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MICRO ASSAY FOR 3-HYDROXY-3-METHYLGLUTARYL-CoA REDUCTASE IN RAT LIVER AND IN L-CELL FIBROBLASTS

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SUMMARY

A rapid, highly reproducible, micro-incubation, direct thin-layer chromatographic assay for 3-hydroxy-3-methylglutaryl-CoA reductase is described The isolation and quantitation of mevalonic acid is simplified by direct application of the deproteinized, acidified incubation mixture to thin-layer chromatography sheets The tedious and variable extraction of mevalonic acid into ether is eliminated by this procedure Background levels are lower than those obtained with comparable assays and permit quantitation of as little as 30 pmoles of mevalonic acid

The method is illustrated by analysis of 3-hydroxy-3-methylglutaryl CoA reductase from rat liver, cultured L cells, and solubilized microsomal reductase The rapid inactivation of reductase by MgATP and the low levels of reductase activity in cholesterol-treated L cells are accurately determined by using the micro-incubation, direct thin-layer chromatography method

INTRODUCTION

3-Hydroxy-3-methylglutaryl CoA reductase (Mevalonate NADP$^+$ oxidoreductase (CoA-acylating), EC 1 1 1 34), is the major regulatory site in hepatic sterol synthesis [1] Regulation appears to involve changes in the rate of reductase synthesis [1–3], modulation of catalytic activity [2, 4–6] and the action of hormones [7–9] Defective regulation of 3-hydroxy-3-methylglutaryl CoA reductase has been demonstrated in hepatic tumor cells [10, 11] and has been implicated in familial hypercholesterolemia [12]

Published methods for analysis of reductase activity include column chromatography [13, 14], gas–liquid chromatography [15–19], electrophoresis [20], thin-layer chromatography [21–26], and colorimetric analysis [27] The most widely used method [22] and its modifications [23, 24, 26] generally involve extraction of radioactive mevalonolactone from the incubation mixture into ether [28], separation from unreacted substrate by thin-layer chromatography and radioactivity counting Since extraction into ether is not only time-consuming, but also incomplete, internal standards [23], and parallel controls [24], are employed to correct raw data for incomplete recovery of mevalonic acid

We describe an improved procedure for the assay of 3-hydroxy-3-methylglutaryl CoA reductase The incubation volume is 150 μl, and portions of the deproteinized acidified aqueous incubation mixture are applied directly to thin-layer chromatography sheets This eliminates ether extraction, and raises recovery of mevalonic acid to above 95% Since, as much as 90% of the deproteinized reaction mixture may be used for thin layer chromatography, sensitivity is correspondingly improved

Advantages of this method include a lower background, increased sensitivity and precision, lower cost, and a marked decrease in the time required for each assay The assay procedure readily measures the low 3-hydroxy-3-methylglutaryl-CoA reductase activities present in livers of cholesterol-fed rats, cholesterol-treated L-cells, and rat liver microsomes treated with MgATP in vitro The high reductase activities of microsomal and solubilized preparations from rats killed at midnight also are readily measured

METHODS

Chemicals were from previously listed sources [3] DL-Hydroxymethyl-[3-^{14}C]-glutarate was converted to DL-hydroxymethyl-[3-^{14}C]glutaric acid anhydride as described by Goldfard and Pitot [23] and to DL-hydroxymethyl-[3-^{14}C]glutaryl CoA by the method of Hiltz et al [30] Active microsomal preparations [3] from the livers of 150–250 g female Wistar rats may be prepared in a variety of buffers [1] We initially used 30 mM EDTA–70 mM NaCl–1 0 mM dithiothreitol, pH 7 2–7 4 Later, we adopted 30 mM EDTA–250 mM NaCl–1 0 mM dithiothreitol–50 mM potassium phosphate, pH 7 4, to provide improved buffering and to exploit the enhanced activity observed at higher salt concentrations [28] Each of these buffers may also be used as incubation buffer for the assay of reductase activity Soluble 3-hydroxy-3-methylglutaryl-CoA reductase was prepared from rat liver microsomes using 4 M KCl essentially as described by Brown et al [26] but extracting for only 2 h Mouse fibroblast L-cells, L-929 DC, a gift from Drs M Moscowitz and D Casciano, were maintained as monolayers in Eagle's minimal essential medium (GIBCO) supplemented with 0 35 g of $NaHCO_3$, 2 5 g of Bactopeptone (Difco), 5 10^4 units of penicillin and 5 10^4 μl of dihydrostreptomycin per l Three days prior to use, cells were transferred to suspension culture in minimum essential medium with spinner salts containing Bactopeptone and antibiotics as above plus 2 2 g of $NaHCO_3$, 5 0 g of bovine serum albumin fraction V (GBI) and 0 5 g of Darvan 2 (R T Vanderbilt Co) per l Cells were collected by centrifugation, washed twice in 0 1% glucose in minimum essential medium spinner salts solution, suspended in incubation buffer, ruptured with ultra sonic energy, and centrifuged for 10 min at 12 000 $\times$ g The supernatant liquid was centrifuged for 1 h at 48 000 $\times$ g and the microsomal pellet resuspended for assay in 1 0 ml of buffer per 10^8 original cells

Assay procedure

Incubations are performed in 400-μl capacity Beckman Microfuge tubes in a total volume of 150 μl Microsomes, 5–100 μl, which have been resuspended in buffer by gentle homogenization, are mixed at 4 °C with sufficient buffer to bring the volume in the tubes to 100 μl After a 5-min preincubation at 37 °C [28], 50 μl of cofactor–substrate solution 4 5 μmoles of glucose 6-phosphate, 0 3 I U of glucose-

6-phosphate dehydrogenase, 450 nmoles of $NADP^+$ and 50 nmoles of DL-hydroxymethyl-[3-^{14}C]glutaryl CoA (specific activity 3000 or 11 300 dpm/nmole) and 30 000 dpm of [5-^{3}H]mevalonic acid (specific activity 19 300 dpm/pmole) as an internal standard, all in incubation buffer) is added After 15 min at 37 °C, incubations are terminated by adding 25 μl of 10 M HCl Samples are incubated at 37 °C for at least 30 min to permit mevalonic acid to lactonize and then centrifuged for 1 min in a Beckman 152 Microfuge to sediment denatured protein Portions generally 100 μl of the protein-free supernatant solution are then applied to activated silica gel thin-layer chromatography sheets (Eastman Chromagram) ruled vertically into 4–8 channels The chromatograph is developed in benzene–acetone (1 1, v/v) [22], and then air-dried The region R_F 0 6–0 9 is removed by scraping with a razor blade and added to 10 ml of dioxane fluor (5 0 g of diphenyloxazole and 100 g of naphtalene plus dioxane to 1 l and counted for both ^{14}C and ^{3}H Raw ^{14}C data are corrected for recovery by use of the [^{3}H]mevalonic acid internal standard Corrected recovery typically ranges from 96–99% Enzyme activities are expressed as picomoles mevalonic acid synthesized per min per mg protein (pmoles/min per mg)

RESULTS

Composition of the assay mixture

Assay conditions were developed to permit analysis of enzyme preparations with widely different activities and protein content The concentrations of cofactors and of 3-hydroxy-3-methylglutaryl-CoA in the assay were established experimentally to be adequate for analysis of highly active reductase preparations Doubling the standard concentration of cofactors did not further increase reductase activity, and even halving their concentration decreased activity only at the higher protein con-

TABLE I

HYDROXYMETHYLGLUTARYL CoA REDUCTASE ACTIVITY AS A FUNCTION OF COFACTOR CONCENTRATION AND OF INCUBATION VOLUME

Analysis was conducted in the usual manner, except that NADP, glucose 6-phosphate and glucose-6-phosphate dehydrogenase were present at the standard concentrations (1×) or at the indicated multiples of these concentrations (1/2×, 2×) The hydroxymethyl-[^{14}C]glutaryl CoA used had a specific radioactivity of 3000 dpm/nmole The incubation volume was either 150 or 75 μl Data are mean values for microsomal preparations from rats killed at the peak of the cyclic rhythm

Incubation volume (μl)	Cofactor concentration	Reductase specific activity (pmoles/min per mg)	
		108 μg microsomal protein assayed	270 μg microsomal protein assayed
150	0	13	5
150	1×	2425	2460
150	2×	2340	2560
150	1/2×	2275	1775
75	0	25	
75	1×	2250	
75	2×	2360	

TABLE II

EFFECT OF HYDROXYMETHYLGLUTARYL CoA CONCENTRATION ON REDUCTASE ACTIVITY

The data are mean values for duplicate analyses of 216 μg of microsomal protein from the livers of rats killed at the peak of the cyclic rythm Analysis was by the standard method except for the indicated changes in the concentrations of DL-hydroxymethyl-[3-^{14}C]glutaryl CoA (specific radioactivity 3000 dpm/nmole)

Hydroxymethylglutaryl CoA added (nmoles)	Reductase specific activity (pmoles/min per mg)
25	2275
50	2300
100	2340

centration tested (Table I) While valid results are obtained using an incubation volume of 75 μl (Table I), we employ 150 μl to permit analysis of a wider range of sample volumes and protein levels

The concentration of DL-hydroxymethylglutaryl CoA used (50 nmoles per assay, or 3 3 10^{-4} M) exceeds by about an order of magnitude the K_m value of 1 10^{-5}–5 10^{-5} M (DL-hydroxymethylglutaryl CoA) for rat liver microsomal reductase [1] Doubling or halving the concentration of hydroxymethylglutaryl CoA does not greatly alter the microsomal reductase activity observed (Table II) The radioactivity present as background in samples from which reductase or cofactors are omitted, and hence the resolving power of the analysis, is directly related to the total quantity of hydroxymethyl-[^{14}C]glutaryl CoA used as substrate The background is never greater than 1 dpm per 3000 dpm of added 3-hydroxy-3-methylglutaryl-CoA (Shapiro, D J , unpublished observations)

Comparison of direct thin-layer chromatography and ether extraction of mevalonic acid

The accuracy and reproducibility of direct thin-layer chromatography is compared to results obtained following ether extraction of mevalonic acid in Table III

TABLE III

COMPARISON OF DIRECT THIN-LAYER CHROMATOGRAPHY AND ETHER EXTRACTION OF MEVALONIC ACID

Microsomes were prepared from rats sacrificed at the peak of the daily cyclic rhythm Microincubations and direct thin layer chromatography were carried out as described in Methods Ether extractions and thin-layer chromatography of ether extracts were carried out as described previously [22]

Microsomal protein (μg)	Reductase activity (pmoles/min per mg)	
	Ether extracted	Direct thin-layer chromatography
80	1417	1425
160	1503	1481
320	1439	1454
640	1462	1473

Reductase activities determined after ether extraction, or following direct thin-layer chromatography, are identical The reproducibility of the direct thin-layer chromatography method is also evident

Effect of protein concentration and incubation time

The assay conditions chosen are widely applicable The reductase activity from rat liver or from L-cells is linear with protein concentration within the ranges tested (Fig 1) and with incubation time up to 30 min (Fig 2) The identity of the reaction product (mevalonic acid) was confirmed by rechromatography as described previously [22] Assay conditions initially developed for assay of rat liver microsomal 3-hydroxy-3-methylglutaryl-CoA reductase thus appear suitable for assay of L-cell mouse fibroblast microsomal reductase and of solubilized reductase

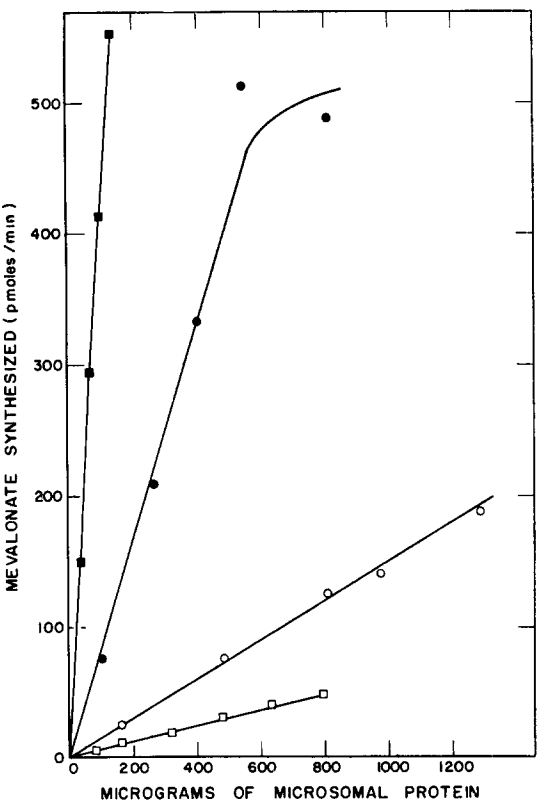

Fig 1 Mevalonate synthesis as a function of microsomal protein concentration Analyses were conducted at the indicated concentrations of microsomal protein from the livers of rats killed at noon (○—○) or at midnight (●—●), of microsomal preparations from L-cells in suspension culture (□—□) or of solubilized, partially purified rat liver hydroxymethylglutaryl CoA reductase (■—■)

Cholesterol regulation of 3-hydroxy-3-methylglutaryl-CaA reductase

In cultured cells, limited quantities of cellular material and low basal activities are frequently encountered [31–35] Exogenous sterols severly decrease L-cell sterol synthesis [31–33], and this decrease is paralleled by a decrease in reductase activ-

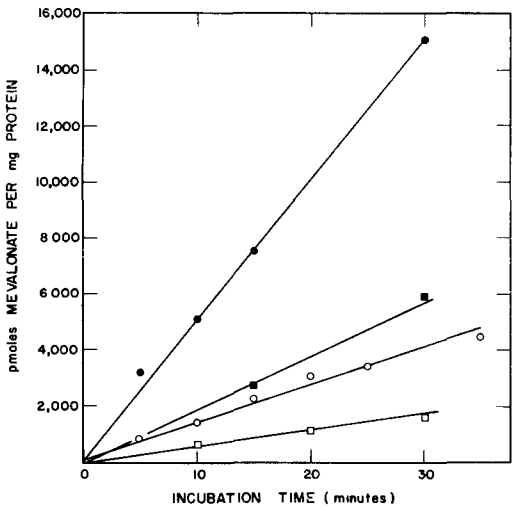

Fig 2 Mevalonate synthesis as a function of incubation time Analyses were conducted for the indicated times using microsomal preparations from livers of rats killed at noon (○—○) or at midnight (●—●), microsomal preparations from L-cells in suspension culture (□—□), or hydroxymethylglutaryl CoA reductase solubilized from rat liver microsomes (■—■)

ity [35] Assay of reductase in cultured cells, especially under conditions of such low activity, requires long incubation times of 1–2 h [12, 35] in order to produce sufficient mevalonic acid for detection To test the limits of the assay system using a 20-min incubation, reductase activity was measured 15 h after the addition of unpurified cholesterol (U S P Grade, 10 μg/ml) to the culture medium of L-cells (Table IV)

TABLE IV

EFFECT OF CHOLESTEROL ON L-CELL HYDROXYMETHYLGLUTARYL CoA REDUCTASE ACTIVITY

Rats fed chow diet (control) or chow diet plus 5% cholesterol for 30 h (cholesterol) were killed at midnight Cholesterol and lecithin, added as a solution in ethanol [31] were present in the medium of L-cells in suspension culture for 15 h Both control and experimental flasks contained 0 5% ethanol and 2 0 μg of lecithin per ml The experimental flask contained, in addition, 10 μg unpurified of cholesterol (U S P Grade) per ml Microsomal preparations from rat liver and from L-cells were then isolated and assayed for reductase activity Data are mean values and standard deviations for duplicate (rat) and for triplicate analyses (L-cells)

Source of reductase	Specific activity of hydroxymethylglutaryl CoA reductase (pmoles/min per mg)		Mevalonate produced during incubation (pmoles)	
	Control	Cholesterol	Control	Cholesterol
Rat*	365 ± 30	18 ± 1	4215 ± 450	228 ± 14
L-cells**	135 ± 5	6 ± 1	529 ± 20	27 ± 2

* Specific radioactivity of hydroxymethyl-[^{14}C]glutaryl CoA = 3000 dpm/nmole
** Specific radioactivity of hydroxymethyl-[^{14}C]glutaryl CoA = 11 300 dpm/nmole

For comparison, reductase activity from livers of rats fed a 5% cholesterol diet for 30 h was also measured (Table IV) The method is capable of detecting the synthesis of as little as 27 pmoles of mevalonic acid with good precision

Inactivation of rat liver 3-hydroxy-3-methylglutaryl-CoA reductase by MgATP

Beg et al [6] have recently shown that preincubation of unwashed rat liver microsomes with MgATP profoundly reduces hydroxymethylglutaryl-CoA reductase activity The time course of the MgATP-dependent reduction of hydroxymethylglutaryl-CoA reductase activity in rat liver microsomes isolated at the nadir of the diurnal cycle is shown in Fig 3* The flexibility of the assay system is shown by the detection of this rapid in vitro change in reductase activity To eliminate the possibility that the observed decrease in reductase activity is due to the conversion of mevalonic acid to 5-phosphomevalonic acid or other intermediates of isoprenoid

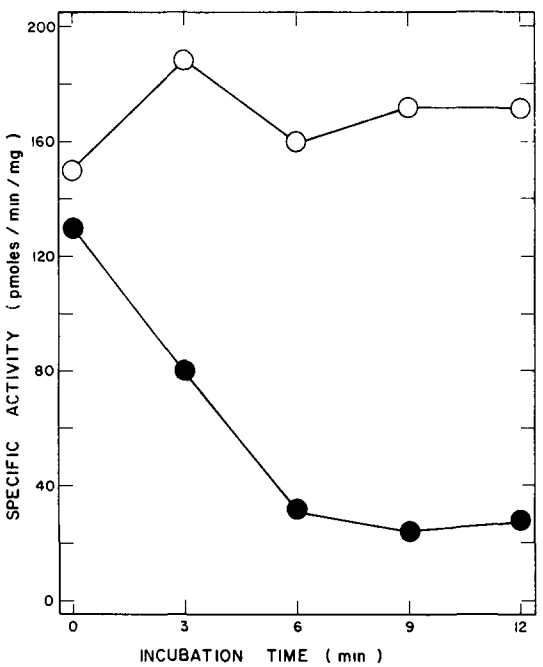

Fig 3 Time course of inactivation of hydroxymethylglutaryl-CoA reductase by MgATP Unwashed microsomal preparations from rats killed at the nadir of the daily cyclic rhythm (noon) were isolated and resuspended in buffer containing 1 0 mM EDTA, 250 mM NaCl, 1 0 mM dithiothreitol, 50 mM potassium phosphate, pH 7 4 Initially, experiments were attempted in the absence of EDTA in the buffer However, 1 0 mM EDTA was required to stabilize microsomal hydroxymethylglutaryl CoA reductase activity during the incubation period Microsomal suspension (0 76 mg in 90 μl) was mixed, on ice, with 10 μl of water (without MgATP, ○—○) or with 10 μl of 40 mM MgATP (with MgATP, ●—●) and incubated at 37 °C for the indicated times The concentration of MgATP during the initial incubation was thus 4 0 mM Cofactor substrate solution, 50 μl, containing sufficient EDTA to bring the total concentration to 15 mM (to chelate Mg^{2+} and block further inactivation during the assay) was then added and incubations continued for an additional 15 min

* A similar effect has been observed with L-cell hydroxymethylglutaryl CoA reductase (Mitschelen, J , unpublished observations)

biosynthesis, unwashed microsomes were incubated with [^{14}C]mevalonic acid in the absence of hydroxymethyl-[^{14}C]glutaryl-CoA Using the direct assay, recovery of added [^{14}C]mevalonic acid after incubations in the presence or absence of MgATP was 96–100% Addition of small amounts of carrier [^{3}H]mevalonic acid does not effect reductase activity

DISCUSSION

Assay of 3-hydroxy-3-methylglutaryl-CoA reductase has been complicated by the tedious, non-quantitative ether extraction of mevalonic acid The micro-incubation, direct thin-layer chromatography method described is sensitive, rapid and reproducible Application of the deproteinized acidified incubation mixture to the chromatogram yields reductase activities which are indistinguishable from those obtained following ether extraction of mevalonic acid (Table III) However, the micro-incubation direct thin-layer chromatography method is both more rapid and more sensitive than ether extraction of 1-ml incubations The direct thin-layer chromatography method is far more rapid than gas–liquid chromatography [14] or anion-exchange chromatography [14] The background obtained using the direct assay is equal to 3–15 pmoles of mevalonic acid and is far lower than the 40–500 pmoles reported for other thin-layer chromatographic [3, 22] enzymatic [7] or spectrophotometric [27] assays for hydroxymethylglutaryl-CoA reductase The low background level in the direct thin-layer chromatographic assay permit detection of as little as 30 pmoles of mevalonic acid compared to about 500 pmoles of product for the spectrophotometric assay recently described by Hulcher and Oleson [27]

The direct thin-layer chromatographic method is versatile and applicable to assay of microsomal and solubilized 3-hydroxy-3-methylglutaryl-CoA reductase 3-Hydroxy-3-methylglutaryl-CoA reductase activity is known to vary widely under diverse physiological conditions [1] For example, low activities are observed in cholesterol-fed rats or cholesterol-treated L-cells, while high activities are observed in rats killed at midnight The cofactor concentrations used in the micro-incubation, direct thin-layer chromatography assay permit reliable quantitation of 3-hydroxy-3-methylglutaryl-CoA reductase over its entire activity range without modification of assay conditions

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