-CoA reductase was determined at 37°C in a total volume of 0.5 ml using a Beckman spectrophotometer (Model 25). The cell path length was 1.0 cm. The rate of oxidation of NADPH was initially determined in the absence of HMG-CoA and this blank value, if any, was subtracted from the rate obtained with both substrates.

Maximum activity was obtained by assaying the enzyme in buffer C (0.2 M KCl, 0.16 M potassium phosphate, 0.004 M EDTA, and 0.01 M DTT, pH 6.8) together with 0.2 mM NADPH and 0.1 mM *RS*-HMG-CoA. One unit of enzyme activity is defined as the amount required to oxidize 2 nmol NADPH per minute. Hence, one unit is equivalent to the synthesis of 1 nmol mevalonate per minute.

Protein determinations

The protein content of microsomal suspensions and solubilized enzyme was determined by the biuret method (13) and the method of Lowry et al. (14), respectively. Suspensions containing DTT were first treated with 10% trichloroacetic acid as described by Heller and Gould (7).

Soluble proteins were also measured using Coomassie brilliant blue G-250 by a minor modification

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TABLE 1. Effect of various agents on solubilization of HMG-CoA reductase

Method of Freezing Microsomes	Homogenizing Buffer	Additions to Homogenizing Buffer	Relative Amount of Solubilized HMG-CoA Reductase
			%
I	B		40
I	B	NaF (0.25 M)	76
I	B	NaF (0.5 M)	62
I	B	KBr (0.5 M)	62
I	B	NaI (0.5 M)	56
I	B	LIS (0.005 M)	0
I	B	DOC (0.25%)[a]	36
I	B	Triton X-100 (5%)	0
I	B	Glycerol (50%)	100
I	B (0.25×)	Glycerol (50%)	40
I	B (0.125×)	Glycerol (50%)	19
I	C	Glycerol (50%)	95
II	B	Glycerol (50%)	94

A concentrated suspension of hepatic microsomes was prepared from cholestyramine-fed rats as described in Materials and Methods. Equal aliquots (0.6 ml) were placed in conical centrifuge tubes and frozen either slowly at 6–8°C per min (Method I) or rapidly by immersing the tube in liquid nitrogen (Method II). After overnight storage at −20°C, the microsomes were allowed to thaw at room temperature and were homogenized with 0.6 ml of the glycerol-containing buffers or 3.0 ml of buffer containing the indicated chaotropic agents or detergents. After 10 min at room temperature, 2.4 ml of buffer B were added to the tubes containing glycerol. All samples were then homogenized and centrifuged at 100,000 *g* for 60 min at 25°C. Reductase activities in the supernatants are given as a percentage of the method using 50% glycerol in buffer B. Concentrations of the chaotropic agents and detergents are those in the 3.6 ml of the microsomal suspension. The composition of buffers B and C are given under Materials and Methods. Where indicated, buffer B was used after a 4-fold (0.25×) or 8-fold (0.125×) dilution with water. LIS, lithium diiodosalicylate.

[a] After microsomes had been exposed to DOC for 10 min, cholestyramine (22.5 mg Questran/ml) was added to the suspension and the suspension was incubated for another 10 min before centrifugation. Cholestyramine binds the DOC and removes the detergents from solution (18).

of the method of Bradford (15). Briefly, 0.5-ml samples containing either 1–10 μg or 10–100 μg of protein were mixed on a vortex mixer with 1.0 ml or 5.0 ml, respectively, of the Coomassie brilliant blue solution (15) and the absorption at 595 nm was determined after 10 min (15). Bovine serum albumin was used as a standard in all protein assays.

Analyses of the protein contents of a number of partially and completely purified reductase preparations by the method of Lowry et al. and the method of Bradford (15) gave similar protein concentrations.

RESULTS

Solubilization of HMG-CoA reductase

The amount of active HMG-CoA reductase solubilized from frozen–thawed microsomes was dependent on the composition of the buffer in which the thawed microsomes were homogenized (**Table 1**). In agreement with the results of Heller and Gould (7), buffer B solubilized approximately 30% of the microsomal enzyme. Addition to buffer B of a number of chaotropic agents, agents that promote solubilization of membrane proteins (16), increased the solubilization of the enzyme (Table 1). However, lithium diiodosalicylic acid, a potent solubilizer of membrane proteins (17), inactivated the reductase in both microsomal membranes (Table 1) and in solubilized enzyme preparations.[3] The detergent Triton X-100 at a concentration of 5% also inactivated the reductase (Table 1).

At room temperature optimal solubilization of the reductase was obtained after 1 volume of 50% glycerol in buffer B was added to each volume of thawed microsomes and the microsomes were resuspended with 10 downward passes of a glass homogenizer. After 10 min, 4 volumes of buffer B were added and the mixture was homogenized again with 10 downward passes (Table 1).

However, maximal solubilization of the reductase was obtained when microsomes were homogenized with an equal volume of the glycerol buffer and incubated at 37°C for 60 min before addition of the 4 volumes of buffer B (**Fig. 1**). Under these conditions, approximately 194 ± 39% (mean ± SD; $n = 8$) of the reductase originally assayed in the microsomes was solubilized during the 60-min incubation. The amount of enzyme solubilized was not affected by increasing the pH of buffer B to pH 8.0.

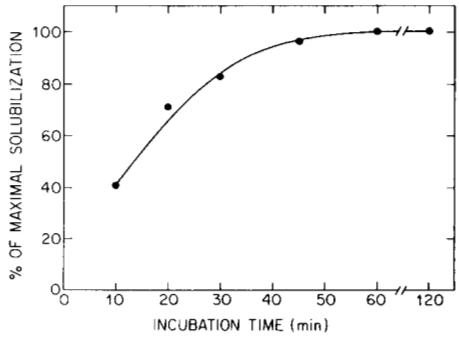

Fig. 1. Effect of the time of incubation of rat liver microsomal membranes on the solubilization of HMG-CoA reductase. Microsomes were prepared, frozen at a rate of 6–8°C per min, and stored overnight at −20°C as described in Materials and Methods. The microsomes were thawed and homogenized with 50% glycerol in buffer B as described under Materials and Methods and the suspension was incubated at 37°C. At the times indicated, 1.0-ml aliquots were removed, homogenized with 2.0 ml of buffer B, and centrifuged at 100,000 *g* for 60 min. The 100,000 *g* supernatant was incubated for a further 45 min at 37°C before determination of the reductase activity. Reductase activities are given as a percentage of the maximum obtained at 60 min.

The solubilized enzyme obtained in the 100,000 *g* supernatant after incubation of the microsomes at 37°C for 0–120 min (Fig. 1) was re-incubated at 37°C for up to 60 min before assaying for reductase activity. Hence, we conclude that the increase in solubilized reductase activity observed in Fig. 1 was a result of increased solubilization of the enzyme and not simply the result of activation of reductase solubilized before the incubation at 37°C.

The amount of enzyme solubilized by the technique described above was the same for microsomes initially frozen at rates of 4–6°C, 6–8°C, 8–10°C, or 10–12°C per minute or after quick freezing in liquid nitrogen (Table 1). However, the concentration of microsomes at the time of freezing did affect the amount of reductase solubilized by the glycerol buffer; maximum solubilization was obtained when the protein concentration of the frozen microsomes was approximately 82 mg protein/ml (**Table 2**). More concentrated microsomal suspensions were not examined since it was difficult to homogenize such suspensions by hand.

Spectrophotometric assay for solubilized HMG-CoA Reductase

HMG-CoA reductase partially purified by affinity chromatography[3] to a specific activity of 2200 units per mg protein was used to determine optimal assay conditions. Most investigators have determined the activities of microsomal HMG-CoA reductase in buffer B (4, 9, 12). However, solubilized reductase activities were enhanced significantly if either 0.2 M KCl or 0.16 M phosphate was added to buffer B or if buffer B was used at four times the normal concentration (**Table 3**). Enzyme activities decreased significantly if 10% sucrose or 1.0 M KCl was added to buffer B (Table 3). Maximal activities were observed in assays performed in buffer C (Table 3) at the pH optimum, pH 6.8 (**Fig. 2**), and under these conditions the activity of HMG-CoA reductase was increased 220% above those obtained in buffer B alone. A similar increase has been shown to occur when enzyme prepared from rats fed a diet without cholestyramine was assayed in buffer C compared to buffer B. The spectrophotometric assay has been used to follow the solubilized reductase activity during all stages of enzyme purification (**Table 4**).

Using purified HMG-CoA reductase the stoichio-

TABLE 2. Effect of microsomal concentration on solubilization of the reductase

Concentration of Microsomal Suspension	Relative Amount of Reductase Solubilized
mg protein/ml	%
82	100
59	83
48	77

Hepatic microsomes were prepared from 12 rats as described in Materials and Methods. Microsomes were resuspended in buffer B to the concentration indicated, frozen at a rate of 6–8°C per minute, and stored at −20°C overnight. The microsomes were thawed at room temperature, homogenized with an equal volume of 50% glycerol in buffer B, and the reductase was solubilized by the standard method given in Materials and Methods.

TABLE 3. Optimal conditions for the assay of soluble HMG-CoA reductase

Buffer	Relative Reductase Activity
	%
B	100
B (4×)	164
B + 10% sucrose	79
B + 16.8% sucrose	76
B + 0.2 M KCl	163
B + 0.4 M KCl	142
B + 0.6 M KCl	103
B + 0.9 M KCl	63
B + 2.0 M KCl	18
B + 0.06 M phosphate	137
B + 0.16 M phosphate	140
B + 0.24 M phosphate	134
C	222
C (0.5×)	218
C (2×)	220

Enzymic activities were determined spectrophotometrically as described under Materials and Methods except that the 0.5-ml assay contained the indicated buffer. In each case 5 μl of enzyme (sp act 2200 units per mg protein) was preincubated for 10 min at 37°C before the assay was started by addition of *RS*-HMG-CoA. All buffers were pH 6.8 and the compositions of buffers B and C are given in Materials and Methods.

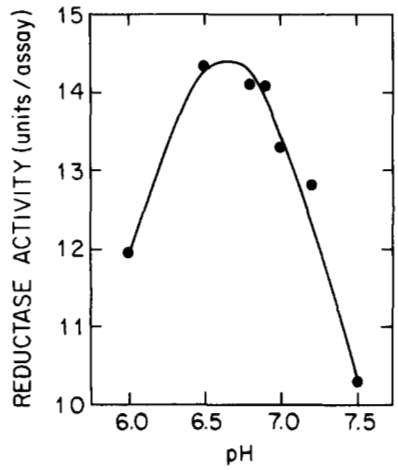

Fig. 2. Optimal pH for the assay of solubilized HMG-CoA reductase. HMG-CoA reductase was solubilized as described in Materials and Methods and partially purified to a specific activity of 3000 units/mg protein. The standard spectrophotometric assay was used to determine the enzyme activity of a 10-μl sample except that the pH of the phosphate buffer was adjusted to the values shown.

TABLE 4. Spectrophotometric assay of HMG-CoA reductase

Fraction Assayed	Rate of Oxidation of NADPH −HMG-CoA	Rate of Oxidation of NADPH +HMG-CoA	Reductase Specific Activity
	nmol/min		*nmol NADPH oxidized/min/mg protein*
Solubilized enzyme	0.47	6.98	76.6
Purified enzyme	0.0	11.07	17,405

HMG-CoA reductase was solubilized from the microsomal membranes as described in Materials and Methods and 50 μl of the 100,000 *g* supernatant was assayed spectrophotometrically at 37°C for HMG-CoA reductase activity. Enzyme (5 μl) was also assayed after purification by affinity chromatography.[3] Each 0.5-ml assay contained buffer C, pH 6.8, 0.2 mM NADPH, and enzyme. The reference cell contained no enzyme. The reaction was started by addition of 0.1 mM *RS*-HMG-CoA to the experimental cell and the rate of NADPH oxidation was followed on a Beckman recorder (Model 24–25). The reductase activity was determined after subtracting the rate of oxidation of the nucleotide in the absence of HMG-CoA from the rate obtained with both substrates present.

metric ratio between nmol NADPH oxidized and nmol mevalonate formed from HMG-CoA was 1.89 (**Table 5**). This value approximates the theoretical value of 2.0 and provides further evidence for the validity of the spectrophotometric assay for determining the activity of purified enzyme.

Enzyme purification and kinetics

HMG-CoA reductase was solubilized by the standard method described in Materials and Methods and purified either by the method of Kleinsek et al. (6) to a final specific activity of 10,600 units per mg protein (range 10,200–11,200; $n = 3$) and a yield of 6% or by affinity chromatography to an average specific activity of 10,859 units per mg protein (range 7,200–19,500; $n = 8$) and an average yield of 20%.[3] The method of enzyme solubilization described in this report and subsequent purification by affinity chromatography have been applied successfully to HMG-CoA reductase obtained from rats fed a normal diet in the absence of cholestyramine.[3]

Enzyme kinetics performed with purified enzyme having a specific activity of 17,400 nmol NADPH oxidized per min per mg protein gave a K_m for NADPH of 51.0 μM and for *RS*-HMG-CoA of 5.2 μM (**Fig. 3**). The K_m for *S*-HMG-CoA, the active isomer, is 2.6 μM. The activity of HMG-CoA reductase was inhibited by concentrations of NADPH or *RS*-HMG-CoA above 200 μM and 150 μM, respectively (Fig. 3).

DISCUSSION

We have described an improved method for solubilizing HMG-CoA reductase from hepatic microsomes and defined the optimal conditions for a spectrophotometric assay of the solubilized enzyme.

The concentration of the microsomes at the time of freezing, the composition of the homogenizing buffer, and the incubation at 37°C of the microsomal suspension for 60 min were all critical factors in solubilizing maximal amounts of the reductase in the absence of detergents (Tables 1 and 2, Fig. 1).

We have routinely solubilized approximately twice the amount of HMG-CoA reductase activity that was originally assayed in the microsomal fraction. Whether this increase is due to activation of enzyme after its slow release from the membrane during the 60-min incubation at 37°C or to solubilization of enzyme that is not accessible to the [3-^{14}C]HMG-CoA substrate when the enzyme is bound to the membrane has yet to be determined.

Although 2.5-fold more enzyme was solubilized with buffer B containing 50% glycerol than buffer B alone (Table 2), the specific activity of the solubilized enzyme was the same both immediately after solubilization and after partial purification by affinity chromatography.[3]

Brown et al. (8) have previously reported that buffers containing glycerol and 1.0 M KCl solubilized as much as 68% of the reductase. However, such high levels of solubilization are only observed in this laboratory after incubating the microsomes with glycerol at 37°C.

The spectrophotometric assay for the reductase described here greatly simplifies the assay for the solubilized enzyme. During the course of this work, Kleinsek et al. (6) reported a spectrophotometric assay for the reductase but with conditions significantly different from the optimal conditions described in this paper.

The apparent decrease in reductase activity observed with enzyme assayed in buffer containing 2.0

TABLE 5. Stoichiometry between NADPH oxidized and mevalonate formed

Assay	nmol NADPH oxidized/min	nmol mevalonate formed/min	Ratio: NADPH oxidized / mevalonate formed
1	1.55	0.79	1.96
2	1.82	0.88	2.07
3	1.53	0.75	2.04
4	1.21	0.67	1.81
5	1.22	0.71	1.73
6	1.26	0.71	1.77
			Mean + SEM = 1.89 ± 0.06

Purified HMG-CoA reductase was assayed spectrophotometrically as described in the legend to Table 4, except that [3-^{14}C]HMG-CoA was used. Each assay was stopped after 4 min with 50 μl of 33% KOH and the ^{14}C content of the mevalonate was determined as described in Materials and Methods.

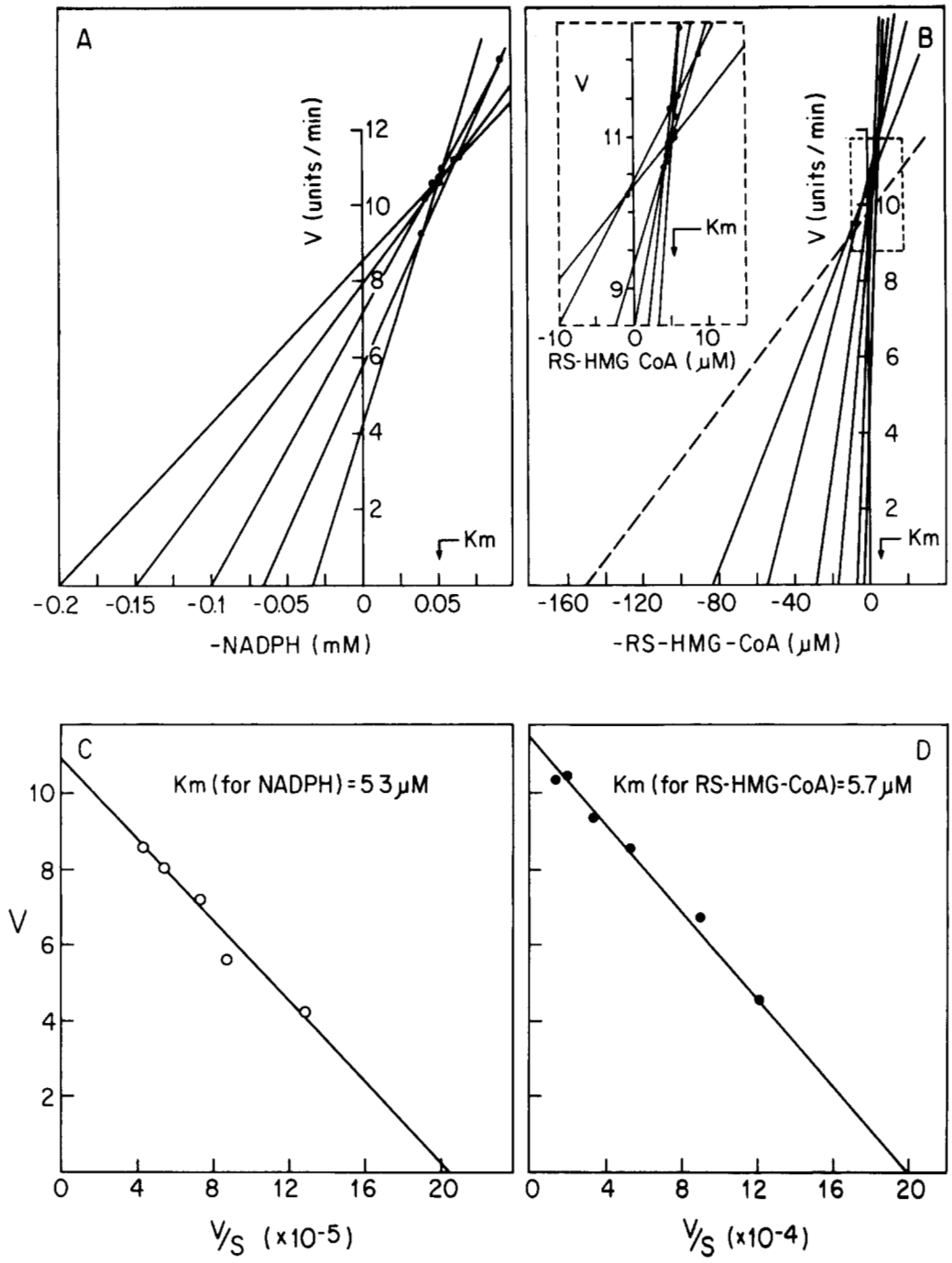

Fig. 3. Determination of K_m for HMG-CoA reductase. HMG-CoA reductase, purified to homogeneity and with a specific activity of 17,405 nmol NADPH oxidized/min per mg protein, was used to determine K_m values using the spectrophotometric assay described in Materials and Methods. In Fig. 3*A* the concentration of *RS*-HMG-CoA was 0.1 mM and in Fig. 3*B* that of NADPH was 0.2 mM. The data were plotted as described by Eisenthal and Cornish-Bowden (21) and Cornish-Bowden and Eisenthal (22). In Fig. 3*B* the area enclosed within the dotted rectangle (which includes all the intercepts) has been expanded and is shown in the inset. In Fig. 3*A* the velocity at 0.4 mM NADPH was 7.36 units/min (data not shown). This represented a 14% inhibition of the reaction rate compared to the value obtained at 0.2 mM NADPH. In Fig. 3*B*, 150 μM *RS*-HMG-CoA was inhibitory (– – –) and the data were not used to determine the K_m. K_m for NADPH was 51 μM and for *RS*-HMG-CoA was 5.2 μM. When the data were plotted according to Eadie–Hofstee (v against v/s), as in Fig. 3*C* and 3*D*, a straight line was obtained when inhibitory concentrations of substrate were omitted. The K_m for NADPH was 53 μM and for *RS*-HMG-CoA was 5.7 μM. In Figs. 3*C* and 3*D*, v is given as units/min and s is given in nM. All the HMG-CoA concentrations studied were above the K_m.

M KCl (Table 2) could be overcome by dilution of the buffer to the same ionic strength as buffer C.[3] It is possible that high salt concentrations dissociate the enzyme into inactive subunits.

HMG-CoA reductase purified from cholestyramine-fed rats and with a specific activity of 8700 units per mg protein had a K_m for NADPH of 51.0 μM and for *S*-HMG-CoA of 2.6 μM. These values are similar to those reported by Kawachi and Rudney (3) for enzyme purified from normally fed rats (87 μM for NADPH, 6 μM for *S*-HMG-CoA) but significantly less than the values of 320 μM for NADPH and 34.5 μM for *S*-HMG-CoA reported by Srikantaiah et al. (5). It is possible that enzyme purified from animals housed and fed under different conditions will have different kinetic properties. Langdon and Counsell (19) have demonstrated that a number of the reported values for the Michaelis–Menten constant for microsomal HMG-CoA reductase may have been in error as a result of nonlinear conversion of substrates to products. However, in the present report, the linearity of all spectrophotometric assays could be ascertained immediately. It is of interest that the lowest reported K_m values for *S*-HMG-CoA for the microsomal enzyme, 1.01–2.0 μM (12, 19, 20), are similar to that reported here (2.6 μM) for the purified enzyme.

We believe that the improved methods for solubilization and assay of HMG-CoA reductase described in this report will aid in the further investigation of this important regulatory enzyme.

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