Measurement of human leukocyte microsomal HMG-CoA reductase activity

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Abstract Methods were developed for determination of microsomal HMG-CoA reductase activity from freshly isolated human lymphocytes, monocytes, and granulocytes or cultured human lymphoid cells. Reductase activity in monocytes is approximately twice that in lymphocytes or granulocytes. The activity in cultured cells is approximately 34-fold greater than that in freshly isolated cells. Assay conditions were such as to preclude formation of HMG-CoA cleavage products. Leukocyte reductase activity was inhibited by dichloroacetate, a noncompetitive inhibitor of rat liver reductase and a serum cholesterol-lowering agent in man. Measurement of microsomal reductase activity from freshly isolated leukocytes may prove useful in assessing in vivo regulation of cholesterol synthesis in man.—**Harwood, H. J., Jr., M. Schneider, and P. W. Stacpoole.** Measurement of human leukocyte microsomal HMG-CoA reductase activity. *J. Lipid Res.* 1984. **25:** 967–978.

Supplementary key words monocytes • lymphocytes • granulocytes • dichloroacetate • cholesterol synthesis • cultured lymphoid cells

Under virtually all physiologic conditions, the rate of cellular cholesterol synthesis is determined by the activity of the microsomal enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase, ref. 1, 2). Accurate assessment of changes in the catalytic activity and/or total quantity of this enzyme in man should, in theory, provide insight into fluctuations in endogenous rates of cholesterolgenesis. To date, however, studies of the regulation of reductase have primarily been restricted to the use of either whole animals (3), animal tissues (1, 4–9), or cultured human cells (2, 10–12).

Several workers have studied reductase activity in human peripheral blood leukocytes cultured for several days (13–17) or incubated for several hours (18–21) under various experimental conditions. These investigators have noted changes in the rate of leukocyte reductase synthesis upon incubation of cells with lipoprotein-deficient serum (13, 15, 16, 18, 20), or in media containing oxygenated sterols (14, 21) or phytohemagglutinin (14, 17, 21). It has been difficult, however, to accurately relate these in vitro enzyme measurements to in vivo activity.

Currently, many of the limitations involved in the measurement of reductase activity from freshly isolated leukocytes are due to the very low activity of the enzyme (18) relative to that of rat liver or intestine (1). This has lead investigators to estimate enzyme activity in mitochondria-free whole cell extracts, rather than in microsomal suspensions (14–22). Young and Berger (22) have pointed out, however, that the specific activity of the human leukocyte enzyme measured in cell homogenates may be overestimated as a result of contamination with unidentified HMG-CoA cleavage products which migrate with or near mevalonate during its chromatographic isolation (22). These products, produced even in the absence of NADPH, preclude accurate measurement of reductase activity.

While most cleavage activity is associated with the low-speed mitochondrial pellet (22), substantial activity is released by the freeze-thaw homogenization required to disrupt cells (22). The non-ionic detergent KYRO-EOB has been used for cell disruption in place of freeze-thawing (18), but it also increases cleavage activity in the post-mitochondrial supernatant fraction (22). Although elaborate chromatographic techniques have been developed to separate mevalonate from HMG-CoA cleavage products (22), these methods do not remove or inhibit cleavage activity. Thus, reductase activity may be underestimated due to substrate depletion (22) by cleavage activity. Finally, while cleavage activity may be decreased when leukocyte extracts are incubated for extended periods of time in phosphate buffer (22), reductase activity is also reduced and examination of rapid changes in leukocyte reductase activity becomes impractical.

To more accurately measure human leukocyte reductase activity, therefore, it is necessary to separate the activity of reductase from the activity of those enzymes responsible for the generation of cleavage products. In the present report, we describe procedures for isolation

Abbreviations: HMG, hydroxymethylglutaryl; TLC, thin-layer chromatography; DCA, dichloroacetate.

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of microsomes from freshly isolated or cultured human leukocytes and determination of microsomal reductase activity. The reductase-catalyzed reaction is linear with respect to time of incubation and microsomal protein concentration and is free of HMG-CoA cleavage activity. In addition, leukocyte microsomal reductase activity can be modulated by pharmacologic inhibitors of the rat liver enzyme.

MATERIALS AND METHODS

Chemicals

Chemicals from commercial sources included: Histopaque and an α-naphthylacetate esterase staining kit (#90-A1) (Sigma Chemical Co., St. Louis, MO), HEPES (Research Organics, Inc., Cleveland, OH), glucose and NH_4Cl (Mallinckrodt, Inc., St. Louis, MO), phenol red, Wright's staining solution, and Giordano buffer (Fisher Scientific Co., Orlando, FL), heat-inactivated fetal bovine serum, RPMI-1640, and an antibiotic/antimyotic solution (Cat.# 600-5245) containing penicillin, streptomycin, and fungizone (Gibco Laboratories, Grand Island, NY) and Percoll (Pharmacia Fine Chemicals, Piscataway, NJ). All other chemicals were from previously listed sources (4, 23–26).

Buffered solutions

TEDK buffer contains 50 mM Tris-HCl (pH 7.5), 1.0 mM EDTA, 5.0 mM dithiothreitol, and 70 mM KCL. TEDF buffer contains 50 mM Tris-HCl (pH 7.5), 1.0 mM EDTA, 5.0 mM dithiothreitol, and 70 mM NaF. Hank's balanced salt solution contains 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). Double-strength phosphate-buffered saline contains 17.6 g/l NaCl and 3.55 g/l Na_2HPO_4 (pH 7.4). Fifty-five percent iso-osmotic Percoll contains 55 ml of Percoll, 0.48 g of NaCl, and 45 ml of 0.15 M NaCl. Seventy-four percent iso-osmotic Percoll contains 74 ml of Percoll, 0.65 g of NaCl, and 26 ml of 0.15 M NaCl. Fifty-five percent Percoll in phosphate-buffered saline is prepared by mixing 105 ml of Percoll with 90 ml of double-strength phosphate-buffered saline.

Isolation of peripheral blood mononuclear leukocytes

Peripheral blood mononuclear leukocytes from one unit of blood, obtained from normal volunteers, are isolated by leukophoresis on a Haemonetic Model 30 semicontinuous cell separator. Mononuclear cells are separated from erythrocytes and granulocytes by isopynknic centrifugation over Histopaque (specific gravity 1.078) by the method of Böyum (27). Virtually 100% of the granulocytes are removed by this procedure. Mononuclear cells at the interface (buffy coat) are removed using a plastic pipette and washed twice in 100 ml of Hank's balanced salt solution (pH 7.4) at 10°C to remove residual Histopaque. Contaminating red blood cells are lysed during a 10-min incubation at 0°C in 50 ml of 0.87% NH_4Cl. Following centrifugation, (400 *g*; 15 min), the cell pellet was washed twice in 50 ml of TEDK at 10°C and frozen in liquid N_2 in a volume of 0.5 ml TEDK per 10^8 cells [freezing cells in liquid N_2 aids in subsequent cell homogenation (5)]. This fraction typically contained 77% ± 1.3% (SE; n = 38) lymphocytes and 23% ± 1.2% (n = 38) monocytes, based on α-naphthylacetate esterase staining, using the method outlined in Sigma Technical Bulletin #90 (1983).

Isolation of enriched populations of lymphocytes, monocytes, and granulocytes

Isolation of lymphocytes, monocytes and granulocytes from a single blood sample is based on the methods of Hjorth, Jonsson, and Vretblad (28) for separation of granulocytes from erythrocytes and mononuclear leukocytes, and of Gmelig-Meyling and Waldmann (29) for separation of lymphocytes from monocytes. Peripheral blood leukocytes from one unit of blood, isolated and concentrated by leukophoresis, (30–50 ml leukocyte suspension/unit blood), are diluted to 120 ml with Hank's BSS. Fifteen-ml aliquots of the diluted suspension are added to each of eight 50-ml conical centrifuge tubes. The suspension is underlayered first with 15 ml of 55% iso-osmotic Percoll (d 1.065 g/ml) and subsequently with 15 ml of 74% iso-osmotic Percoll (d 1.103 g/ml). Discontinuous gradients are centrifuged for 40 min at 350 *g* (1,500 rpm) in an IEC PR-6000 clinical centrifuge. Mononuclear leukocytes band at the upper interface, while granulocytes and eosinophils band at the lower interface. Erythrocytes are pelleted. The lower band (granulocytes and eosinophils) is removed and washed twice in RPMI-1640 tissue culture medium supplemented with 10% fetal bovine serum. The resulting cell pellet is washed in 0.87% NH_4Cl and TEDK and frozen in liquid N_2, as described above. The upper band, containing mononuclear cells, is removed, washed twice in RPMI-1640 tissue culture medium supplemented with 10% fetal bovine serum, and resuspended in 10 ml of the same medium. An aliquot (1.25 ml) of the mononuclear cell population is applied to each of eight 24-ml (7 × 2.2 cm) iso-osmotic continuous Percoll gradients (d 1.010 to d 1.160 g/ml), prepared by centrifuging 55% Percoll in phosphate-buffered saline for 40 min at 21,000 *g* (15,000 rpm) with a 50.2 Ti Beckman fixed-angle ultracentrifuge rotor. Gradients containing mononuclear cells are centrifuged for 20 min at 1,000 *g* (2,500 rpm) in an IEC PR-6000 clinical centrifuge.

The upper band of leukocytes, migrating to d 1.060 g/ml, contains monocytes and large lymphocytes and is washed three times in RPMI-1640 supplemented with 10% fetal bovine serum, and twice in TEDK. The final cell pellet is resuspended in a minimal volume (0.1 ml) of TEDK and frozen in liquid N_2. The lower band of leukocytes, migrating to d 1.075 g/ml, and containing lymphocytes and dead monocytes, is washed and frozen in liquid N_2.

Growth and isolation of IM-9 lymphocytes

Lymphoid cells from the IM-9 cell line (30) are 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 (30). Cell viability is ≥90%, as assessed by Trypan Blue dye exclusion. IM-9 cells from late-log phase cultures are 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. IM-9 cells are stable frozen in liquid N_2 for at least 1 month.

Enzyme isolation

Frozen cell suspensions are incubated at room temperature until just thawed. Subsequent operations are at 0–5°C. Suspensions are homogenized 15 times with a motor-driven ground-glass pestle in a 1-ml Potter-Elvehjem Tissue Homogenizer, then 15 times manually. Suspensions are transferred to a 5-ml Potter-Elvehjem Tissue Homogenizer and homogenized 10 times with a motor-driven Teflon pestle. The homogenate is diluted to 8 ml with TEDK and centrifuged at 2,000 *g* for 10 min. In initial experiments the supernatant was decanted and centrifuged at 105,000 *g* for 90 min. Subsequently, however, we noted that microsomal reductase specific activity could be substantially increased by raising the centrifugation speed to 172,000 *g* (see Results). Unless otherwise noted, therefore, all experiments reported below employed microsomal sedimentation at 172,000 *g*. Following centrifugation, the cytosol is discarded and the microsomal pellet is resuspended in a volume of 0.16 ml TEDK per 10^8 cells. Peripheral blood leukocyte microsomal reductase activity is stable frozen in liquid N_2 for at least 3 months. IM-9 cell microsomal reductase is stable frozen in liquid N_2 for 2 months.

A preparation of rat liver mevalonate kinase, which also contains phosphomevalonate kinase, was prepared through the $(NH_4)_2SO_4$ fractionation, as described by Beytia et al. (31) for hog liver mevalonate kinase.

Assay of enzyme activity

Freshly isolated leukocyte or IM-9 cell microsomal reductase activity was measured essentially as described for rat liver microsomal reductase (4) and for rat leukocyte microsomal reductase (5). Up to 0.2 mg of microsomal protein are incubated at 37°C for 30 min in a final volume of 75 μl of TEDK containing 67 μM [3-^{14}C]HMG-CoA (7.8 cpm/pmol), [5-^{3}H]mevalonolactone (11,000 cpm; 629 Ci/mol) as an internal standard, 3.4 mM $NADP^+$, 30 mM glucose-6-phosphate, 0.2 U of glucose-6-phosphate dehydrogenase, and 68 mM EDTA to prevent conversion of mevalonate to phosphomevalonate during incubation. Following incubation, 10 μl of 6 M HCl is added to terminate the reductase reaction and convert mevalonate to mevalonolactone. The complete incubation mixture is then applied as a 2-cm-diameter spot to a 20 × 20 cm silica gel sheet, scored in ten channels. Following development in toluene–acetone 1:1 (v/v) the region corresponding to R_f 0.5 to 1.0 is cut from the sheet and counted in 4.0 ml of Scintelene liquid scintillation fluid. The quantity of [^{14}C]mevalonate produced is calculated, using the ^{3}H cpm as an internal standard to correct for variation in recovery of product. For determination of the effects of DCA or glyoxylate on reductase activity, microsomes and drugs were incubated together for either 0 or 20 min at 37°C, as previously described for rat liver microsomes (24), prior to measurement of enzyme activity.

Measurement of cholesterol synthesis

Measurement of incorporation of ^{3}H from tritiated water into nonsaponifiable, digitonin-precipitable lipid is based upon previously described methods for rat hepatocytes (32). Leukocyte suspensions are washed once (0–4°C) with 0.5 ml of chilled, 2 N $HClO_4$ and once with 5.0 ml of chilled 0.5 N $HClO_4$. The precipitate is resuspended in 0.25 ml of water and 3.75 ml of chloroform–methanol 2:1 and stored overnight at 5°C. Following centrifugation at 3,000 *g*, the supernatant liquid is removed and the wet pellet is mixed with 4.75 ml of chloroform–methanol–water 10:20:8. After a second centrifugation at 3,000 *g*, the supernatant liquid is removed and added to the chloroform–methanol extract. An equal volume of water is added to the extract and, after separation, the aqueous phase is discarded. Benzene is added to the organic phase and the resulting fraction is evaporated to dryness at 60°C. Five ml of 0.3 M NaOH in 90% methanol is added to the precipitate and the suspension is incubated (75°C, 2 hr). Three 5-ml portions of petroleum ether are added successively to the mixture to extract the nonsaponifiable lipids. The extract (approximately 15 ml) is mixed with 2.0 ml of benzene and evaporated to dryness at 60°C under N_2. The residue is dissolved in 4.0 ml of acetone–95% ethanol 1:1 and 2.0 ml of 0.5% digitonin in 50% ethanol is added. After overnight incubation, samples are centrifuged and the supernatant is removed. The precipitate is washed once with 3.0 ml of diethyl ether–acetone 1:1 and twice with 3.0 ml of diethyl ether. After the final

centrifugation, the precipitate is dried under N_2 at 40°C and quantitatively transferred with methanol to scintillation vials for counting. The rate of incorporation of ^3H_2O into digitonin-precipitable sterols and the mevalonate equivalents formed were calculated as previously described (24).

Analysis of protein

Protein was determined by the method of Bradford (33), using bovine serum albumin as standard.

RESULTS

Assay parameters

Peripheral blood mononuclear leukocyte or IM-9 cell microsomal reductase activity is dependent on the time of incubation at 37°C (**Fig. 1, B** and **D**) and on the amount of microsomal protein assayed (Fig. 1; A and C). Changes in reductase activity are linear up to 200 μg of microsomal protein and up to 30 min of incubation at 37°C. Leukocyte reductase activity exhibits a broad temperature optimum between 35°C and 45°C (data not shown). Assay of up to 150 μg of microsomal protein for 30 min at 37°C thus assures measurement of enzyme activity at its temperature optimum under conditions where it is a linear function of both incubation time and protein concentration. Under these conditions, and when microsomes are isolated by centrifugation at 105,000 *g* for 90 min, reductase activity obtained from microsomes of freshly isolated peripheral blood mononuclear leukocytes of healthy subjects (n = 15) averages 4.08 ± 0.18 (SE) pmol of mevalonate formed/min per mg microsomal protein, while that from IM-9 cells (n = 12) equals 134 ± 9 pmol/min per mg. When microsomes were isolated by centrifugation at 172,000 *g* for 90 min, however, reductase activity of freshly isolated mononuclear leukocytes from healthy subjects (n = 18) averaged 9.60 ± 0.18 pmol/min per mg and that from IM-9 cells (n = 6) averaged 324 ± 39 pmol/min per mg (**Table 1**). Thus, increased centrifugal force results in a 2.5-fold increase in the specific activity of microsomal reductase, presumably by increasing the yield of sedimented microsomes relative to other non-microsomal

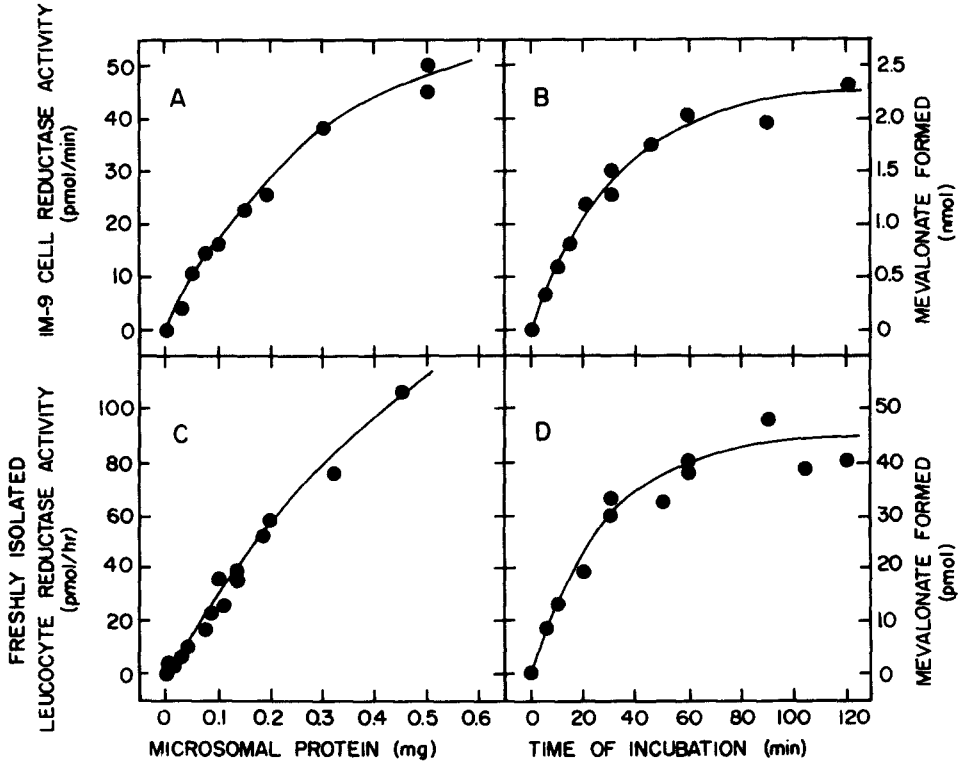

Fig. 1. Time and protein dependence of mononuclear leukocyte microsomal reductase activity. Protein dependence: up to 0.5 mg of IM-9 cell (A) or peripheral blood mononuclear leukocyte (C) microsomal protein (isolated by centrifugation at 105,000 *g* for 90 min), in a final volume of 75 μl, was assayed for reductase activity in a 30-min incubation at 37°C. Time dependence: microsomal protein (isolated by centrifugation at 105,000 *g* for 90 min), 140 μg from peripheral blood mononuclear leukocytes (D) for 500 μg from IM-9 cells (B), in a final volume of 75 μl, was assayed for reductase activity for up to 120 min at 37°C. Time and protein dependence profiles were similar whether microsomes were isolated by centrifugation at 105,000 *g* or 172,000 *g* for 90 min.

TABLE 1. Increased specific activity of microsomal reductase activity sedimented with increased centrifugal force[a]

Centrifugation Conditions	Reductase Activity from IM-9 Cells	Reductase Activity from Mononuclear Leukocytes
	pmol/min per mg	
105,000 *g*; 90 min (A)	134 ± 9 (n = 12)	4.08 ± 0.18 (n = 15)
172,000 *g*; 90 min (B)	325 ± 39 (n = 6)	9.60 ± 0.18 (n = 15)
Ratio B/A	2.43	2.35

[a] Microsomes from IM-9 cells grown for 3 days in culture (approximately 2 × 10^6 cells/ml) or freshly isolated peripheral blood mononuclear leukocytes were isolated by centrifugation at either 105,000 *g* (40,000 rpm; Beckman 50 Ti rotor) or 172,000 *g* (50,000 rpm; Beckman 50 Ti rotor) for 90 min. Microsomal reductase activity was then determined, as described in Methods. Data are expressed as mean ± SE. N, number of microsomal preparations prepared using the indicated conditions.

subcellular particles. Intraassay variation for reductase activity obtained under these improved conditions was 7.1% ± 0.8% for both freshly isolated and cultured cell populations. Intrasubject variability for freshly isolated leukocyte reductase averaged 6.2 ± 1.4%. Intraassay variation of the lymphocyte:monocyte ratio in the mononuclear cell population (n = 38) was 1.3%.

Absence of HMG-CoA cleavage activity from microsomal preprations

In previous reports, human leukocyte reductase activity was assayed in cell homogenates following low-speed centrifugation (1,000 *g*) to remove cell debris (14–22). Difficulties have been reported, however, in quantitating mevalonate formation due to HMG-CoA cleavage during incubation (22). As shown in **Table 2,** apparent reductase activity, measured in microsomal suspensions from IM-9 cells, is 14.6-fold and 4.7-fold greater, respectively, than that measured when phosphate-treated (22) cell-free supernatants or cell lysates are used as sources of reductase activity. This compares well with the difference observed between mononuclear leukocyte microsomal reductase activity (9.60 ± 0.18 pmol/min per mg; this report) and reductase activity measured in cell-free extracts (0.622 ± 12 pmol/min per mg; refs. 16, 17, 19, 20, 34–37). The apparent increase in enzyme activity when microsomes are used as the source of reductase is due, in part, to removal of HMG-CoA cleavage enzymes from microsomal preparations. As shown in **Fig. 2,** HMG-CoA cleavage activity remains in the supernatant liquid following high-speed centrifugation (172,000 *g*) and is therefore separated from microsomal reductase. Hence, when microsomes from either IM-9 cells or freshly isolated leukocytes are incubated in the absence of NADPH, no radiolabeled products are generated from [^{14}C]HMG-CoA (**Fig. 3**). The elaborate TLC systems described by Young and Berger (22) for separation of [^{14}C]mevalonate from ^{14}C-labeled cleavage products are unnecessary, therefore, when microsomes are used as the source of reductase activity.

Identification of the reductase reaction product as [^{14}C]mevalonate

In the presence of NADPH, [^{14}C]HMG-CoA, and either IM-9-cell microsomes or peripheral blood mononuclear leukocyte microsomes, NADPH-dependent ^{14}C-labeled reaction products comigrated with [^{3}H]meva-

TABLE 2. Comparison of IM-9 leukocyte reductase activity measured in cell lysates, cell-free supernatants, and microsomal suspensions[a]

Fraction Used for Reductase Determination	"Apparent" Reductase Activity Contained in Original Homogenate: Total Reductase Activity	"Apparent" Reductase Activity Contained in Original Homogenate: Percentage of Microsomal Activity	Specific Activity of Fraction
	pmol/min	%	*pmol/min per mg*
Microsomal suspension	1,420 ± 12	⟨100⟩	395 ± 3.5
Cell-free supernatant	97 ± 6	6.8	7.7 ± 0.5
Cell lysate	301 ± 11	21.2	17.4 ± 0.5

[a] IM-9 cells grown for 3 days in culture were harvested and homogenized, as described in Methods. The homogenate was diluted to 1.8 ml and divided into two equal portions. Each portion received 100 μl of 1.0 M potassium phosphate (pH 7.5). One portion was incubated for 90 min at room temperature then rehomogenized with five strokes of a motor-driven Potter-Elvehjem Tissue Homogenizer, as previously described by Young and Berger (22). Following homogenization, this fraction was divided into two equal portions. One portion remained untreated (cell lysate fraction) while the other was centrifuged at 2,000 *g* for 15 min (cell-free supernatant fraction). Microsomes isolated from the second portion of the original homogenate were resuspended in 0.9 ml of TEDK plus 100 ml of 1.0 M potassium phosphate (pH 7.5). Fifty μl of each fraction (corresponding to 100 μg, 350 μg, and 475 μg of microsomal suspension, cell-free supernatant fraction, and cell lysate protein, respectively) were assayed for reductase activity. Data are represented as mean ± SE for triplicate determinations.

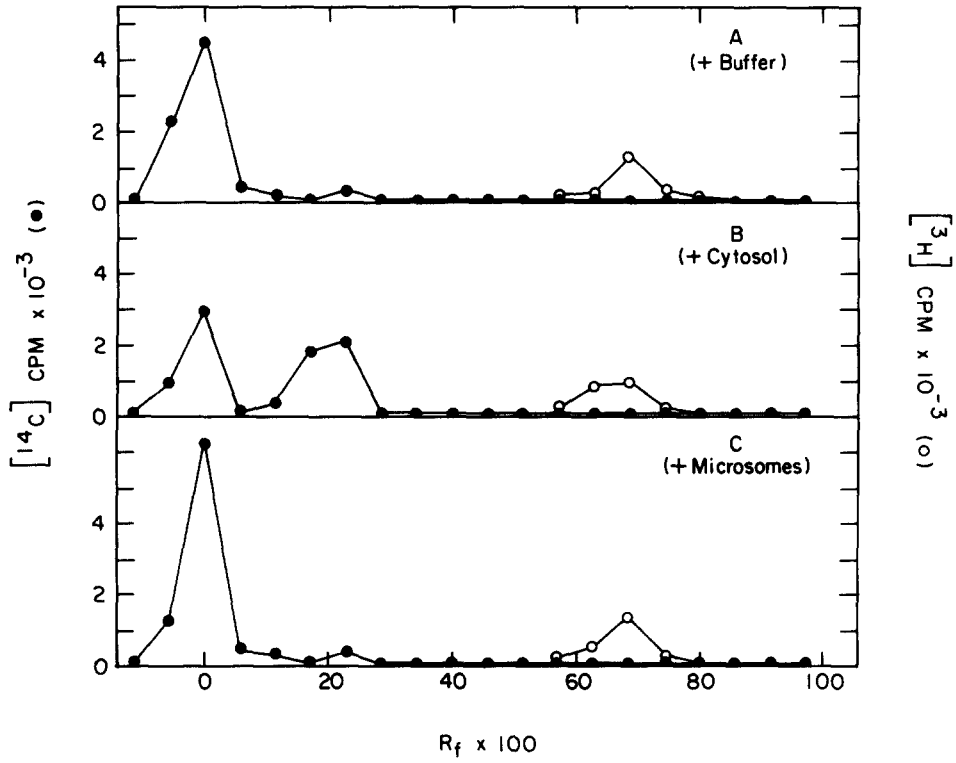

Fig. 2. HMG-CoA cleavage activity remains in the cytosolic fraction following high speed centrifugation. TEDK (A), 215 μg of IM-9 cell cytosolic protein (B), or 208 μg of IM-9 cell microsomal protein (C) was incubated in a final volume of 75 μl of TEDK containing 67 μM [^{14}C]HMG-CoA (sp act 7.8 cpm/pmol), 11,000 cpm [^{3}H]mevalonate (629 Ci/mol), 0.2 U of glucose-6-phosphate dehydrogenase, and 68 mM EDTA, for 30 min at 37°C. Following incubation the reaction was terminated by addition of 10 μl of 6 M HCl. Thirty μl of incubation mixtures was applied to 2 × 20 cm channels of a silica gel TLC plate, dried, and chromatographed in toluene–acetone 1:1. Following chromatography, channels were dried, sectioned into 1-cm slices, and assessed for radioactivity. Shown are the ^{14}C (●) and ^{3}H (○) cpm as a function of R_f value. R_f values for HMG-CoA, HMG-CoA cleavage products, and mevalonate are 0, 0.20, and 0.68, respectively.

lonolactone to R_f 0.63 when silica gel TLC sheets were developed in toluene–acetone (Fig. 3). The ratio of [^{3}H] cpm (○) to [^{14}C] cpm (●) was constant throughout the mevalonolactone peak, indicating that presumed [^{14}C]mevalonolactone was the only ^{14}C-labeled reaction product migrating with [^{3}H]mevalonolactone (**Fig. 4A**). Using [^{14}C]HMG-CoA isolated by the method of Williamson and Rodwell (25), no other peaks of radioactivity migrated between R_f 0.5 and 1.0 in this TLC system.[2] In addition, following base hydrolysis of mevalonolactone to mevalonate, neither the ^{14}C-labeled reaction products nor [^{3}H]mevalonate migrated further when rechromatographed in the same solvent (data not shown).

In two other TLC systems, i.e., those employed by Young and Rodwell (5) to identify [^{14}C]mevalonate produced by rat liver leukocyte reductase, presumed [^{14}C]mevalonate comigrated with authentic [^{3}H]mevalonate. The ratios of ^{3}H to ^{14}C were again constant through each mevalonate peak (Fig. 4B and 4C), strongly suggesting that the ^{14}C-labeled reaction product was [^{14}C]mevalonate.

To provide further evidence that the ^{14}C-labeled reaction product was [^{14}C]mevalonate, [^{3}H]mevalonolactone and the associated ^{14}C-labeled reaction product were isolated following TLC on silica gel in toluene–acetone. The isolate was treated with base to convert mevalonolactone to mevalonate and incubated with MgATP and rat liver mevalonate kinase (which also contains phosphomevalonate kinase) at 37°C for 30 min to convert mevalonate to phosphomevalonates. Eighty-nine percent of the presumed [^{14}C]mevalonate and 47% of the [^{3}H]mevalonate (94% of the active isomer) were converted to phosphomevalonates by this procedure (**Table 3**).

[2] Use of a commercial [3-^{14}C]HMG-CoA (Amersham) purified by the paper chromatography method of Goldfarb and Pitot (48) generated, in addition to [^{14}C]mevalonate, a ^{14}C-labeled contaminant migrating to R_f 0.8. The contaminant was present even in the absence of microsomes during incubation and its migration was independent of addition of 6 M HCl (data not shown). This contaminant appears to be one of the two proposed HMG-CoA "cleavage products" reported by Young and Berger (22).

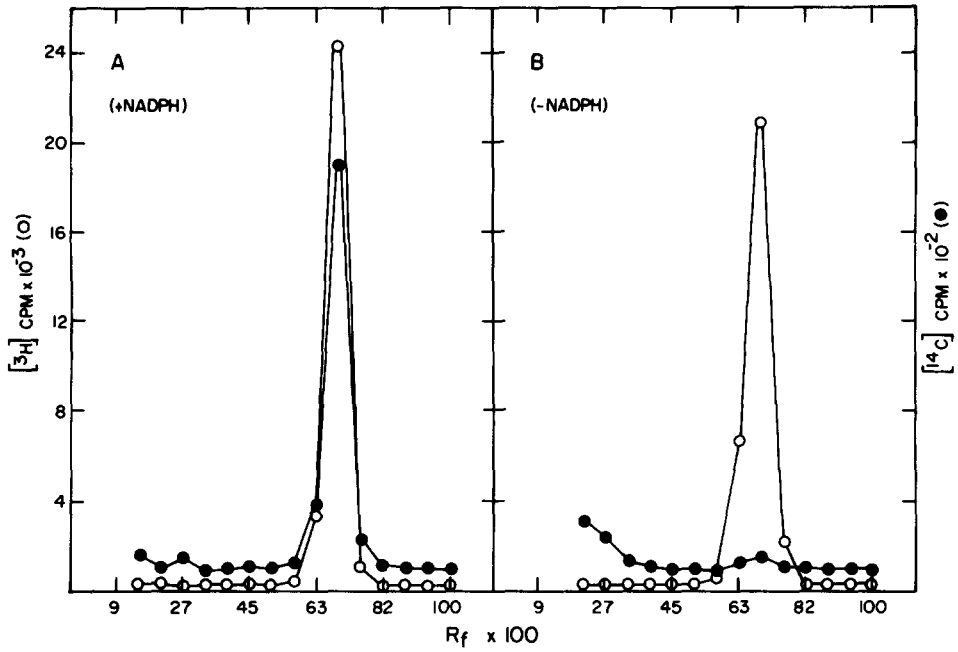

Fig. 3. NADPH-dependence of leukocyte microsomal reductase activity. IM-9 cell microsomal protein, 630 μg, was assayed for reductase activity in the presence (A) or absence (B) of NADPH, for 30 min at 37°C in a final volume of 300 μl. Following chromatography in solvent system I (toluene–acetone 1:1), the TLC sheet was cut into 1-cm sections and each section was counted for [^{3}H]mevalonate standard and [^{14}C]HMG-CoA reaction products; shown are [^{14}C] cpm $\times 10^{-2}$ (●) and [^{3}H] cpm $\times 10^{-3}$ (O).

Comparison of the rate of cholesterol synthesis in cultured IM-9 cells

Cultured IM-9 cells actively synthesize cholesterol. As shown in **Table 4,** the rate of mevalonate formation was approximately twofold greater than that derived from measurement of ^3H_2O incorporation into cholesterol. This ratio is similar to that observed by Fogelman et al. (18) for cultured peripheral blood mononuclear leuko-

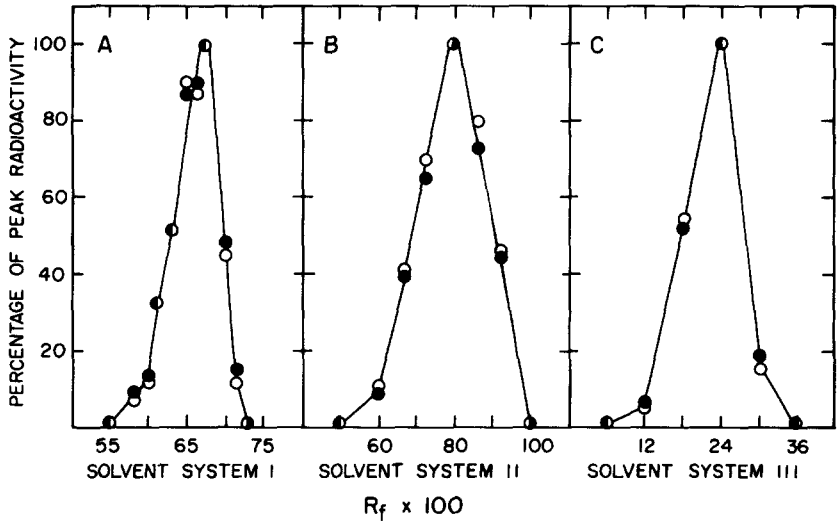

Fig. 4. Comigration of the ^{14}C-labeled reaction product with [^{3}H]mevalonic acid. IM-9 cell microsomal protein, 3.16 mg, was assayed for reductase activity in a 30-min incubation at 37°C in a final volume of 300 μl. Radioactive products migrating between R_f 0.5 to 1.0 on silica gel TLC sheets in solvent system I (toluene–acetone 1:1, A) were extracted from the silica gel with toluene–acetone 1:1. Radioactive products were then chromatographed on silical gel TLC sheets in solvent system II (n-butanol–propionic acid–water 10:4:1, B) or hydrolyzed with 1.0 M NH_4OH, and chromatographed on cellulose TLC sheets in solvent system II (n-butanol–NH_4OH–water 20:1:1, C). Following chromatography, TLC sheets were cut into 1-cm sections and each section was counted for [^{14}C]HMG-CoA reaction products and [^{3}H]mevalonate standard. Shown are the [^{14}C] (●) and [^{3}H] (O) cpm normalized to the peak radioactivity.

TABLE 3. Conversion of [^{3}H]mevalonate and the ^{14}C-labeled reaction product to phosphomevalonates[a]

Compound	^{3}H-Labeled Products[b]		^{14}C-Labeled Products	
	cpm	*% of total*	*cpm*	*% of total*
Before incubation:				
Mevalonate	3,155	100	2,861	100
Phosphomevalonates	0	0	0	0
After incubation:				
Mevalonate	167	5	323	11
Phosphomevalonates[c]	2,988	95	2,538	89

[a] In a final volume of 200 μl of TEDK were mixed the remainder of the hydrolyzed extract described in Fig. 3 containing 6,310 cpm R,S-[^{3}H]mevalonate standard (3,155 cpm R-[^{3}H]mevalonate) and 2,861 cpm [^{14}C]HMG-CoA reaction products, 1.2 mg of rat liver mevalonate kinase $(NH_4)_2SO_4$ fraction, 10 μl of 150 mM ATP, and 10 μl of 100 mM $MgCl_2$. The mixture was incubated for 60 min at 37°C. Following incubation, the reaction was terminated by immersion in a 100°C water bath for 10 min. After centrifuging to remove denatured protein, the entire sample was applied to a 3 × 20 cm channel of a cellulose thin-layer chromatogram, dried, and chromatographed in butanol–formic acid–water 77:10:13, as previously described (23). Following chromatography, the TLC sheet was dried, cut into 0.5-cm sections, and counted for radioactivity. R_f values for mevalonate, phosphomevalonate, and pyrophosphomevalonate were 0.89, 0.51, and 0.23, respectively.

[b] Data are for the active (R) isomer only.

[c] Data are the sum of both the phosphomevalonate and pyrophosphomevalonate produced from mevalonate during incubation. Twenty-four percent and 76% of the [^{3}H]phosphomevalonate produced, and 27% and 73% of the [^{14}C]phosphomevalonates formed were present as phosphomevalonate and pyrophosphomevalonate, respectively.

cytes obtained from healthy donors. Compared to rat liver, of course, leukocyte cholesterol synthesis is very low. Based upon our previous data (38), rat liver incorporates approximately 1,330 pmol of ^3H_2O into digitonin-precipitable sterols/min per mg microsomal protein, compared to only 44.1 pmol ^3H_2O/min per mg by IM-9 cells.

TABLE 4. HMG-CoA reductase activity and cholesterol synthesis as a function of mevalonate equivalents formed by cultured IM-9 cells[a]

Index	Rate of Mevalonate Formation
	pmol mevalonate equivalents/min per mg
Reductase activity (A)	57.3
[^{3}H]Cholesterol synthesis (B)	26.5
A/B	2.16

[a] IM-9 cells from late log phase cultures (3×10^7 cells) were incubated in a final volume of 0.4 ml containing 0.2 ml of original culture medium and 0.1 ml of ^3H_2O (sp act 100 Ci/g) for 2 hr at 37°C. Following incubation, cells were either homogenized in the presence of 50 mM NaF (TEDF) and assayed for reductase activity, or were treated as described in Methods for determining incorporation of radioactivity into digitonin-precipitable sterols. The presence of NaF during homogenization and assay prevents activation (dephosphorylation) of reductase by inhibiting the action of reductase phosphatase (4, 29). Thus, the data presented reflect the activity of only that portion of reductase capable of forming mevalonate during the 2 hr in which cholesterol was being formed.

Pharmacologic regulation of leukocyte microsomal reductase activity by dichloroacetate and glyoxylate

The response of leukocyte microsomal reductase to dichloroacetate (DCA) and glyoxylate, noncompetitive inhibitors of the rat liver enzyme (24), was also examined. As shown in **Table 5,** both drugs inhibited IM-9 cell and freshly isolated mononuclear leukocyte reductase in a dose-dependent manner. Glyoxylate was a more potent inhibitor than DCA for both cell types. A concentration of 2 mM glyoxylate inhibited freshly isolated leukocyte reductase approximately 47% and IM-9 cell reductase about 36%. The inhibitory effect of glyoxylate was seen with both fresh and frozen microsomes and was present even if glyoxylate was added without preincubation. In contrast, a modest inhibitory effect of DCA on reductase was noted only when fresh microsomes were preincubated with the drug.

Measurement of microsomal reductase activity in enriched populations of granulocytes, lymphocytes, and monocytes

Table 6 summarizes the extent of homogeneity, cell yield, and microsomal reductase activity in enriched cell populations obtained from healthy donors. The granulocyte-enriched population was 95% homogeneous (based on Wright's staining), with the remaining 5% being lymphocytes. Granulocyte yield was 55% and viability exceeded 99% (based on Trypan Blue dye exclusion). The monocyte-enriched fraction averaged 71% homo-

TABLE 5. Inhibition of human leukocyte HMG-CoA reductase activity by DCA and glyoxylate[a]

Drug Added	Drug Concentration	Mononuclear Leukocytes		IM-9 Cells	
		Reductase Activity	Inhibition	Reductase Activity	Inhibition
	mM	*pmol/min per mg*	*%*	*pmol/min per mg*	*%*
None		1.66[b]		262	
DCA	1.0	1.48	11	258	2
DCA	2.0	1.40	16	255	3
DCA	10	1.13	32	230	12
Glyoxylate	1.0	1.08	35	227	13
Glyoxylate	2.0	0.88	47	168	36
Glyoxylate	10	0.20	88	80	69

[a] Freshly isolated human leukocyte microsomes (200 μg of microsomal protein isolated by centrifugation at 105,000 *g* for 90 min; initial sp act 3.95 pmol/min per mg) or IM-9 cell microsomes (158 μg microsomal protein; initial sp act 262 pmol/min per mg) were incubated (20 min, 37°C) with DCA or glyoxylate in a final volume of 50 μl. Following incubation, reductase activity was measured, as described in Methods.

[b] Mononuclear leukocyte reductase activity decreased from initial sp act of 3.95 pmol/min per mg to 1.66 pmol/min per mg during the 20-min preincubation at 37°C. This decrease occurred in controls as well as drug-treated microsomes. Similar activity loss was not observed for IM-9 reductase activity.

geneity (based on α-naphthylacetate esterase staining), with the remaining cells being large lymphocytes. Monocytes were recovered in 75% overall yield, with viability exceeding 99%. The lymphocyte-enriched fraction was 96% homogeneous, with remaining cells being dead monocytes. Overall yield was 91% and viability was 95%. Dead monocytes accounted for most, if not all, nonviable cells. Microsomal reductase activities for both lymphocyte (9.60 ± 0.68 pmol/min per mg) and granulocyte (8.82 ± 0.48 pmol/min per mg) populations were similar. Mean enzyme activity for the monocyte-enriched fraction was 15.2 ± 0.9 pmol/min per mg. When calculated on the basis of a 100% pure monocyte population, reductase activity (17.6 pmol/min per mg) was approximately twice that of either lymphocytes or granulocytes.

DISCUSSION

Measurement of human leukocyte HMG-CoA reductase activity in whole cell homogenates has been complicated by the presence of enzyme activities that produce cleavage products from HMG-CoA (22). These cleavage

TABLE 6. Microsomal HMG-CoA reductase activity in enriched populations of granulocytes, lymphocytes, and monocytes[a]

Enriched Cell Fraction	Cell Population			Yield of Major Cell Type[b]	Reductase Activity
	Monocytes	Lymphocytes	Granulocytes		
	%				*pmol/min per mg*
Granulocyte	0.7 ± 0.3	4.0 ± 0.9	95.3 ± 1.2	52.7 ± 1.7	8.82 ± 0.48[c]
Lymphocyte	3.1 ± 0.8	95.8 ± 0.4	1.1 ± 0.5	91.0 ± 10.7	9.60 ± 0.68[d]
Monocyte	71.4 ± 4.8	27.8 ± 5.3	0.8 ± 0.8	74.7 ± 12.6	15.2 ± 0.9[d]

[a] Lymphocyte- monocyte- and granulocyte-enriched leukocyte populations were prepared and homogenized as described in Methods. Microsomal reductase of each enriched cell population was isolated and assayed in triplicate. Leukocyte differential counts were conducted using Wright's stain (granulocytes) and α-naphthylacetate esterase stain (lymphocytes and monocytes).

[b] Data are presented as mean ± SE for three granulocyte-, five monocyte-, and five lymphocyte-enriched fractions isolated from blood samples obtained from five healthy donors.

[c] Data are presented as mean ± SE for triplicate determinations of granulocyte reductase activity for each of five granulocyte-enriched fractions obtained from healthy donors.

[d] Data are presented as mean ± SE for triplicate determinations of lymphocyte or monocyte reductase activity for each of five enriched fractions obtained from healthy donors.

enzymes form products that migrate with or near authentic mevalonate during chromatographic isolation and hinder accurate measurement of newly formed mevalonate (22).

Measurement of reductase activity from isolated leukocyte microsomes, however, limits production of nonmevalonate products from HMG-CoA and thus permits more accurate assessment of leukocyte reductase activity. In the presence of HMG-CoA and leukocyte microsomes, no formation of HMG-CoA cleavage products could be detected.[3] This suggests that cleavage enzymes are absent from our microsomal preparations.

In the presence of HMG-CoA and NADPH, leukocyte microsomes formed a product that comigrated in a 1:1 relationship with authentic [^{3}H]mevalonate in three different TLC systems and was converted to phosphomevalonates in the presence of MgATP and mevalonate kinase. We conclude that this reaction product is mevalonate, the product of the HMG-CoA reductase-catalyzed reaction. Since no other ^{14}C-labeled reaction products were detected between R_f 0.5–1.0 in the silica gel–toluene–acetone TLC system, counting the ^{14}C in this region of the TLC plate is an effective measure of microsomal reductase activity. Reductase activity measured by this method is 14.6-fold greater than reductase activity measured in cell-free supernatants and, hence, more accurately reflects levels of reductase activity present in leukocytes. As noted by Young and Berger (22), there are difficulties associated with isolation and manipulation of leukocyte microsomal fractions from less than 10 ml of whole blood. Microsomal sedimentation is incomplete when centrifugation is conducted for 90 min at 105,000 *g* and microsomal pellets are small. We found that a visible, easily manipulated microsomal pellet, containing protein sufficient for duplicate analyses, may be obtained from the mononuclear leukocytes of 40 ml of whole blood, when microsomes are isolated by centrifugation at 172,000 *g* for 90 min.

With leukocyte microsomes as a source of reductase activity, mevalonate formation was linear with respect to both protein concentration and incubation time. Using these assay conditions, the rate of mevalonate formation by IM-9 cells was approximately twice the rate of mevalonate incorporation into digitonin-precipitable sterols. That only 50% of the newly formed mevalonate is converted to cholesterol suggests that, in contrast to liver (38), a substantial portion of mevalonate synthesized by leukocytes may be directed towards nonsterol products, such as dolichol and ubiquinone. This might be anticipated, since leukocytes have a readily available supply of cholesterol in the form of circulating plasma lipoproteins and, in contrast to liver and steroidogenic tissues, do not synthesize products such as bile acids and steroid hormones.

Granulocytes are unable to synthesize sterols from either acetate or mevalonate (39), but can form squalene and farnesol (40). Lymphocytes and monocytes, on the other hand, can synthesize cholesterol from acetate (39, 40). The inability of granulocytes to produce cholesterol from acetate is reported to be due to their lack of squalene epoxidase (40), the enzyme required for conversion of squalene to lanosterol. Our finding that lymphocyte and granulocyte reductase activities are similar is consistent with the observations that defects in granulocyte cholesterol synthetic capacity are independent of reductase activity (39, 40).

Monocyte reductase activity was approximately twice that of either lymphocytes or granulocytes. These findings are consistent with those of Fogelman et al. (39) for incorporation of [^{14}C]acetate into squalene and cholesterol for these cell types. While measurement of microsomal reductase activity in enriched populations of lymphocytes, monocytes, and granulocytes is clearly possible, the isolation procedures are time consuming and require large amounts of blood (e.g., 240 ml for triplicate analyses of each cell population). In contrast, duplicate measurement of microsomal reductase activity from a mixed population of peripheral blood mononuclear leukocytes may be conducted using 40 ml of blood, with assurances that intraassay variation in the lymphocyte:monocyte ratio is very low.

These studies also reveal that the activity of microsomal reductase derived from actively dividing, cultured IM-9 cells is approximately 34-fold greater than the activity measured in nonproliferating, freshly isolated leukocytes. Cohen, Massoglia, and Gospodarowicz (41) observed a 50-fold higher reductase activity in actively growing, bovine aortic endothelial cells, compared to confluent epidothelial cell cultures. Chen (42) also noted an inverse relationship between reductase activity and cell density, using Chinese hamster ovary and lung cells and mouse L-cells. Thus, the activity of reductase in human leukocytes and other mammalian cells appears to be closely associated with the proliferative state of the cells. The increment in reductase activity seen during cell growth may be due to an increase in enzyme synthesis, a decrease in enzyme degradation, or both. Further experiments are necessary to distinguish among these possible mechanisms.

The activity of microsomal reductase from freshly isolated human leukocytes is considerably less than the

[3] Using [3-^{14}C]HMG-CoA of specific activity 10 cpm/pmol, no counts above background corresponding to HMG-CoA cleavage products were observed (Fig. 3B). The error in the background of 20 cpm, however, was ±16%. Two σ above background, therefore, would be 6.4 cpm or 0.64 pmol. This represents <0.7% of the amount of mevalonate formed in 30 min at 37°C from 186 μg of microsomal reductase.

activity of rat liver microsomal reductase (about 500 pmol/min per mg; ref. 4) and more closely approximates the activity of human liver microsomal reductase (about 40 pmol/min per mg; refs. 43–45 and H. J. Harwood and P. W. Stacpoole, unpublished results). The activity of microsomal reductase from freshly isolated human leukocytes, however, is only one-fourth the activity of the human liver enzyme.

Finally, human leukocyte reductase is subject to pharmacologic regulation by DCA and its active metabolite, glyoxylate, both of which noncompetitively inhibit rat liver reductase (24). These findings provide further evidence that one mechanism of the cholesterol-lowering effect of DCA observed in patients with phenotypes IIa or IIb hyperlipoproteinemia (46, 47) may be through inhibition of HMG-CoA reductase activity.

Since human leukocyte reductase may be inactivated by agents known to inhibit hepatic cholesterolgenesis and reduce circulating cholesterol levels in vivo, measurement of leukocyte microsomal reductase activity may be useful as an indicator of changes in the rate of whole body cholesterol synthesis in man. Further studies exploring this possibility are in progress.

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