Development of a noncompetitive, solid phase, bridged biotin-avidin enzyme immunoassay for measurement of human leukocyte microsomal HMG-CoA reductase protein concentration

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Abstract Methods were developed for determination of human mononuclear leukocyte HMG-CoA reductase protein concentration by a noncompetitive, solid phase, bridged biotin-avidin enzyme immunoassay procedure. Leukocyte microsomal HMG-CoA reductase, first immobilized onto a nitrocellulose filter, is sequentially reacted with *1*) monospecific, polyclonal rabbit anti-rat liver HMG-CoA reductase antiserum, which crossreacts with the human liver and leukocyte enzymes; *2*) biotinylated donkey anti-rabbit immunoglobulin; *3*) a streptavidin-horseradish peroxidase conjugate; and *4*) 4-chloro-1-naphthol and H_2O_2 to visualize the quantity of horseradish peroxidase bound to the immunocomplex. Color development was proportional to the quantity of either purified liver or leukocyte microsomal HMG-CoA reductase applied to the nitrocellulose. Color development was not observed, however, when HMG-CoA reductase was omitted from the nitrocellulose, when one of the reactant species was omitted from the incubation reactions, or when anti-rat liver HMG-CoA reductase antiserum was preabsorbed with either rat liver or human leukocyte HMG-CoA reductase. Immunoreactivity of microsomal HMG-CoA reductase was independent of the phosphorylation state of the enzyme, but was inversely related to the concentration of thiol-reducing agents present in the microsomal preparation up to 4 mM. Further increases in thiol-reductant failed to produce changes in immunoreactivity. Freshly isolated mononuclear leukocyte microsomal HMG-CoA reductase protein concentration in leukocytes from 31 healthy, normocholesterolemic subjects was a linear function of HMG-CoA reductase activity ($R = 0.65$; $P < 0.001$). The catalytic efficiency of the freshly isolated mononuclear leukocyte enzyme was 313 ± 34 pmol of mevalonate formed per min of incubation at 37°C per mg immunoreactive protein. This methodology, in conjunction with that recently developed to measure human leukocyte HMG-CoA reductase activity (1984. *J. Lipid Res.* **25:** 967–978), should prove useful in discriminating between HMG-CoA reductase regulatory mechanisms involving changes in enzyme protein concentration and those resulting from changes in enzyme catalytic efficiency. — **Harwood, H. J., Jr., I. M. Alvarez, Y. J. Greene, G. C. Ness, and P. W. Stacpoole.** Development of a noncompetitive, solid phase, bridged biotin-avidin enzyme immunoassay for measurement of human leukocyte microsomal HMG-CoA reductase protein concentration. *J. Lipid Res.* 1987. **28:** 292–304.

Supplementary key words enzyme activity • enzyme catalytic efficiency • metabolic regulation • phosphorylation • thiol-disulfide formation

HMG-CoA reductase (E.C. 1.1.1.34) is the rate-limiting enzyme in polyisoprenoid biosynthesis (1) and hence plays a critical role in controlling metabolic pathways requiring availability of isoprenoids or isoprenoid derivatives (2, 3). The activity of HMG-CoA reductase is subject to variation by two classes of mechanisms; *1*) those that alter the intracellular concentration of the enzyme molecule by changes in rates of enzyme synthesis or degradation and *2*) those that alter the catalytic efficiency of existing enzyme molecules. Processes that appear to influence primarily HMG-CoA reductase protein concentration most notably include feedback regulation by dietary and plasma cholesterol (4, 5), diurnal variation in enzyme activity (1, 6), responses to fasting and refeeding (7), and responses to cholesterol-lowering drugs (6, 8). Regulatory mechanisms that involve modulation of the catalytic activity of existing enzyme molecules include reversible phosphorylation (9–12), reversible thiol-disulfide formation (13–16), allosteric activation by NAD(P)H (15–19), and membrane fluidity changes (20, 21). In addition, under certain circumstances, both the intracellular concentration of HMG-CoA reductase as well as the catalytic efficiency of the enzyme may be altered (18, 22–25). Hence when studying the regulation of HMG-CoA reductase, it is important to consider not only changes in enzyme activity, but also changes in enzyme protein content and catalytic efficiency.

In the past, investigators have attributed differences in HMG-CoA reductase activity to changes in enzyme protein concentration by determining the activity of the fully dephosphorylated enzyme (7, 12, 26) or by examining differences in enzyme activity in the presence and absence

Abbreviations: HMG, 3-hydroxy-3-methylglutaryl; TBS, Tris-buffered saline.

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of cycloheximide (1, 27) which inhibits nascent protein synthesis. More recently, the preparation of monospecific, polyclonal anti-HMG-CoA reductase antiserum (22, 24, 28–30) has permitted investigators to determine the intracellular concentration of HMG-CoA reductase directly by immunotitration (22, 24, 28–30). Milligram quantities of microsomal protein are required, however, for quantitation of HMG-CoA reductase by immunotitration (28, 29).

We now report the development of a noncompetitive, solid phase enzyme immunoassay for measurement of microsomal HMG-CoA reductase protein concentration that permits determination of enzyme protein concentration using less than 2 μg of microsomal protein per analysis. We also report its applicability to measurement of HMG-CoA reductase protein concentration in both freshly isolated and cultured human leukocytes.

MATERIALS AND METHODS

Chemicals

Gelatin, Tween-20, 4-chloro-1-naphthol, electrophoresis grade Tris, Coomassie brilliant blue R-250, sodium dodecyl sulfate, glycine, acrylamide, bis-acrylamide, ammonium persulfate, N,N,N',N'-tetramethylenediamine, bromophenol blue, and Affi-Gel Blue were from Bio-Rad Laboratories (Richmond, CA). Hydrogen peroxide and β-mercaptoethanol were from Sigma Chemical Co. (St. Louis, MO). Biotinylated donkey anti-rabbit immunoglobulin (Cat. #RPN 1004) and a streptavidin-horseradish peroxidase conjugate (Cat. #RPN 1231) were from Amersham Corp. (Arlington Heights, IL). Cholestyramine (Questran) was from Mean Johnson and Co. (Evansville, IN). HMG-CoA agarose was from Pharmacia/P-L Biochemicals (Piscataway, NJ). BA-85 nitrocellulose sheets were from Schleicher and Schuell (Keene, NH). Prestained protein molecular weight standards were from Bethesda Research Laboratories (Gaithersburg, MD). All other chemicals were from previously listed sources (31–34).

Solutions

Hank's Balanced Salts Solution (10 ×) contained 80 g/l NaCl, 4 g/l KCl, 10 g/l glucose, 600 mg/l KH_2PO_4, 475 mg/l Na_2HPO_4, and 170 mg/l phenol red (pH 7.4). Tris-buffered saline (TBS) contained 2.4 g/l Tris (pH 7.5) and 29.1 g/l NaCl. TEDK buffer contained 50 mM Tris (pH 7.5) 1.0 mM EDTA, 5.0 mM dithiothreitol, and 70 mM KCl. TEDF buffer contained 50 mM Tris (pH 7.5), 1.0 mM EDTA, 5.0 mM dithiothreitol, and 70 mM NaF.

Isolation of peripheral blood mononuclear leukocytes

Forty-ml blood samples were obtained using tourniquet pressure from an antecubital vein with a 19-gauge needle and a 60-ml syringe. After removing the needle from the syringe, the blood was discharged into a 50-ml conical polystyrene centrifuge tube containing 1.0 ml of 250 mM EDTA (pH 7.4) and inverted several times. Care was taken to empty the syringe over 5 to 10 sec with the jet of blood coursing along the side of the tube. This was done to minimize hemolysis which may artifactually elevate leukocyte HMG-CoA reductase activity (35). Within 10 min of blood drawing, the sample was diluted with an equal volume of room temperature Hank's Balanced Salts Solution. Aliquots (20 ml) of the diluted blood sample were added to 50-ml plastic conical centrifuge tubes and underlayered with 20 ml of room temperature Histopaque 1077. Following centrifugation at 400 *g* for 40 min to sediment erythrocytes and granulocytes, the mononuclear leukocyte population ("buffy coat") was isolated, washed twice in Hank's Balanced Salts Solution, once in 0.87% NH_4Cl to lyse contaminating erythrocytes, and once in TEDK buffer. The resulting leukocyte pellet was resuspended in 0.5 ml of TEDK buffer and frozen in liquid N_2.

Growth and isolation of IM-9 lymphocytes

Lymphoid cells from the IM-9 cell line (36) were cultured in sterile RPMI-1640 medium containing 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 0.25 μg/ml fungizone, and 100 μg/ml streptomycin (36). Cell viability was $\geq$ 90%, as assessed by Trypan Blue dye exclusion. IM-9 cells from late-log phase cultures were washed twice in 50 ml of TEDK and frozen in liquid N_2 in a volume of 0.5 ml TEDK per 10^8 cells.

Isolation of leukocyte microsomes

Leukocyte microsomes were isolated as described by Harwood, Schneider, and Stacpoole (33). Frozen cell suspensions were incubated at room temperature until just thawed. Subsequent operations were at 0–5°C. Suspensions were homogenized 15 times with a ground-glass pestle in a 1-ml Potter-Elvehjem tissue homogenizer. Suspensions were transferred to a 5-ml Potter-Elvehjem tissue homogenizer and homogenized five times with a motor-driven Teflon pestle. The homogenate was diluted to 8 ml with TEDK and centrifuged at 2,000 *g* for 10 min. The pellet was discarded and the resulting supernatant liquid was centrifuged at 172,000 *g* for 90 min. Following centrifugation, the microsomal pellet was resuspended in 0.16 ml of TEDK per 10^8 cells.

Partially phosphorylated, and hence partially inactive, HMG-CoA reductase was obtained, as previously described (32), by isolating leukocytes in fluoride-containing buffer (TEDF) to prevent complete dephosphorylation during microsomal isolation (26). Alternatively, partially phosphorylated enzyme was prepared by incubating active microsomal HMG-CoA reductase (microsomes also contain a reductase kinase (32)) with 8 mM $MgCl_2$, 2 mM MgATP, 2 mM ADP, and 50 mM NaF for 30 min at 30°C, and terminating the inactivation reaction by addition of 125 mM EDTA (37).

Isolation and purification of rat and human liver HMG-CoA reductase

Liver microsomal HMG-CoA reductase, from rats fed 3% cholestyramine for 5 days, was solubilized from the microsomal membrane by limited proteolysis and purified through the heat fractionation, as described by Rogers, Panini, and Rudney (28). Liver microsomal HMG-CoA reductase from a brain-dead human organ donor was solubilized from the microsomal membrane by limited proteolysis and purified through the heat fractionation as described by Harwood, Greene, and Stacpoole (31). The resulting catalytically active, proteolytic fragment of rat or human liver HMG-CoA reductase, contained in the heat fraction, was further purified to apparent homogeneity by affinity chromatography first using Affi-Gel Blue and then HMG-CoA agarose. Heat fraction, containing between 4 mg and 12 mg of protein, was diluted 1:11 with TEDK to decrease the KCl concentration to below 0.2 M (34), and applied to a 4 × 0.8 cm column of Affi-Gel Blue equilibrated in TEDK. Following sample application, the column was washed first with 100 ml of TEDK and then with 25 ml of TEDK containing 6% (v/v) glycerol and 0.2 M KCl. HMG-CoA reductase protein was then eluted with 20 ml of TEDK containing 15% (v/v) glycerol and 0.5 M KCl. Five-ml fractions were collected. Fractions 1 and 2 contained the eluted protein and were retained for subsequent analysis.

Both rat liver and human liver HMG-CoA reductases, purified through the Affi-Gel Blue column step, migrated as a single Coomassie blue-staining and immunoreactive band when subjected to nondenaturing polyacrylamide gel electrophoresis (data not shown) and as a single Coomassie blue-staining and immunoreactive band of apparent M_r 52,000 when subjected to denaturing polyacrylamide gel electrophoresis (see Fig. 3). These proteins were determined, by scanning transmission densitometry of the Coomassie blue-stained denaturing polyacrylamide gels to be 88% and 94% homogenous, respectively. Subsequent chromatography of the Affi-Gel Blue fractions on HMG-CoA agarose (39) did not result in further purification of the enzyme, as judged by immunoreactivity per mg of protein (see Fig. 4).

Preparation of rabbit anti-rat liver HMG-CoA reductase antiserum

Antiserum specific for the proteolytic fragment of rat liver microsomal HMG-CoA reductase was prepared as previously described (40). Approximately 500 μg of proteolytically modified rat liver HMG-CoA reductase, purified to homogeneity as described by Ness, Spindler, and Moffler (39), was concentrated to a volume of 400 μl in 50 mM potassium phosphate buffer, pH 7.1, emulsified with 1 ml of Freund's complete adjuvant and injected into the toe pads of a 3-month-old male New Zealand White rabbit. Two weeks later, 100 μg of purified enzyme in a volume of 400 μl was mixed with 1 ml of Freund's incomplete adjuvant and injected subcutaneously in several sites along the rabbit's back. Two weeks later and at weekly intervals thereafter, blood was collected from an ear vein, and IgG was prepared (41) and stored at −20°C. Ouchterlony double-diffusion analysis of this serum revealed a single precipitin line when diffused against reductase obtained from all stages in the purification procedure, indicating that the antiserum specifically recognized HMG-CoA reductase (**Fig. 1**).

Measurement of human leukocyte HMG-CoA reductase activity

Leukocyte HMG-CoA reductase activity was measured as described by Harwood et al. (33), with the following ex-

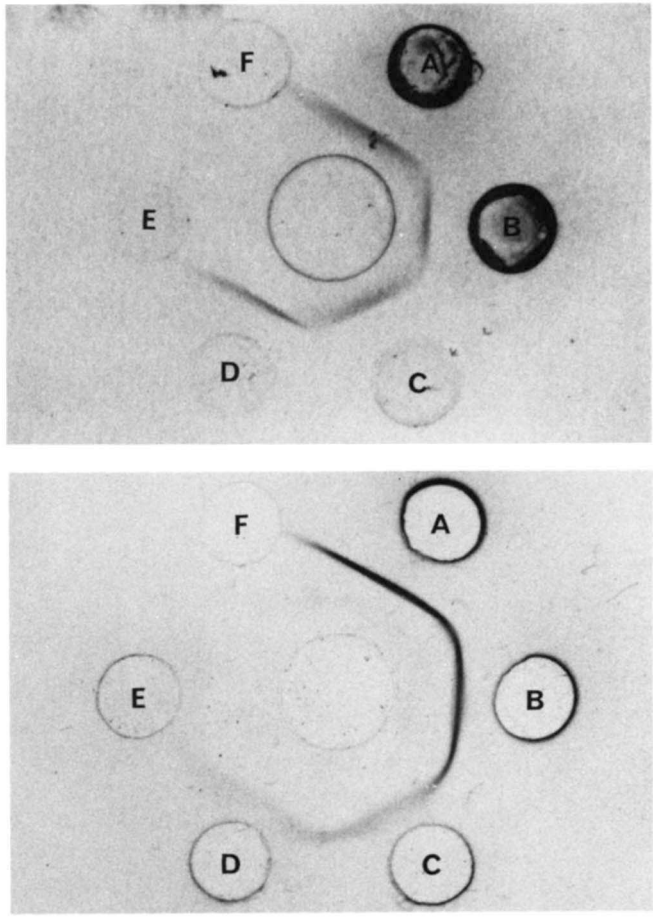

Fig. 1. Ouchterlony double diffusion analysis of anti-HMG-CoA reductase antisera. Top: The center well contained 8 μg of rabbit anti-rat liver HMG-CoA reductase IgG. The outer wells contained rat liver protein isolated after the indicated stages in the purification procedure; A, ammonium sulfate precipitation, 0.5 mg; B, heat treatment, 0.1 mg; C, Affi-Gel Blue affinity chromatography, 12 μg; and D, HMG-CoA agarose affinity chromatography, 1 μg. Bottom: The center well contained 3 μg of purified rat liver HMG-CoA reductase. The outer wells contained A, 16 μg; B, 8 μg; C, 4 μg; and D, 2 μg of anti-rat liver HMG-CoA reductase IgG.

ceptions. The specific activity of [3-^{14}C]HMG-CoA used for determining enzyme activity in freshly isolated leukocytes was 30 cpm/pmol, compared to a specific activity of 10 cpm/pmol employed for measuring HMG-CoA reductase activity in liver or cultured leukocytes (31). EDTA, 68 mM, was included in all incubations to prevent conversion of mevalonate to phosphomevalonate. HMG-CoA reductase activity is expressed as pmol of mevalonate formed per min of incubation at 37°C per mg of microsomal protein. Under the standard isolation conditions described above (33), HMG-CoA reductase is fully activated through dephosphorylation (32, 35). No further increases in enzyme activity occur upon treatment with either *E. coli* alkaline phosphatase or a crude preparation of rat liver phosphoprotein phosphatase (35).

Measurement of leukocyte microsomal HMG-CoA reductase protein concentration

Human leukocyte microsomal HMG-CoA reductase protein concentration was quantitated using a noncompetitive, solid phase, bridged biotin-avidin enzyme immunoassay procedure as described below.

Application of leukocyte microsomes to BA 85 nitrocellulose filters. Portions of leukocyte microsomal preparations containing 1.5 μg of microsomal protein were applied to BA 85 nitrocellulose filters in 0.75 mm × 8.0 mm rectangular areas (6.0 mm^2 surface area) under vacuum using a Schleicher and Schuell Minifold-II slot-blot apparatus. When the entire sample had been filtered through the nitrocellulose, the sheet was removed from the blotting apparatus, dried, immersed in 100 ml of TBS containing 3% gelatin, and incubated for 30 min at room temperature with gentle shaking.

Reaction between bound microsomal HMG-CoA reductase and anti-HMG-CoA reductase antiserum. Following incubation, the nitrocellulose sheet was removed from the blocking solution, immersed, without rinsing, into 50 ml of TBS containing 1% gelatin and 0.5 ml rabbit anti-rat HMG-CoA reductase antiserum, and incubated at 45°C for 2 hr with gentle shaking. The nitrocellulose was then removed from the first antibody solution, rinsed three times with 200 ml each of distilled water, washed twice at room temperature for 15 min each with 100 ml of TBS containing 0.05% Tween-20, and rinsed once at room temperature for 15 min with 100 ml of TBS.

Reaction of the primary antibody conjugate with biotinylated donkey anti-rabbit immunoglobulin. The washed nitrocellulose sheet was next immersed in 40 ml of TBS containing 1% gelatin and 240 μl of biotinylated donkey antirabbit immunoglobulin (Amersham, Cat. #RPN 1004), and incubated at room temperature for 60 min with gentle shaking. Following incubation, the nitrocellulose was rinsed in distilled water, washed in TBS containing 0.05% Tween-20, and washed in TBS as described above.

Reaction of the second antibody conjugate with streptavidin-horseradish peroxidase. The washed nitrocellulose sheet was next immersed in 40 ml of TBS containing 1% gelatin and 80 μl of a streptavidin-horseradish peroxidase conjugate (Amersham, Cat. #RPN 1231) and incubated at room temperature for 30 min with gentle shaking. Following incubation, the nitrocellulose was rinsed in distilled water, washed in TBS containing 0.5% Tween-20, and washed in TBS as described above.

Measurement of bound horseradish peroxidase activity. During the final 3 min of the last TBS wash, 40 mg of 4-chloro-1-naphthol was dissolved in 10 ml of room temperature methanol, and 50 μl of 30% H_2O_2 was mixed with 50 ml of room temperature TBS containing 1% gelatin. After the final TBS wash, the two solutions were mixed and immediately poured onto the still damp nitrocellulose sheet, which had been removed from the final washing solution just prior to mixing the solutions. The reaction was permitted to incubate at room temperature for 15 min with gentle shaking or until maximal or desired color development was observed. The nitrocellulose sheet was washed three times with 200 ml each of distilled water and allowed to stand at room temperature in distilled water overnight. The next morning, the nitrocellulose was air-dried and the intensity of color formation was quantitated by reflectance densitometry. Relative reflectance, measured in units of pen deflection, was converted to μg of HMG-CoA reductase protein by comparison to known amounts of purified human liver HMG-CoA reductase protein standards included on each nitrocellulose sheet. Leukocyte microsomal HMG-CoA reductase protein concentration is expressed as μg immunoreactive protein per mg microsomal protein.

Measurement of protein concentration

Protein was determined by the method of Bradford (42), using bovine serum albumin as standard.

RESULTS

Cross-reactivity of anti-rat liver HMG-CoA reductase antiserum with the human enzyme

Polyclonal antiserum, monospecific for rat liver HMG-CoA reductase (Fig. 1), inactivated cultured human leukocyte (IM-9 cell line) microsomal HMG-CoA reductase in a dose-dependent manner (**Fig. 2**). The decrease in enzyme activity was a linear function of the $\log_{10}$ antiserum concentration, with 50% inhibition occurring upon addition of 0.28 μl of antiserum. The equivalence point for IM-9 leukocyte microsomal HMG-CoA reductase, calculated by extrapolation of the linear portion of the curve to the abscissa (volume of antiserum required

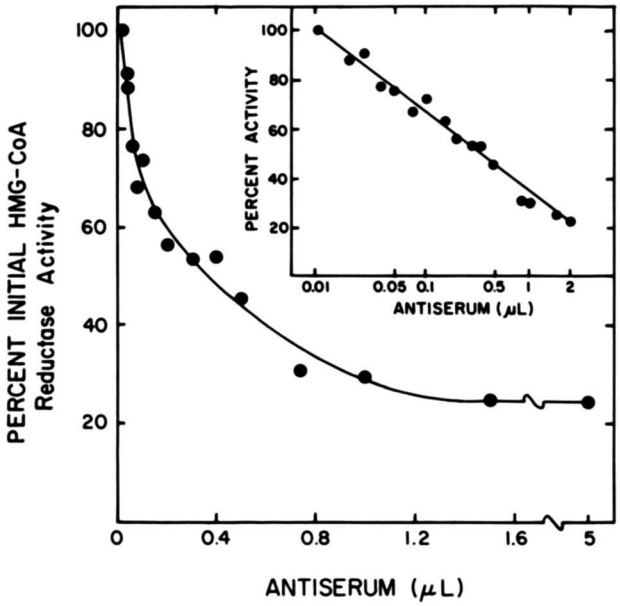

Fig. 2. Inactivation of human leukocyte HMG-CoA reductase by anti-rat liver HMG-CoA reductase antiserum. IM-9 leukocyte microsomes (20 μl, 8.0 mg/ml) were incubated with 80 μl of saline containing the indicated volume of anti-rat liver HMG-CoA reductase IgG for 5 min at room temperature. Remaining HMG-CoA reductase activity was then measured in a 30-min incubation at 37°C. Shown is the percentage of control HMG-CoA reductase activity (105 pmol/min per mg) as a function of the volume of antiserum added. Inset: Percentage of control HMG-CoA reductase activity as a function of $\log_{10}$ antiserum volume added.

for complete inhibition) and using least squares analysis of the appropriate data points, was 134 pmol/min per μl of antiserum.

The antiserum produced against purified, proteolytically modified rat liver HMG-CoA reductase also cross-reacted with purified, proteolytically modified human liver HMG-CoA reductase (**Fig. 3**). The antigenicity of the purified human liver enzyme, however, was only 1/22nd that of the purified rat liver enzyme (**Fig. 4**).

Development of the enzyme immunoassay methodology

The activity of freshly isolated human leukocyte HMG-CoA reductase is approximately 50-fold lower than that of rat liver (33). Reduced enzyme activity, in combination with the 22-fold lower antigenicity of the human enzyme relative to the rat enzyme, made it necessary to modify standard ELISA methodology (i.e., reaction with specific antiserum followed by reaction with anti-IgG antiserum conjugated with horseradish peroxidase (43)) in order to visualize the human leukocyte enzyme.

The first modification involved addition of a biotin-avidin bridge between the anti-IgG molecule and the horseradish peroxidase molecule (44). This involved reaction of the primary conjugate with anti-IgG immunoglobulin bound to biotin, followed by reaction with a streptavidin-horseradish peroxidase conjugate (44). This modification increased the sensitivity of the immunoassay about 5-fold (data not shown). The second major modification involved increasing the incubation temperature of the primary antibody reaction to 45°C. This produced an additional 5-fold enhancement in sensitivity (data not shown). Attempts to increase incubation times or incubation temperatures of subsequent steps were either without effect or severely increased background color development. The sequence of incubations resulting in maximal color development with optimal signal to background ratio is described in Materials and Methods. Using this

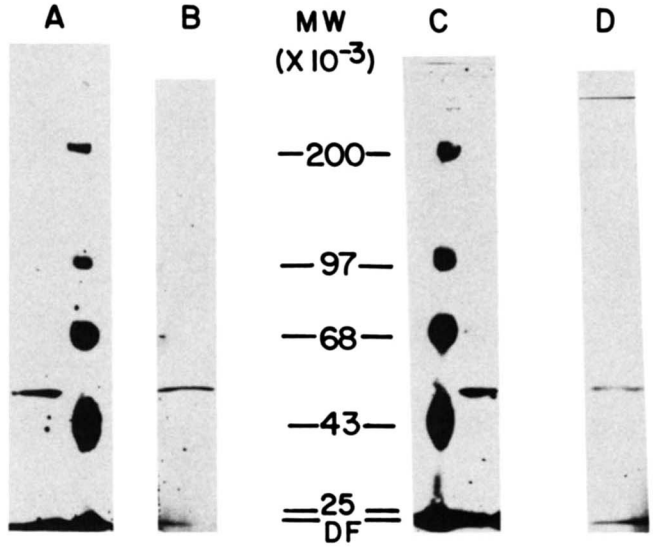

Fig. 3. Denaturing polyacrylamide gel electrophoresis and Western blot analysis of purified rat and human liver HMG-CoA reductase. Electrophoresis in 7.5% (w/w) polyacrylamide slab gels containing 0.1% sodium dodecyl sulfate was conducted at room temperature with a constant current of 30 mA/gel, as described by Laemmli (52). Portions of either purified, proteolytically modified rat liver (Affi-Gel Blue fraction), or purified, proteolytically modified human liver (Affi-Gel Blue or HMG-CoA agarose fraction) HMG-CoA reductase, or a mixture of prestained standards containing 10 μg each of myosin (M_r = 205,000), phosphorylase b (M_r = 97,400), BSA (M_r = 66,000), ovalbumin (M_r = 45,000), chymotrypsinogen (M_r = 26,000), β-lactoglobulin (M_r = 18,000) and cytochrome c (M_r = 12,000) in 100 μl of sample buffer (80 mM Tris (pH 6.8), 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 200 mM sucrose, and 0.001% bromophenol blue) were heated in a boiling water bath for 3 min before application to the gel. Protein fractions were applied to the gel such that each well containing HMG-CoA reductase protein was adjacent to a well containing prestained protein molecular weight standards. Following electrophoresis, all proteins in one portion of the gel were stained with Coomassie blue, as previously described (53). Proteins from a second portion of the gel were electrophoretically transferred to BA 85 nitrocellulose paper in 25 mM Tris, 192 mM glycine buffer (pH 8.3) containing 20% (v/v) methanol at 0–5°C with a constant voltage of 55V (0.18A) for 16–18 hr. The nitrocellulose paper was then incubated as described in Materials and Methods to visualize anti-HMG-CoA reductase immunoreactive protein. Left: A, 11 μg of purified rat liver HMG-CoA reductase stained with Coomassie blue; B, 0.9 μg of purified rat liver HMG-CoA reductase stained for immunoreactive protein. Right: C, 6.8 μg of purified human liver HMG-CoA reductase stained with Coomassie blue; D, 8.1 μg of purified human liver HMG-CoA reductase stained for immunoreactive protein. For clarity, only the prestained standards associated with the Coomassie blue-stained portions of the gel (A and C) are shown.

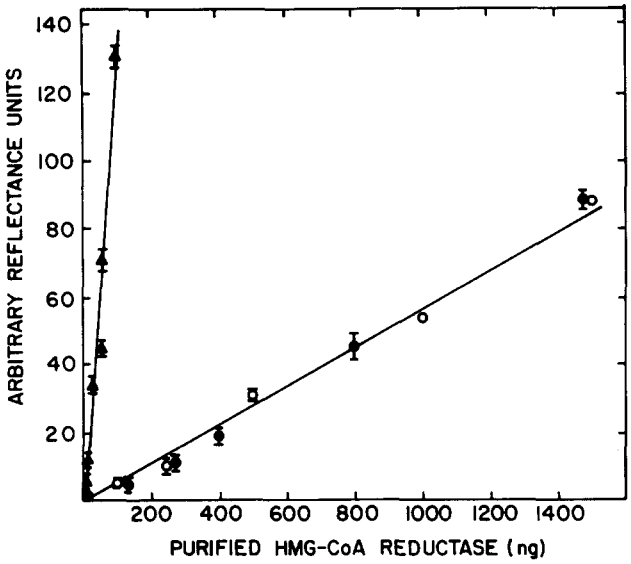

Fig. 4. Relative antigenicity of purified rat and human HMG-CoA reductase. The indicated amounts of purified rat liver HMG-CoA reductase protein (▲) or purified human liver HMG-CoA reductase protein (●, ○) were applied to a BA 85 nitrocellulose sheet using a Schleichter and Schuell Minifold-II slot-blot apparatus. Anti-HMG-CoA reductase immunoreactive protein was visualized as described in Materials and Methods and quantitated by reflectance densitometry. Shown are arbitrary reflectance units as a function of the quantity of purified rat (▲) or human (Affi-Gel Blue fraction ●, HMG-CoA agarose fraction ○) liver HMG-CoA reductase protein applied to the nitrocellulose.

method, immunoreactivity (as judged by color development and quantitated by reflectance densitometry) was a linear function of the amount of either purified rat or human liver HMG-CoA reductase applied to the nitrocellulose (Fig. 4).

Color development was also a linear function of the amount of either freshly isolated mononuclear leukocyte (**Fig. 5**) or cultured IM-9 leukocyte (data not shown) microsomal protein applied to the nitrocellulose, up to approximately 1.5 μg of microsomal protein.[2] Similar results were obtained for the rat liver microsomal enzyme (data not shown). A quantity of 1.5 μg of microsomal protein was thus chosen as the optimal amount of microsomal protein to apply to the nitrocellulose to assure maximal color development in a range where color development was a linear function of protein concentration. Intra-assay variation for human leukocyte HMG-CoA reductase protein concentration using 1.5 μg of microsomal protein was 4.4%.

Specificity of the enzyme immunoassay procedure for HMG-CoA reductase protein

To determine the degree of nonspecific binding of the primary antiserum, biotinylated second antibody, and streptavidin-horseradish peroxidase conjugate to the applied microsomal protein, immunoreactivity of IM-9 leukocyte and freshly isolated mononuclear leukocyte microsomes was assessed under conditions in which one complexing reagent was omitted from the assay. No color development was observed when the primary antiserum was omitted from the first incubation. Likewise, no color development was observed when the biotinylated anti-rabbit immunoglobulin preparation was omitted from the second incubation or when the streptavidin-horseradish peroxidase conjugate was omitted from the third incubation. Color development was only observed when all incubation reactants were included. Thus, color development is not the result of nonspecific interaction between the components of the immunoassay. Furthermore, no color development occurred when microsomes were applied to the nitrocellulose and immediately reacted with color development reagents. Thus, endogenous leukocyte peroxidases (45) are either absent from the leukocyte microsomal preparations or do not interfere with the immunoassay.

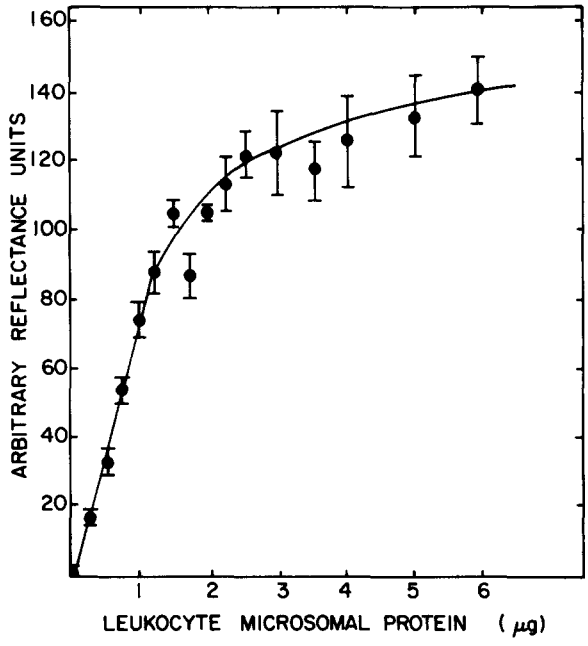

Fig. 5. Color development is dependent on the amount of immunoreactive protein applied to the nitrocellulose paper. The indicated amounts of freshly isolated mononuclear leukocyte microsomal protein (HMG-CoA reductase activity = 14.6 ± 0.3 pmol/min per mg) were applied to a BA 85 nitrocellulose sheet using a Schleichter and Schuell Minifold-II slot-blot apparatus. Anti-HMG-CoA reductase immunoreactive protein was visualized as described in Materials and Methods and quantitated by reflectance densitometry. Shown are arbitrary reflectance units as a function of the quantity of leukocyte microsomal protein applied to the nitrocellulose. The arbitrary reflectance units shown here are not directly comparable to those shown in Fig. 4 since the strip chart recorder was adjusted to be approximately 28 times more sensitive in this experiment than in the experiment described in Fig. 4.

[2]The binding capacity of BA 85 nitrocellulose for protein in approximately 250 μg/cm^2 or 2.5 μg/mm^2 (51). The maximal protein binding capacity of the 0.75 mm × 8.0 mm rectangular area (6.0 mm^2 surface area) to which sample is applied is thus approximately 15 μg.

TABLE 1. Preabsorption of anti-HMG-CoA reductase antiserum with rat liver microsomal HMG-CoA reductase reduces immunoreactivity with both the rat and human enzymes[a]

Enzyme Source	Color Development using as Primary Antibody: Anti-Rat Liver HMG-CoA Reductase Antiserum (A)	Preabsorbed Anti-Rat Liver HMG-CoA Reductase Antiserum (B)	Ratio B/A
	arbitrary reflectance units		
Rat liver Affi-Gel Blue fraction	179 ± 41	111 ± 31	0.62
Human liver Affi-Gel Blue fraction	167 ± 12	80 ± 18	0.48
Cultured human leukocyte microsomes	64 ± 14	26 ± 5	0.41
Freshly isolated human mononuclear leukocyte microsomes	48 ± 5	18 ± 1	0.38

[a]Rat liver microsomes isolated from five rats killed at the mid-dark phase of their diurnal cycle (200 mg microsomal protein; 500 pmol/min per mg) were washed once in TEDK buffer and then resuspended in 10 ml of TBS plus 1% gelatin containing 100 μl of anti-rat liver HMG-CoA reductase antiserum. Following a 30-min incubation at room temperature, the suspension was centrifuged at 172,000 *g* for 90 min to resediment the microsomes. Following centrifugation, the microsomal pellet was discarded and the supernatant liquid was retained as the preabsorbed anti-rat liver HMG-CoA reductase antiserum preparation. A second 100-μl portion of anti-rat liver HMG-CoA reductase antiserum was mixed with 10 μl of TBS plus 1% gelatin and stored at 4°C for 120 min. During microsomal sedimentation, 0.2 μg of purified rat liver HMG-CoA reductase (Affi-Gel Blue fraction), 1.5 μg of purified human liver HMG-CoA reductase (Affi-Gel Blue fraction), 1.5 μg of freshly isolated mononuclear leukocyte microsomal protein, and 1.5 μg of cultured human leukocyte microsomal protein were applied in 0.75 × 8 mm rectangular areas to each of two, 1 × 1 cm strips of BA 85 nitrocellulose. Following application, the nitrocellulose strips were incubated for 30 min at room temperature in TBS plus 3% gelatin. One strip was then incubated for 2 hr at 45°C with anti-rat liver HMG-CoA reductase antiserum in TBS plus 1% gelatin, whereas the second strip was incubated for 2 hr at 45°C with the preabsorbed anti-rat liver HMG-CoA reductase antiserum preparation in TBS plus 1% gelatin. Subsequent operations leading to quantitation of immunocomplex formation were as described in Material and Methods. Data are the average of the results of two independent experiments ± difference from the mean.

To estimate the extent to which the primary antiserum was specific for HMG-CoA reductase, anti-rat liver HMG-CoA reductase antiserum was preabsorbed with either rat liver microsomes (**Table 1**) or partially purified human liver HMG-CoA reductase (heat fraction; **Fig. 6**). As shown in Fig. 6, preabsorbing anti-HMG-CoA reductase antiserum with varying amounts of partially purified human liver HMG-CoA reductase (heat fraction) decreased the intensity of color formation for purified rat liver HMG-CoA reductase (shown), purified human liver HMG-CoA reductase, and human leukocyte microsomal HMG-CoA reductase (data not shown) in a concentration-dependent manner to less than 5% of control values. Furthermore, partial preabsorption of the antiserum with rat liver microsomes resulted in a partial reduction in color development for the rat liver, human liver, and human leukocyte enzymes (Table 1). Thus the anti-rat liver HMG-CoA reductase antiserum has a high degree of specificity for both the rat and human enzymes.

Anti-HMG-CoA reductase antiserum recognizes both the phosphorylated and unphosphorylated forms of the human leukocyte enzyme

The catalytic activity of the HMG-CoA reductase molecule is known to be modulated by a variety of mechanisms that include reversible phosphorylation (9–12) and essential thiol-disulfide formation (13–16). To be a useful probe for measuring human leukocyte HMG-CoA reductase protein concentration, therefore, anti-HMG-CoA reductase antiserum must equally recognize both active and inactive forms of the enzyme. As shown in **Fig. 7** (left panel and inset), fully active IM-9 leukocyte HMG-CoA reductase and that inactivated 32% by phosphorylation in the presence of MgATP plus ADP produced identical antibody endpoints when subjected to immunotitration. Thus, the antiserum recognizes equal amounts of immunoreactive protein in both fully active (dephosphorylated) and partially active (partially phosphorylated) preparations. Further, as shown in Fig. 7 (right panel), the antigenicity of a preparation of IM-9 leukocyte microsomes remained unaltered as enzyme activity was decreased 80% in a time-dependent manner, following addition of MgATP and ADP.

Isolation of microsomes in the presence of 100 mM NaF, which prevents dephosphorylation of HMG-CoA reductase by phosphoprotein phosphatase (26), also permits isolation of partially phosphorylated, and hence partially inactive, HMG-CoA reductase. As shown in **Table 2**, antigenicity of rat liver and IM-9 leukocyte microsomal preparations, isolated in the presence of 100 mM NaF (TEDF), was similar to that of microsomes isolated in the presence of 100 mM NaCl (TEDK). HMG-CoA reductase activity of microsomes isolated with TEDF, however, was only 20–30% that of enzyme activity in microsomes isolated with TEDK (Table 2). Thus, anti-rat liver HMG-CoA reductase antiserum equally recognizes both the phosphorylated and unphosphorylated forms of both the rat and human enzymes.

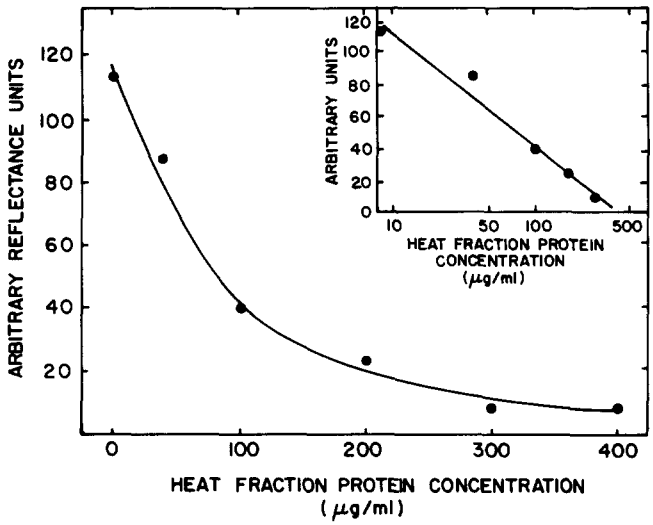

Fig. 6. Preabsorption of anti-HMG-CoA antiserum with partially purified human liver HMG-CoA reductase prevents immunocomplex formation. Anti-HMG-CoA reductase antiserum (28 μl) was incubated for 90 min at room temperature in a final volume of 4.0 ml TBS plus 1% gelatin with the indicated amounts of partially purified human liver HMG-CoA reductase (heat fraction). During this incubation, 0.2 μg of purified rat liver HMG-CoA reductase (Affi-Gel Blue fraction), 1.5 μg of purified human liver HMG-CoA reductase (Affi-Gel Blue fraction), and 1.5 μg of freshly isolated or cultured leukocyte microsomal protein were applied in 0.75 × 8 mM rectangluar areas to each of six 1 × 7 cm strips of BA 85 nitrocellulose. Following application of the samples to the nitrocellulose, the nitrocellulose strips were incubated for 30 min at room temperature in TBS plus 3% gelatin. Following incubations, the nitrocellulose strips were immersed in the preabsorbed antibody solutions and incubated for 2 hr at 45°C. Subsequent operations leading to visualization of the quantity of immunocomplex formation were as described in Materials and Methods. Shown are the arbitrary reflectance units measured for 0.2 μg of purified rat liver HMG-CoA reductase (Affi-Gel Blue fraction) as a function of the quantity of partially purified human liver HMG-CoA reductase used to preabsorb the antiserum. Inset: Arbitrary reflectance units as a function of $\log_{10}$ preabsorbing protein concentration.

Thiol-disulfide reduction increases leukocyte HMG-CoA reductase activity and decreases antigenicity

Reduction of an internal thiol-disulfide linkage in the HMG-CoA reductase molecule results in a conformational change that increases the catalytic efficiency of the enzyme (14–16). As shown in **Fig. 8,** these changes also decrease the antigenicity of the enzyme molecule. Isolation of mononuclear leukocyte microsomes in the absence of the thiol-disulfide agent, dithiothreitol, resulted in a preparation that possessed maximal antigenicity and minimal enzyme activity. Increasing dithiothreitol concentrations between 0 and 4 mM decreased antigenicity and increased enzyme activity (Fig. 8). Further increases in dithiothreitol concentration between 4 and 20 mM had no further effect on either antigenicity or enzymatic activity (Fig. 8). Although maximal antigenicity occurs when microsomes are isolated in the absence of thiol-reducing agents, slight differences in thiol-disulfide oxidation states resulting from differences in environmental oxidation could cause artifactual differences in measured HMG-CoA reductase protein concentrations. Isolation of leukocyte microsomes in buffer containing greater than 4 mM dithiothreitol, however, permits measurement of enzyme protein concentration under conditions where oxidation-induced changes in antigenicity are minimal. Further, isolation of microsomes in the presence of greater than 4 mM dithiothreitol allows measurement of both HMG-CoA reductase activity and protein concentration in the same microsomal preparation.

Leukocyte HMG-CoA reductase activity is dependent on enzyme protein concentration

When microsomes were isolated from peripheral blood mononuclear leukocytes of 33 healthy normocholesterolemic individuals in the presence of 5 mM dithiothreitol, and under conditions that fully dephosphorylate the enzyme, HMG-CoA reductase activity was directly proportional to the concentration of microsomal HMG-CoA reductase protein ($R = 0.65$; $P < 0.001$; **Fig. 9**). The specific activity (catalytic efficiency) of the freshly isolated mononuclear leukocyte HMG-CoA reductase molecule was 313 ± 34 pmol/min per mg of immunoreactive protein.

DISCUSSION

In this report, we describe the development of a noncompetitive, solid phase enzyme immunoassay for quantitation of microsomal HMG-CoA reductase protein concentration. This method may be applied to either solubilized or membrane-bound forms of the enzyme. The procedure is specific for HMG-CoA reductase, recognizes both the phosphorylated and unphosphorylated forms of the enzyme, and, in the presence of 5 mM dithiothreitol, permits measurement of both enzyme activity and enzyme protein concentration in a single microsomal preparation.

We employed a polyclonal, monospecific anti-serum prepared against the proteolytically solubilized, purified rat liver enzyme as the source of anti-HMG-CoA reductase antibody. This antiserum preparation crossreacted with both the human liver and leukocyte enzymes and was capable of inactivating the enzyme. This is not surprising, however, since the rodent (hamster) and human enzymes show a high degree of sequence homology (46, 47). Interestingly, the total antigenicity of the human enzyme with respect to this antiserum is only 1/22nd that of the rat liver enzyme, suggesting that the rat and human enzymes may exhibit subtle, but significant differences in primary or secondary structure. In contrast, however, the quantity of human leukocyte HMG-CoA reductase activity inactivated by this antiserum (134 pmol/min per μl of

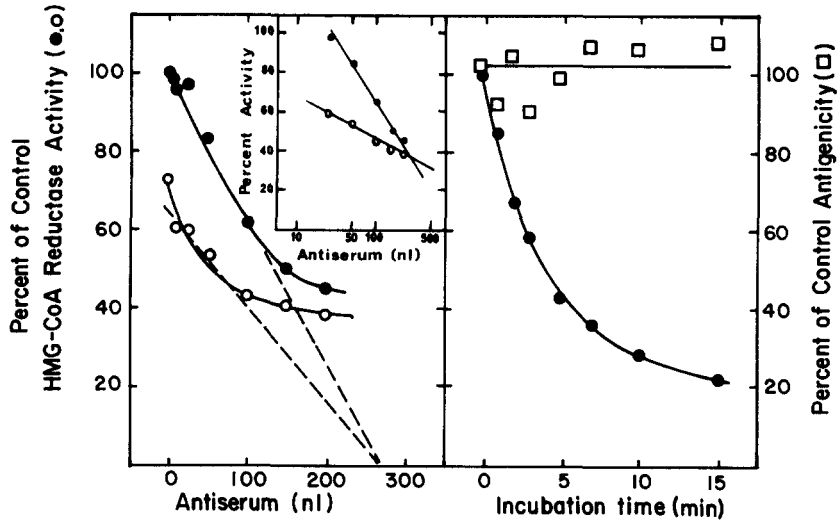

Fig. 7. Anti-HMG-CoA reductase antiserum recognizes both the phosphorylated and unphosphorylated forms of the human leukocyte enzyme. Left: IM-9 leukocyte microsomes (120 μl; 6.8 mg/ml) which contain both HMG-CoA reductase and HMG-CoA reductase kinase (32) were incubated with 2.0 mM ATP, 2.0 mM ADP, 8 mM $MgCl_2$, and 50 mM NaF in a final volume of 210 μl of TEDF for 5 min at 37°C to partially inactivate HMG-CoA reductase activity (○). A parallel incubation lacked ATP and ADP (●). Following incubation, EDTA (pH 7.5) was added to the incubation mixture at a final concentration of 96 mM to terminate the inactivation reaction and inhibit mevalonate kinase activity. Thirty μl of each incubation mixture (containing 94 μg microsomal protein) was diluted with 70 μl of saline containing the indicated volume of anti-rat liver HMG-CoA reductase antiserum and incubated for 5 min at room temperature. Remaining HMG-CoA reductase activity was then measured in a 30-min incubation at 37°C. Shown is the percentage of initial IM-9 leukocyte HMG-CoA reductase activity (161 ± 15 pmol/min per mg) as a function of the antiserum volume added. Extrapolation of the linear portion of the curves to the abcissa was done using least squares analysis of the appropriate data points. Inset: Percentage of control HMG-CoA reductase activity as a function of $\log_{10}$ antiserum volume added. Right: IM-9 leukocyte microsomes (200 μl; 30 mg/ml) were incubated with 2.0 mM ATP, 2.0 mM ADP, 8.0 mM $MgCl_2$, and 118 mM NaF in a final volume of 1150 μl of TEDF for up to 15 min at 37°C. At the indicated times, two 25-μl portions (containing 130 μg microsomal protein) were removed and assessed for remaining HMG-CoA reductase in the presence of 68 mM EDTA to terminate the kinase reaction and inhibit mevalonate kinase activity. A third 25-μl portion was also removed and mixed with 25 μl of 250 mM EDTA (pH 7.5) to terminate the inactivation reaction. Three aliquots of this mixture, each containing 1.5 μg of microsomal protein, were applied to BA 85 nitrocellulose and assessed for relative immunoreactivity of the partially inactivated enzyme. Shown are the percentages of initial HMG-CoA reductase activity (107 ± 2 pmol/min per mg; ●) and relative immunoreactivity (69 ± 6 μg/mg; □) as a function of time of incubation with Mg-nucleotides.

antiserum) is similar to that reported by Edwards et al. (140 pmol/min per μl antiserum) for the rat liver enzyme (22). Thus, while a significant fraction of the antibody mixture directed against epitopes on the rat liver enzyme fails to recognize the human enzyme, the inhibitory antibody, presumably recognizing a portion of the enzyme active site, recognizes HMG-CoA reductase from both species equally. Thus the primary and secondary struc-

TABLE 2. Measurement of leukocyte HMG-CoA reductase protein concentration is independent of the phosphorylation state of the enzyme[a]

Source of Microsomes	Isolation Buffer	HMG-CoA Reductase Activity	HMG-CoA Reductase Protein Concentration
		pmol/min mg microsomal protein	*μg immunoreactive protein mg microsomal protein*
Cultured human leukocytes (IM-9 cells)	TEDK (a)	216 ± 11 (n = 4)	48.2 ± 1.5 (n = 24)
	TEDF (b)	65 ± 4 (n = 4)	53.3 ± 1.5 (n = 23)
	b/a	0.30 ($P < 0.001$)	1.10 (NS)
Rat liver	TEDK (a)	671 ± 23 (n = 3)	4.4 ± 0.3 (n = 12)
	TEDF (b)	147 ± 8 (n = 3)	4.3 ± 0.2 (n = 12)
	b/a	0.22 ($P < 0.001$)	0.97 (NS)

[a]Cultured IM-9 cells from 800 ml of a late log-phase culture were divided into two portions, centrifuged to remove culture medium, and washed twice in either TEDK (to isolate fully active, dephosphorylated enzyme) or TEDF (to isolate partially active, partially phosphorylated enzyme). All subsequent operations were conducted with the same buffer used to wash the cells. Liver tissue from rats killed at the mid-dark phase of their diurnal cycle was divided into two equal portions. One portion was homogenized in TEDK and the other homogenized in TEDF. Following microsomal isolation, HMG-CoA reductase activity was measured using 50 μg of microsomal protein. HMG-CoA reductase protein concentration was determined using 1.5 μg of microsomal protein as described in Materials and Methods. Data are the averages of the indicated number of determinations ± SEM; NS, not significant.

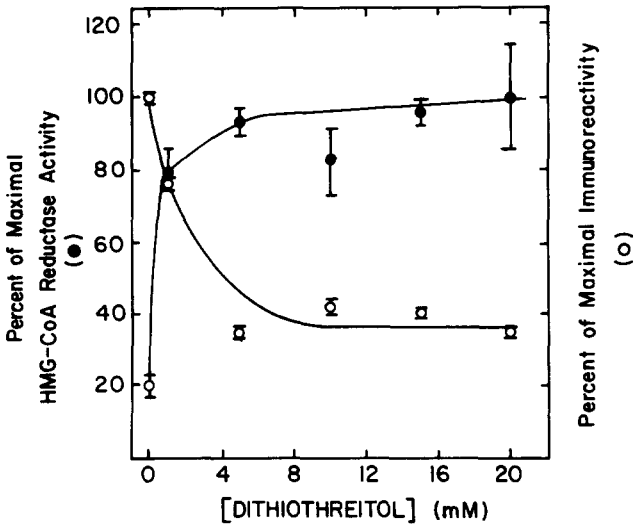

Fig. 8. Thiol-disulfide reduction increases leukocyte HMG-CoA reductase activity and decreases antigenicity. Freshly isolated mononuclear leukocytes from 1 unit of blood were isolated through the 0.87% NH_4Cl step. The mononuclear leukocyte cell pellet was resuspended in TEK buffer (TEDK buffer lacking dithiothreitol) and separated into six portions. Each portion received dithiothreitol at the indicated final concentrations. Microsomal HMG-CoA reductase concentration was presumed to be unaltered by this treatment. All subsequent procedures were conducted using TEK containing the indicated dithiothreitol concentrations. Shown are the percentage of maximal mononuclear leukocyte HMG-CoA reductase activity (●) and relative immunoreactivity (○) as functions of dithiothreitol concentration.

tures of this portion of the enzyme must be highly conserved between the two species. Such conservation of the purported active site domains of the hamster and human enzymes has recently been reported (47).

As has been previously shown for other anti-HMG-CoA reductase antisera (22, 29, 30), this antiserum equally recognizes both the phosphorylated and unphosphorylated forms of the enzyme. Thus, this method is applicable to quantitation of HMG-CoA reductase protein concentration in partially active, as well as fully active, enzyme preparations and therefore, may be useful in assessing the ratio of active (unphosphorylated) to total (phosphorylated + unphosphorylated) enzyme.

Since conformational changes in the catalytic portion of the enzyme would be expected to alter the antigenicity of the molecule, our findings suggest that major conformational changes in this portion of the enzyme molecule are not responsible for enzyme inactivation by phosphorylation. The antiserum, however, was prepared against the proteolytically modified form of the rat liver enzyme (39) which contains only the catalytic domain of the holoenzyme molecule (46) and exhibits a M_r of 50,000–54,000 (29, 39). The holoenzyme, however, exhibits a M_r of 90,000–100,000 (40, 48) and contains additional linking, membrane spanning, and inter-lumenal domains (46, 47). These additional regions are presumed to contain the allosteric activation site for NAD(P)H (15–18) and may also contain the essential thiol-disulfide bridge (16). Conformational changes in these regions, as a result of phosphorylation, could potentially have dramatic effects on enzyme activity without exhibiting differences in antigenicity using antibodies directed against the catalytic domain of the holoenzyme.

In contrast to the lack of antigenic differences between the phosphorylated and unphosphorylated forms of the enzyme, antigenicity of the leukocyte microsomal enzyme is sensitive to the thiol-disulfide status of the enzyme. Dotan and Shechter (14), Roitelman and Shechter (15, 17), and Ness et al. (16) have recently observed that essential sulfhydryl residues on the enzyme molecule are important in determining the catalytic efficiency of the enzyme. These studies suggest that oxidation of vicinal sulfhydryl residues in the enzyme molecule to their corresponding thiol-disulfide bridge results in a conformational change in the enzyme that reduces enzyme activity

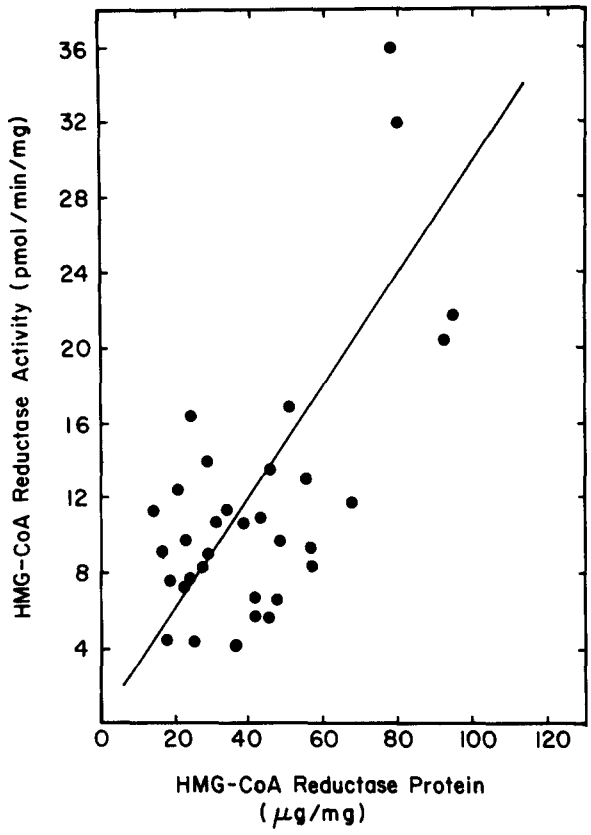

Fig. 9. Linear correlation between mononuclear leukocyte HMG-CoA reductase activity and HMG-CoA reductase protein concentration in healthy, normocholesterolemic individuals. Mononuclear leukocytes were isolated from 40 ml of peripheral blood obtained at 8:00 AM from 33 healthy normocholesterolemic subjects following an overnight fast. Leukocyte microsomes were isolated and HMG-CoA reductase activity and enzyme protein concentration were quantitated as described in Materials and Methods. Shown is mononuclear leukocyte HMG-CoA reductase activity as a function of anti-HMG-CoA reductase immunoreactive protein concentration.

(15, 16). Our results are consistent with these observations. Dithiothreitol, which reduces this thiol-disulfide bridge (14, 15), increases the activity of the human leukocyte enzyme and, at the same time, reduces its relative antigenicity. Since the antiserum was prepared against an enzyme fraction that lacked dithiothreitol (39, 40), antibodies are most likely directed against the oxidized form of the enzyme. Thus the conformational changes associated with thiol-disulfide reduction by dithiothreitol confer activity to the enzyme molecule and also reduce its antigenicity, presumably by altering some epitopes against which antibodies were formed. Our studies further implicate a conformational change in the catalytic domain of the HMG-CoA reductase molecule produced during activity modulation by thiol-disulfide reduction. For microsomal HMG-CoA reductase isolated in the presence of greater than 4 mM dithiothreitol, however, antigenicity is constant. This suggests that, as previously observed for rat liver (14) and human leukocyte (31, 35) HMG-CoA reductase, a dithiothreitol concentration of 5 mM is sufficient to fully reduce this essential thiol-disulfide and fully activate the enzyme molecule.

There are two major advantages of our method over that of immunotitration. First, HMG-CoA reductase protein concentration may be determined in triplicate with as little as 5 μg of microsomal protein. This quantity is considerably less than that required for immunotitration (28), and this becomes extremely important when examining microsomal samples possessing less than 500 μg of microsomal protein (i.e., the mononuclear leukocyte microsomal fraction from a 40-ml blood sampling). Secondly, using this method, together with that for measuring microsomal HMG-CoA reductase activity (33), it is possible to determine the catalytic efficiency of the enzyme molecule directly by comparing enzyme activity and enzyme protein concentration in the same microsomal preparation. Thus, alterations in HMG-CoA reductase activity that are due to changes in enzyme protein concentration, enzyme catalytic efficiency, or both may be ascertained. In this regard, application of these methodologies to investigate the regulation of HMG-CoA reductase in leukocytes from patients with familial hypercholesterolemia (49) or hematologic malignancies (50) has recently been reported.

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