

Regulation of hydroxymethylglutaryl-CoA reductase in rat leukocytes

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Abstract Methods were developed for the assay of hydroxymethylglutaryl-CoA reductase (NADPH) activity in microsomes from rat leukocytes. The activity in freshly isolated leukocytes is low compared to rat liver but can be assayed reliably. The patterns of response of leukocyte reductase in the assay to variation in substrate concentration, protein concentration, and time mimic those of rat liver reductase. Reductase activity in leukocyte microsomes, as in liver microsomes, is depressed by dietary cholesterol and by fasting and is elevated by dietary cholestyramine. Unlike liver reductase, leukocyte reductase activity does not exhibit a detectable diurnal rhythm. We conclude that the assay of reductase in freshly isolated leukocytes holds promise as a technique for detecting the effects of various factors on cholesterol synthesis *in vivo*.

Supplementary key words cholesterol biosynthesis · dietary cholesterol · cholestyramine · diurnal rhythm · enzyme regulation · atherogenesis · atherosclerosis · heart disease

Techniques for studying *in vivo* regulation of cholesterologenesis in humans include sterol balance, turnover measurements after injection of isotopically labeled cholesterol or its precursors (1), and assay of liver biopsy tissue (2). While fruitful, these methods either require steady-state metabolic conditions, and are time consuming and expensive, or are potentially hazardous. These limitations preclude sampling large populations and repetitive sampling of individuals. Safe, quick, and inexpensive techniques for studying the regulation of human cholesterol synthesis *in vivo* by physiological processes and the effect of diet or drugs are clearly needed.

Assay of HMG-CoA reductase (hydroxymethylglutaryl-CoA reductase (NADPH), EC 1.1.1.34) in freshly isolated leukocytes may fulfill these criteria. In rat liver, the rate of cholesterol synthesis usually is directly proportional to the activity of HMG-CoA reductase (3,4). In human leukocytes *in vitro*, reductase activity and cholesterologenesis also vary in parallel, and both are subject to negative feedback regulation by cholesterol in the medium (5–8). Cholesterol synthesis in freshly isolated splenic lymphocytes of mice is reduced by dietary cholesterol (9).

As a preliminary test of the utility of leukocyte reductase for monitoring *in vivo* changes in the rate of cholesterol synthesis in humans, we investigated the effect on reductase in freshly isolated leukocytes of several physiological factors known to affect rat liver reductase.

EXPERIMENTAL PROCEDURES

Materials

Materials from commercial sources included NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase, EC 1.1.1.49, from Sigma Chemical Co., St. Louis, MO; CoA from PL Laboratories, Milwaukee, WI; Dextran T500 and Ficoll 400 from Pharmacia Fine Chemicals, Inc., Piscataway, NJ; Hypaque-M from Winthrop Laboratories, New York, NY; fluorescamine from Roche Diagnostics, Nutley, NJ; *N,N'*-dicyclohexylcarbodiimide (ultra pure) from Schwarz/Mann, Orangeburg, NY; [5-³H]-mevalonate (dibenzylethylenediammonium salt), [3-¹⁴C]HMG, and [3-¹⁴C]HMG-CoA from New England Nuclear Corp., Boston, MA; 2-(4'-*t*-butylphenyl)-5-(4''-biphenyl)-1,3,4-oxadiazole (butyl-PBD) from Research Products International Corp., Elk Grove Village, IL; 3-methoxyethanol from Pierce Chemical Co., Rockford, IL; 6-ml scintillation vials from Brockway Glass Co., Indianapolis, IN; cholesterol standard and cholesterol assay reagents from Boehringer Mannheim, Indianapolis, IN; hemoglobin assay reagents and cyanmethemoglobin standard from Hycel, Inc., Houston, TX; antibiotic Tylan from Elanco, Indianapolis, IN; silica gel and cellulose TLC sheets from Eastman Organic Chemicals, Rochester, NY; 2.0-ml screw cap Nunc serum tubes

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Abbreviations: HMG, 3-hydroxy-3-methylglutaric acid; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; SEM, standard error of the mean; TLC, thin-layer chromatography; butyl-PBD, 2-(4'-*t*-butylphenyl)-5-(4''-biphenyl)-1,3,4-oxadiazole; *R_f*, mobility relative to the solvent front; sp act, specific activity.

from Polysciences, Inc., Warrington, PA; 0.4- and 1.5-ml polyethylene centrifuge tubes from Walter Sarstedt Inc., Princeton, NJ; and the Potter-Elvehjem tissue grinder, size 22 and Kel-F pestle from Kontes Glass Co., Vineland, NJ. Kryo-EOB, a nonionic detergent with membrane solubilizing properties (10) was a gift from Dr. D. H. Hughes of Procter and Gamble Co., Cincinnati, OH.

R,S-[3-¹⁴C]HMG-CoA, sp act 22.8 Ci/mol, synthesized and purified by paper chromatography as subsequently described (11), was a gift from J. Nordstrom.

Buffered solutions

Buffer I contained 250 mM NaCl, 5.0 mM Na₂-EDTA, 5.0 mM dithiothreitol, and 50 mM K₂HPO₄ at pH 7.5. Buffer II contained 0.5% Kryo-EOB (v/v) in Buffer I.

Animals

Wistar strain rats from our department colony, weighing 300–400 g, were housed in a room with a fixed schedule of 12 hr of light and 12 hr of darkness. They were fed, ad libitum, a powdered chow diet consisting of 51% ground yellow corn, 16.4% soybean meal, 14.5% wheat middlings, 7.3% meat meal, 3.9% dried whey, 1.4% liver meal, 1.9% dried yeast, 0.7% alfalfa meal, 0.75% corn oil, 1.9% mineral mixture, and 0.48% Tylan (an antibiotic).

Isolation of leukocytes

A rat was anesthetized with ether and a blood sample of about 10 ml was withdrawn from the heart through a 0.5-in 21 gauge needle into a 12-ml polypropylene syringe containing 1.0 ml of 50 mM Na₂EDTA in 50 mM K₂HPO₄, pH 6.5, as anticoagulant. Subsequent steps were conducted at 0–5°C to minimize changes in HMG-CoA reductase activity. To facilitate sedimentation of erythrocytes, the blood sample was added to a 50-ml polycarbonate centrifuge tube containing 10 ml of 3% Dextran T500 in 0.9% NaCl. The erythrocytes were allowed to clump and settle for 30–60 min and were then sedimented further by centrifugation² (25 g, 10 min, swinging bucket rotor). The supernatant liquid was removed by aspiration through polyethylene tubing and centrifuged (300 g, 30 min, swinging bucket rotor) to sediment the leukocytes. The pellet was resuspended by vortex mixing in 1.0 ml of Buffer II. The suspension was transferred to screw-cap serum vials and stored in liquid N₂.

² Relative centrifugal forces are those calculated for the middle of the centrifuge tube.

Isolation of leukocyte microsomes³

The frozen leukocyte suspension was incubated at 37°C until just thawed. Subsequent operations were at 0–5°C. The suspension was homogenized for 15 sec in a Potter-Elvehjem tissue grinder using three or four strokes of a Kel-F pestle driven at 4660 rpm. The homogenate was centrifuged (8,000 g, 10 min) and the supernatant liquid was decanted into a 2.0-ml centrifuge tube. The tube was filled with Buffer I, capped, inverted several times to mix, and centrifuged (113,000 g, 3 hr). The microsomal pellet was suspended in 250 μl of Buffer I by homogenization in the centrifuge tube for one min using a Teflon pestle driven at 7500 rpm.

Assay of HMG-CoA reductase activity in leukocyte microsomes

A 100 μl portion of microsomal suspension containing about 0.5 mg of protein was added to 50 μl of a substrate-cofactor solution in Buffer I to give final concentrations or amounts as follows: 20 μM [3-¹⁴C]HMG-CoA; 20 nM (40,000 dpm) [5-³H]mevalonic acid (6.7 Ci/mmol) as internal standard; 3.0 mM NADP, 30 mM glucose-6-phosphate, and 0.3 units of glucose-6-phosphate dehydrogenase as an NADPH generating system. The mixture was incubated at 37°C for 90 min in a 0.4-ml polyethylene centrifuge tube. The reaction was stopped by adding 25 μl of 6 N HCl containing 0.2% Kryo-EOB. The detergent facilitated subsequent application of samples to TLC sheets. After incubating for 15 min at 37°C or overnight at room temperature to lactonize the mevalonate, the sample was centrifuged (10,000 g, 2 min) to sediment protein. A 150 μl portion of the supernatant liquid was applied as a 2 cm diameter spot on a 20 × 20 cm sheet of activated silica gel scored in eight channels. After development with benzene-acetone 1:1 (v/v) (12), the mevalonolactone region (*R_f* 0.5–0.9) was removed and transferred to a 6-ml vial to which 4 ml of scintillation fluid was added. The scintillation fluid contained 0.7% butyl-PBD and 8% naphthalene (w/v) in 3-methoxyethanol-toluene 2:3 (v/v). Disintegrations per min (dpm) from ³H and ¹⁴C were determined with a scintillation spectrophotometer (Beckman LS-100 or Searle Mark III) using the external standard channel ratio method. The quantity of [¹⁴C]mevalonate produced was calculated from ¹⁴C dpm using ³H dpm to correct for variation in recovery of

³ Microsomes are operationally defined here as the material from a cell homogenate sedimenting during ultracentrifugation between 8,000 and 113,000 g. Leukocyte microsomes may or may not be structurally equivalent to liver microsomes.

product. Microsomes were assayed in duplicate. The background control consisted of 100 μl of Buffer I plus 50 μl of substrate-cofactor solution treated as above.

Rat liver HMG-CoA reductase

Rat liver microsomes were prepared and assayed for HMG-CoA reductase activity as previously described (13).

Assay of protein

Protein was assayed by the fluorescamine method (14) modified as follows. Duplicate 10- μl portions of a microsomal suspension were mixed with 100 μl of 1.0 N NaOH and incubated at 37°C for 30 min to dissolve protein. Water, 90 μl , 1.5 ml of 0.2 M Na_2BO_3 , pH 7.5, and 0.5 ml of fluorescamine reagent (0.03% in acetonitrile) were added with vigorous vortex mixing. After 30 min at room temperature, fluorescence at 475 nm (excitation was at 390 nm) was measured using an American Instrument Co. (Silver Spring, MD) model SPF 125 fluorometer. Bovine serum albumin standards, 10–100 μg , were processed concurrently. A microsomal suspension derived from the leukocytes recovered from 10 ml of blood usually contained protein, i.e., fluorescamine-reacting material, equivalent to about 1.25 mg of albumin. This corresponds to about 13% of the protein present in the leukocyte homogenate.

Assay of cholesterol

Total plasma cholesterol was measured by an enzymatic method (15) with a reagent kit from Boehringer-Mannheim. The end product was measured by its

absorbance at 410 nm. A specimen blank was used to correct for color due to plasma constituents. Each plasma sample was assayed in duplicate, and cholesterol standards were processed concurrently.

Assay of hemoglobin

Hemoglobin was measured as cyanmethemoglobin using a Hycel reagent kit and following the procedure described in the pamphlet supplied with the kit.

METHODOLOGICAL EXPERIMENTS

The following experiments delineate conditions for isolating leukocytes and their microsomes that give optimal HMG-CoA reductase activity, and demonstrate the validity of the reductase assay.

Fractionation of blood

Reductase activity was measured in fractions of whole blood obtained by differential centrifugation after treatment with dextran (Table 1). Erythrocytes sedimenting at 1 g were devoid of detectable HMG-CoA reductase activity, and platelet-rich fractions sedimenting above 235 g had moderate reductase activity. The fraction sedimenting between 41 and 235 g had the highest total as well as the highest specific activity of HMG-CoA reductase. While 39% of the protein in this fraction was hemoglobin, it was largely removed during isolation of the microsomal fraction (only 2% of the protein of an erythrocyte homogenate sedimented between 8,000 and 113,000 g). Furthermore, addition of erythrocyte "microsomes" had little or no effect on the reductase activity of leukocyte microsomes (data not shown). Therefore, for our purpose, residual erythrocyte contamination of this fraction was inconsequential.

Subsequently, we occasionally observed a layer of white cells on the 40 g pellet and a small 235 g pellet. Therefore, for routine isolation of the leukocyte fraction from blood, we reduced the force of the first centrifugation to 25 g and increased the force of the second centrifugation to 300 g. As determined by Coulter counting, the 300 g pellet contained 70–80% of the leukocytes and 0.3% of the erythrocytes initially present in blood.

We tested the possibility that the low HMG-CoA reductase specific activity observed in the mixed leukocyte fraction (25–300 g pellet) reflected a few cells with high activity diluted by many cells with little or no activity. Leukocytes were fractionated by centrifugation over a dense Ficoll-Hypaque solution (Table 2). Elements that remained in the upper phase (mostly platelets) had a low but measurable level of reductase activity reminiscent of the 501–1260 g

TABLE 1. Distribution of HMG-CoA reductase in blood fractions^a

RCF Used to Sediment Cells	Reductase Activity $\times 10^3$	
	Total	Specific
g	$\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{ml blood}^{-1}$	$\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$
1	not detected	not detected
2–40	7.4 ± 0.1	84 ± 2
41–235	18.9 ± 1.0	177 ± 12
236–500	9.0 ± 0.9	94 ± 9
501–1260	8.5 ± 0.7	82 ± 7

^a Pooled blood from 11 female rats was added to an equal volume of 3% dextran in 0.9% NaCl and maintained on ice for an hour to permit most of the erythrocytes to settle. The supernatant liquid was decanted and subjected to successive centrifugations, each for 20 min. Each pellet and a portion of the initial erythrocyte pellet (1 g) were then suspended in Buffer II, freeze-thawed, homogenized, assayed for protein and hemoglobin, and diluted with Buffer II to a protein concentration of 10 mg/ml. Microsomes were isolated from 2.0 ml of each diluted homogenate. They were resuspended in 600 μl of Buffer I, and assayed for protein and for HMG-CoA reductase activity. The data are mean values \pm SEM for triplicate assays.

TABLE 2. Distribution of HMG-CoA reductase activity in leukocytes fractionated by centrifugation over Ficoll-Hypaque

	Differential White Cell Counts ^c			Reductase Activity × 10 ^{4d}	
	Lymphocytes	Granulocytes	Monocytes	Total	Specific
	cells · 10 ⁻⁸ · ml blood ⁻¹			pmol · min ⁻¹ · ml blood ⁻¹	pmol · min ⁻¹ · mg protein
Mixed leukocytes ^a	279	181	29	34 ± 4	274 ± 32
Fractions after centrifugation over Ficoll-Hypaque ^b					
Upper	3	3	1	3.5 ± 0.1	42 ± 2
Interface	248	22	0	20 ± 1	382 ± 16
Lower	20	46	0	<0.03	<2
Pellet	2	74	0	<0.03	<2
Sum	273	145	1	24 ± 1	

^a Prepared as described in Experimental Procedures.

^b Each of six 17-ml portions of the supernatant liquid obtained after centrifuging a blood-dextran mixture (see Experimental Procedures) was layered over 10 ml of the solution of 6.35% (w/v) Ficoll, 10% (w/v) Hypaque in a 50-ml polycarbonate tube and centrifuged (600 g, 10 min, swinging bucket rotor). The upper (15 ml), interface (5 ml), and lower (7 ml) fractions were successively collected with a Pasteur pipette, divided into two portions (one for cell counts and one for reductase assays) and recentrifuged (1000 g, 30 min, swinging bucket rotor). All operations were at 0–5°C.

^c Cell pellets were resuspended in 0.9% NaCl. Total white cells were counted in a hemocytometer after lysing erythrocytes. For differential counts, cells were spread on a slide with a Cytocentrifuge and stained with Wright-Geimsa.

^d Cell pellets were treated as described in Experimental Procedures. Data are expressed as the mean ± SEM of duplicate determinations.

fraction (Table 1). No activity was detected in cells (97% granulocytes) that sedimented into the Ficoll-Hypaque solution. The fraction that sedimented to the Ficoll-Hypaque interface (92% lymphocytes) contained 83% of the reductase activity recovered after the separation procedure and was enriched for reductase specific activity. Therefore, it appears that, although there is some dilution of reductase specific activity in microsomes from the mixed leukocyte pellet by protein from cells with little or no activity, the activity observed comes from the most numerous cells, the lymphocytes. We chose to work with the mixed leukocyte fraction thereafter because of the higher recovery of reductase activity and because it was technically easier. However, isolation of a lymphocyte-rich fraction would have some advantages, especially for work with human cells. In human blood, lymphocytes are less numerous than in rat blood and so would be subject to greater dilution by granulocytes, which may have little reductase activity. Furthermore, the use of lymphocytes in place of mixed leukocytes would eliminate the ambiguity introduced by variation between individual blood samples in the proportion of lymphocytes in the white cell population.

Conditions for optimizing the yield of HMG-CoA reductase in leukocyte microsomes

Freeze-thawing and/or detergent treatment before homogenization of leukocytes decreased the amount

of material sedimenting at 8,000 g (data not shown) and increased the yield of protein and of reductase in microsomes (Table 3). The combined treatments increased the total reductase activity in microsomes to 15 times and specific reductase activity to 4 times that of controls.⁴

The optimal concentration of Kyro-EOB for homogenization of leukocytes was 0.5% (Fig. 1). At lower concentrations, cells were not broken as well, while at higher concentrations reductase activity tended to remain suspended in the 113,000 g supernatant fraction (data not shown).

Adding detergent to the assay is not advisable. Hepatic microsomes isolated in the absence of detergent and suspended in Buffer I with increasing Kyro-EOB concentrations showed a progressive decrease in reductase activity (Fig. 1).

Verification of the presence of HMG-CoA reductase in rat leukocyte microsomes

Microsomes from rat leukocytes convert [¹⁴C]-HMG-CoA to a ¹⁴C-labeled product that comigrates with [³H]mevalonate during chromatography in an alkaline TLC system or with [³H]mevalonolactone after treatment with acid and chromatography in

⁴ Under some circumstances, Kyro-EOB appears to make HMG-CoA reductase sensitive to inactivation by cold temperatures (A. M. Fogelman, personal communication). This does not occur under our conditions (Table 3).

TABLE 3. Effect of freezing and of detergent treatment of leukocytes on yield of microsomal HMG-CoA reductase^a

Treatment		Microsomal Protein $\mu\text{g} \cdot \text{ml blood}^{-1}$	Reductase Activity $\times 10^3$	
Kyro-EOB	Freeze-Thaw		Total $\text{pmol} \cdot \text{min}^{-1} \cdot \text{ml blood}^{-1}$	Specific $\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$
-	-	42	3.1 \pm 0.4	74 \pm 9
-	+	93	21.0 \pm 1.1	225 \pm 12
+	-	103	30.4 \pm 0.4	295 \pm 4
+	+	157	45.3 \pm 0.4	288 \pm 2

^a Leukocyte pellets isolated from the pooled blood of four female rats were suspended in Buffer I (no Kyro-EOB) or in Buffer II (0.5% Kyro-EOB), maintained on ice (Freeze-Thaw -), or frozen in liquid N₂ and then thawed immediately (Freeze-Thaw +). Microsomal pellets were then isolated, suspended in Buffer I or II, and assayed for protein and for HMG-CoA reductase activity. Reductase activity is expressed as mean values \pm SEM for duplicate determinations.

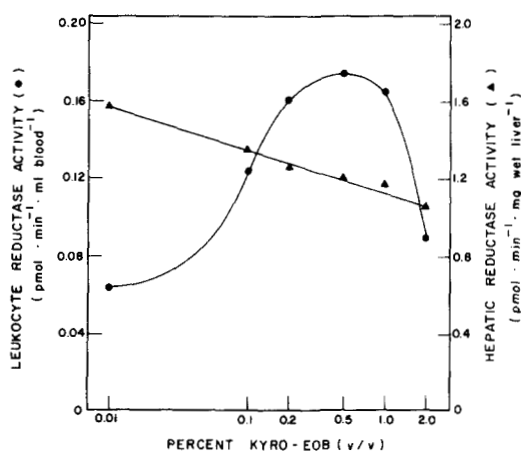


Fig. 1. Effect of Kyro-EOB of various concentrations on the fractionation of leukocyte HMG-CoA reductase and on the activity of hepatic microsomal reductase. Leukocyte pellets from pooled blood were suspended in Buffer I containing Kyro-EOB at the concentration indicated. Microsomes were prepared and assayed for HMG-CoA reductase activity without further addition of detergent (●). Microsomes isolated from rat liver in the absence of detergent were resuspended in Buffer I containing Kyro-EOB at the indicated concentration and assayed for reductase activity (▲). The concentration of Kyro-EOB given is, for leukocytes, that in the cell suspension, and for liver, that in the microsomal suspension. Data are mean values for duplicate assays.

neutral or acidic systems (Fig. 2). The formation of this product requires the presence of NADPH (<1% of the ¹⁴C cpm in this region occurred in the absence of the NADPH generating system). Therefore, we conclude that leukocyte microsomes contain HMG-CoA reductase.

Kinetics of mevalonate formation

Production of mevalonate by leukocyte microsomes increased with incubation time (T) and amount of microsomal protein (P) in the assay (Fig. 3, top). Although the response appears, on casual inspection,

to be nearly linear, it actually is curvilinear. This is more easily detected by examination of a plot of specific activity vs. T or P. If mevalonate production were linear, sp act would not change with P or T. However, sp act declines with P and with T (Fig. 3, bottom). This decline is apparent even at the smallest T or P tested, demonstrating that linearity of mevalon-

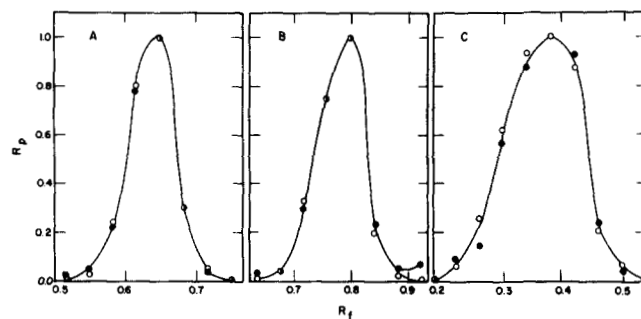


Fig. 2. Chromatography of the product formed from HMG-CoA by leukocyte microsomes. Leukocyte microsomes (600 μl) from female rats were incubated with substrate-cofactor solution for 90 min at 37°C, then with acid for 15 min at 37°C. After removal of precipitated material by centrifugation, the mixture was applied to six channels (each 2.5 \times 17.5 cm) of a silica gel TLC sheet and chromatographed in a sandwich chamber with the usual solvent [benzene-acetone 1:1 (v/v)]. One channel was sectioned at 0.5-cm intervals and the ¹⁴C and ³H in each section were counted (A). The region corresponding to R_f 0.5–0.75 was scraped from the remaining five channels, pooled, and eluted with acetone and methanol. Portions of the eluate were applied to 2.5 \times 17.5 cm channels of silica gel TLC sheets and chromatographed in a chamber equilibrated with an acidic solvent system [butanol-1-propionic acid-water 10:4:1 (v/v)] (B). In addition, a portion was treated with 1.0 N NH₄OH (15 min, 37°C) and then applied to a 2.5 \times 17.5 cm channel of a cellulose TLC sheet and chromatographed in a chamber with solvent-saturated vapor with an alkaline solvent [butanol-1-ammonia-water 20:1:1 (v/v)] (C). Each chromatogram was sectioned and counted as above. Data for the [³H]-mevalonate internal standard (○) and the ¹⁴C-labeled product (●) are expressed as the ratio of dpm in each fraction to dpm in the peak fraction (R_p) versus R_f. The peak fraction contained approximately 800 ¹⁴C dpm and 6000 ³H dpm in each case.

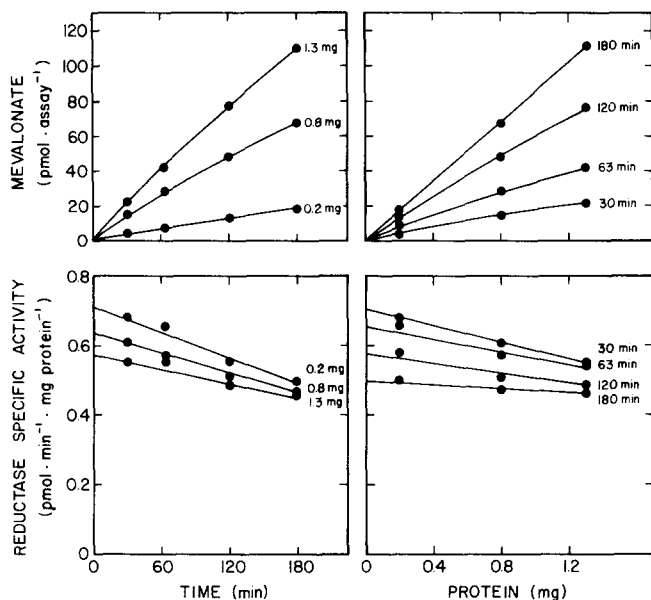


Fig. 3. Kinetics of mevalonate production as a function of incubation time and of microsomal protein concentration. Portions of a suspension of leukocyte microsomes containing the amount of protein indicated were incubated under standard conditions except that time was varied as shown. The data (means of duplicate determinations) are plotted in four different ways: mevalonate produced (top) or specific activity (bottom) vs. time (left) or vs. protein (right). The lines drawn for specific activity vs. time or protein (bottom) were determined by a fitted function (see Appendix).

ate production does not obtain even then. This poses a dilemma: reductase activity is usually defined as sp act, but sp act is not a constant for a given microsomal preparation since it will vary depending on the T and P used in the assay. However, if the relative rates of decline of sp act with T and P are the same for all microsomes, then measurements of sp act of different microsomes obtained for a given P and a given T may be compared. In two experiments where the response to P and T were studied, the value of sp act at a T of 90 min and a P of 0.5 mg was 78% (Fig. 3) and 73% (data not shown) of the value of initial sp act. (Initial sp act is defined in the Appendix.) Therefore, our routine procedure for estimating sp act was to measure the mevalonate produced by 0.5 mg of P in 90 min. We assume that this sp act is about 75% of initial sp act, but we have not corrected the data accordingly.

Substrate dose response of HMG-CoA reductase in leukocyte microsomes

The substrate dose response of HMG-CoA reductase in leukocyte microsomes shows typical saturation kinetics with a K_m for (R,S)-HMG-CoA of $7 \mu\text{M}$

(Fig. 4). Substrate inhibition was observed at $30 \mu\text{M}$ and above.

PHYSIOLOGICAL EXPERIMENTS

In order to compare the regulation of reductase activity in leukocytes with that in liver, we examined the response of leukocyte reductase to factors known to alter liver reductase. These included time of day, dietary cholesterol or cholestyramine, and fasting.

Regulation by fasting and by dietary cholestyramine

When female rats were fasted for 24 hr, mean HMG-CoA reductase activity in leukocyte microsomes declined to 45% of normal while mean plasma cholesterol level remained constant (Table 4). In rats fed 2% cholestyramine for 3 days, mean reductase activity rose to 155% of normal while mean plasma cholesterol declined to 88% of normal. Although the effect of cholestyramine feeding on cholesterol level is not statistically significant ($P < 0.3$), the changes in reductase activity are significant ($P < 0.01$).

Regulation by dietary cholesterol

When female rats were fed a diet containing 1% cholesterol for 3 days, mean HMG-CoA reductase activity in leukocytes declined exponentially with a half-time of about 1.8 days (Fig. 5). Plasma cholesterol levels were elevated on day 1, then declined to nearly normal by day 3.

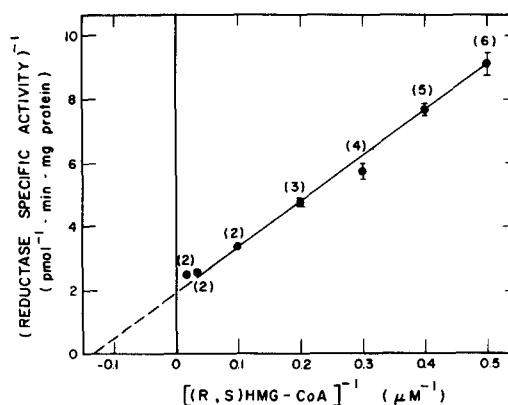


Fig. 4. Double-reciprocal plot of the substrate dose response of HMG-CoA reductase in leukocyte microsomes. Microsomal suspensions were incubated for 90 min under standard assay conditions except that the concentration of (R,S)-[^{14}C]HMG-CoA was varied as shown. The utilization of substrate for mevalonate production varied from 1.5% at $2 \mu\text{M}$ HMG-CoA to 0.2% at $60 \mu\text{M}$ HMG-CoA. Data shown are mean \pm SEM for the number of replicate assays given in parentheses.

TABLE 4. Variation of HMG-CoA reductase activity with cholestyramine feeding, fasting, and gender^a

Expt.	Gender	Diet	n	Body Weight	Plasma Cholesterol	Reductase Specific Activity	
						Liver	Leukocytes
				<i>g</i>	<i>mg · dl⁻¹</i>	<i>pmol · min⁻¹ · mg protein⁻¹</i>	
1	Female	2% Cholestyramine for 3 days	13	354 ± 4	73 ± 4	0.80 ± 0.05	
	Female	Normal	10	350 ± 5	83 ± 6	0.51 ± 0.04	
2	Female	Fasted 24 hr	11	324 ± 6	70 ± 5	0.24 ± 0.03	
	Female	Normal	11	354 ± 5	72 ± 7	0.52 ± 0.06	
3	Male	Normal	11	398 ± 16	48 ± 2	135 ± 45	1.33 ± 0.12
	Female	Normal	11	368 ± 10	59 ± 5	167 ± 38	0.61 ± 0.10

^a Diets were fed ad libitum. All rats were killed at the midpoint of the light phase. Data from duplicate experiments with a total of n rats were pooled and are presented as mean values ± SEM.

Comparison of males and females

Mean HMG-CoA reductase activity in leukocytes was higher in males than in females, although mean activity in liver was about the same in both sexes (Table 4). In this experiment, the male rats were somewhat younger and heavier and had lower plasma cholesterol levels than the females.

Absence of a diurnal rhythm

As a preliminary test, we assayed reductase activity in leukocytes from female rats killed at the midpoint of the dark and of the light phases. We observed no significant difference in mean activity at

these times (data not shown). We next measured activity in leukocyte microsomes and, as a control, in liver microsomes, at 4-hr intervals for 24 hr (Fig. 6). Although liver reductase activity varied over 6-fold from a minimum of 65 ± 12 at 1600 hours to a maximum of 413 ± 95 pmol · min⁻¹ · mg protein⁻¹ at 2400 hours, neither HMG-CoA reductase activity in leukocyte microsomes nor plasma cholesterol varied significantly during the 24-hr period. The mean reductase activity in leukocyte microsomes was 0.50 ± 0.07 pmol · min⁻¹ · mg protein⁻¹ and the mean cholesterol level was 55 ± 3 mg · dl⁻¹.

DISCUSSION

Methods for isolating microsomes and for assaying HMG-CoA reductase activity previously used for rat liver (13) were modified for application to rat leukocytes. Since leukocytes resist breaking, maximal yields of reductase required detergent treatment, freeze-thawing, and homogenization of cells. Because the specific activity of reductase in rat leukocytes is extremely low (10⁻²–10⁻³ that of rat liver), we modified the assay to increase the sensitivity about 500-fold. We used [¹⁴C]HMG-CoA with higher specific activity, more microsomal protein, and a longer incubation time, and we chromatographed a larger portion of the incubation mixture.

We studied leukocyte microsomes rather than fractions containing cytosol, such as whole cell homogenates (7) or postmitochondrial supernatant fractions (6, 8), for two reasons. First, the cytosol as we prepare it contains 0.5% Kyro-EOB, which inhibits

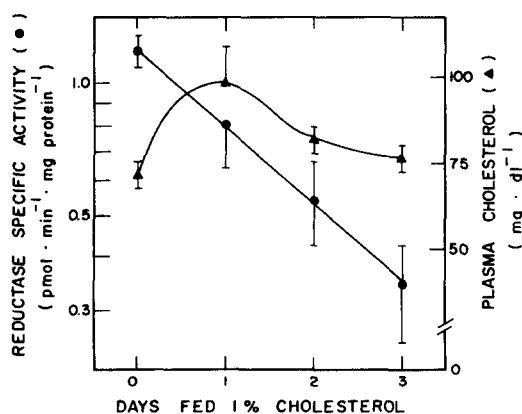


Fig. 5. Regulation of leukocyte HMG-CoA reductase by dietary cholesterol. Twelve female rats weighing 325 ± 5 g were fed a normal diet supplemented with 1% cholesterol for 0–3 days. Plasma cholesterol (▲) and leukocyte microsomal reductase activity (●) were assayed in all rats on the same day at the midpoint of the light phase. Data (mean ± SEM for three rats) for cholesterol are plotted on a linear scale and for reductase activity on a logarithmic scale.

liver reductase activity (Fig. 1). Second, we wished to compare the regulation of leukocyte reductase with that of liver reductase, and all available data for rat liver reductase were obtained using microsomes.

Despite its low activity, HMG-CoA reductase in rat leukocyte microsomes can be assayed reliably. The product formed comigrates with mevalonate in several TLC systems (Fig. 2). The response of leukocyte reductase in the assay to variation in substrate concentration, protein concentration, and time (Figs. 3 and 4) mimics that of rat liver reductase.⁵ The mean coefficient of variation in activity between similarly treated rats was the same in leukocytes and liver (53%). Thus the problem of inter-rat variation is no worse for leukocyte reductase than for liver reductase.

HMG-CoA reductase activity in leukocytes (Fig. 6) like that in brain, testis, lung, and spleen (16) does not vary diurnally. The diurnal reductase rhythm in liver and small intestine (17) may be linked to the rhythms in synthesis, secretion, and circulation of bile acids in which these tissues, but not peripheral tissues, are involved (18, 19).

The observation that leukocyte reductase activity is higher in males than in females (Table 4) is curious. However, the males were somewhat younger and heavier and had lower plasma cholesterol levels than the females, and we do not know how these variables affect leukocyte reductase activity. It therefore is possible that the difference is not due primarily to gender.

Leukocyte reductase activity varies in response to certain treatments that alter hepatic reductase activity. When rats are fed a diet containing 1% cholesterol, leukocyte reductase activity declines exponentially with a half-time of 1.8 days (Fig. 5). On a 5% cholesterol diet, hepatic reductase activity declines exponentially with a half-time of 2.5 hr (20). When rats are fed 2% cholestyramine, leukocyte reductase activity increases to 155% of normal at 3 days (Table 4), and hepatic reductase activity increases to 344% of normal at 2 days (21) and to 530% at 4 days (22). When rats are fasted, leukocyte reductase activity declines to 45% of normal by 24 hr (Table 4), while hepatic reductase activity declines to 8% by 36 hr (20). Thus the changes in reductase activity in leukocytes are less extensive than in liver, but they are in the same direction.

The factors that alter reductase activity in leukocytes *in vivo* have not been identified. While chole-

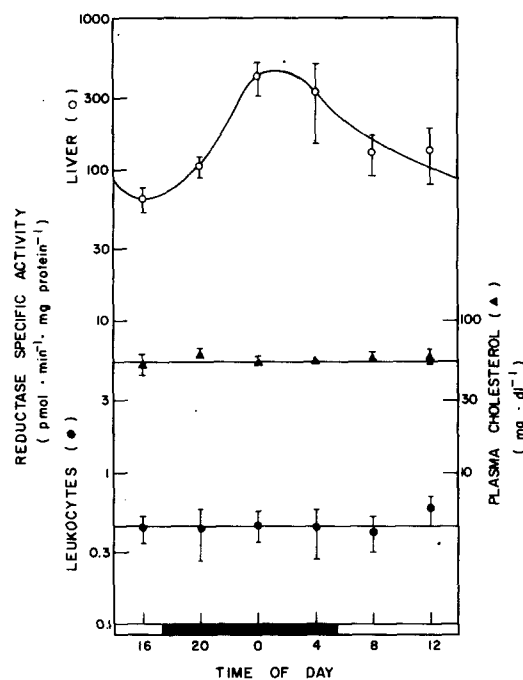


Fig. 6. Activity of liver and of leukocyte HMG-CoA reductase at various times of day. Eighteen female rats weighing 334 ± 10 g were kept on a light-dark schedule as indicated by the horizontal bar on the abscissa. At the time of day indicated, microsomal reductase activity in liver (○) and in leukocytes (●) and plasma cholesterol (▲) were assayed in each of three rats. Data for the mean \pm SEM are plotted on a logarithmic scale.

sterol is an effective feedback inhibitor of cholesterol synthesis in leukocytes *in vitro* (5–8), changes in reductase activity in leukocytes *in vivo* occur under conditions where plasma cholesterol level changes slightly if at all (Table 4). Thus, either leukocyte reductase is sensitive to very small changes in plasma cholesterol or other factors are operating. Such factors might include phospholipids, hydroxylated derivatives of cholesterol and bile acids, and the apportioning of cholesterol to the various density classes of lipoproteins. Some factors may act by affecting the rate of entry of cholesterol into leukocytes.

The study of leukocytes *in vitro* has already proved useful in defining genetic abnormalities of lipid metabolism in humans (5–8, 23) and in pigeons (24). Now we ask whether freshly isolated, unincubated, uncultured leukocytes are likely to be useful for monitoring *in vivo* changes in rates of cholesterol synthesis in humans. Probably they will not be useful for detecting a diurnal rhythm. However, lack of a diurnal rhythm, if such is the case in human leukocyte cholesterol synthesis, facilitates experimentation since blood samples could be obtained at any convenient time of day. Leukocyte reductase may well be useful for detecting *in vivo* changes in response to

⁵ N. Young, unpublished observation.

drugs or diet. The response in leukocytes is likely to be more subtle than in liver. However, unlike rats, whose leukocyte reductase cannot be repetitively sampled, a person can serve as her own control for effects of various treatments. Assuming that the variation in leukocyte reductase activity in one individual over time is less than between individuals, this should make the detection of changes in humans easier than in rats. Ultimately, the advantage of more dramatic effects in reductase in liver must be weighed against the advantage of accessibility of reductase in leukocytes. We are proceeding on the assumption that study of reductase activity in freshly isolated human leukocytes will be useful, and are in the process of modifying the assay for application to human cells. ■■

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APPENDIX

Estimation of initial specific reductase activity

Inspection of the data for specific activity vs. time (T) and microsomal protein (P) in the reductase assay (Fig. 3, bottom) indicated that a function of the form:

$$\text{sp act} = \text{sp act}_0 - m_1T - m_2P + m_3PT$$

would fit the data. This function describes a surface in three dimensions whose intersection at zero T and zero P is initial specific activity (sp act₀). A least squares best fit to the data provided values ± SE for the fitted constants:

$$\text{sp act}_0 = 0.74 \pm 0.02 \text{ (2.4\%)} \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$$

$$m_1 = 0.0014 \pm 0.0002 \text{ (11\%)} \text{ pmol} \cdot \text{min}^{-2} \cdot \text{mg}^{-1}$$

$$m_2 = 0.13 \pm 0.02 \text{ (15\%)} \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-2}$$

$$m_3 = 0.00055 \pm 0.00018 \text{ (32\%)} \text{ pmol} \cdot \text{min}^{-2} \cdot \text{mg}^{-2}.$$

The coefficient of variability for the fit was 3.5%. This function gave a significantly better fit than a planar function (sp act = sp act₀ - m₄T - m₅P).