papers on methodology

A novel assay for cytosolic 3-hydroxy-3-methylglutaryl-coenzyme A synthase activity using reversed-phase ion-pair chromatography: demonstration that Lifibrol (K12.148) modulates the enzyme activity

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Abstract Cytosolic HMG-CoA synthase and microsomal 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase catalyze two sequential steps in the mevalonate pathway. Both enzymes are negatively regulated by cholesterol. Cytosolic HMG-CoA synthase is responsible for the generation of HMG-CoA from acetyl-CoA and acetoacetyl-CoA). We have developed a new method to determine HMG-CoA synthase activity. In this assay, HMG-CoA is formed from acetoacetyl-CoA and labeled acetyl-CoA. The HMG-CoA product is isolated from the reaction mixture by means of reversed-phase ion-pair chromatography. The recovery of the product was always greater than 90%. The average within-batch coefficient of variation for HMG-CoA synthase activity was 5.1%. Using the new assay, we demonstrate that Lifibrol (K12.148), a new hypolipidemic compound, inhibits HMG-CoA synthase. Because our assay is accurate and precise it may become useful in future studies on the regulation and the pharmacological modulation of cytosolic HMG-CoA synthase. **—Scharnagl, H., W. März, M. Schliack, R. Löser, and W. Gross.** A novel assay for cytosolic 3-hydroxy-3-methylglutaryl-coenzyme A synthase activity using reversed-phase ion-pair chromatography: demonstration that Lifibrol (K12.148) modulates the enzyme activity. *J. Lipid Res.* 1995. **36:** 622–627.

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The biosynthesis of sterols proceeds through a complex cascade of reactions. It is mainly regulated by controlling mevalonate production. Two enzymes are subject to end-product feedback suppression, microsomal 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase and cytosolic HMG-CoA synthase. The former performs the rate-limiting step and its inhibitors are potent cholesterol-lowering drugs (1, 2).

Cytosolic HMG-CoA synthase catalyses the generation of HMG-CoA from acetyl-CoA (Ac-CoA) and acetoacetyl-CoA (AcAc-CoA). An isoform of HMG-CoA synthase is located in liver mitochondria (3–5). It is encoded by a different gene and catalyzes the rate-limiting step of ketogenesis.

cDNAs for chicken, rat, hamsters, and human cytosolic HMG-CoA synthase have been cloned and sequenced (6–9). The enzyme is of growing medical interest as it is highly regulated. In hamsters, cholesterol feeding suppressed mRNA for liver cytoplasmic HMG-CoA synthase approximately 18-fold compared to chow diet alone, whereas mRNA for microsomal HMG-CoA reductase decreased only 6-fold in the same livers (7). By raising intracellular HMG-CoA levels, HMG-CoA synthase may help to overcome competitive inhibition of HMG-CoA reductase, as previously demonstrated in a clone of Chinese hamster ovary cells that were adapted to grow in the presence of compactin (10, 11).

HMG-CoA synthase activity has been determined using the method originally described by Clinkenbeard et al. (3) or minor modifications thereof (12–14). In these methods, the conversion of radioactively labeled Ac-CoA

Abbreviations: Ac-CoA, acetyl-CoA; AcAc-CoA, acetoacetyl-CoA; DEAE-cellulose, diethylaminoethyl-cellulose; EDTA, ethylenediaminetetraacetate; HMG, 3-hydroxy-3-methylglutaric acid; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; RP-IPC, reversed-phase ion-pair chromatography; SDS, sodium dodecyl sulfate; TBAP, tetrabutylammonium phosphate; TBAOH, tetrabutylammonium hydroxide.

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to HMG-CoA is determined. After hydrolysis of the CoA-thioesters, acetate is volatilized and the nonvolatile radioactivity is considered to represent the amount of HMG-CoA generated in the reaction. In our work with HMG-CoA synthase, we noticed that this method to separate precursor and product of the reaction resulted in incomplete removal of acetate and/or poor recovery of HMG. Very recently, Mayer et al. (15) published a method in which the CoA-thioesters were hydrolyzed and HMG was recovered by ion exchange chromatography. Here, we present another accurate and precise method for the measurement of HMG-CoA synthase activity, in which HMG-CoA is isolated by reversed-phase ion-pair chromatography (RP-IPC). Using partially purified HMG-CoA synthase from chicken liver, we also demonstrate that Lifibrol, a new, highly effective lipid-lowering agent (16), modulates the enzyme activity.

MATERIALS AND METHODS

Determination of HMG-CoA synthase activity

Method A (conventional method). We used a modification of the method described by Clinkenbeard et al. (3). The reaction mixture (200 μl) consisted of: 0.1 mol/l Tris-HCl (pH 8.0), 0.1 mmol/l EDTA, 20 mmol/l $MgCl_2$ (cf. ref. 7), a total of 70–80 μg protein (column fractions or partially purified HMG-CoA synthase) 60 μmol/l AcAc-CoA, 400 μmol/l Ac-CoA (cf. ref. 15) plus 17 μmol/l [1-^{14}C]Ac-CoA (sp act 5000–6000 cpm/nmol). The reaction mixture was preincubated for 2 min at 30°C and the reaction was started by adding Ac-CoA. After 15 min, the reaction was stopped with 100 μl of 6 mol/l HCl, to hydrolyze CoA-thioesters. Acetic acid was volatilized at 95°C for 2 h. The amount of HMG-CoA formed during incubation was determined from the nonvolatile radioactivity. As blanks, we used incubation mixtures from which the enzyme source had been omitted.

Method B (reversed-phase ion-pair chromatography based method). The composition of the reaction mixture was identical to method A. The reaction was stopped by adding 0.5 ml TBAP (tetrabutylammonium phosphate) buffer, which consisted of 50 mmol/l TBAP, pH 5.5, dissolved in methanol–water 47.5:52.5 (by volume). To prepare TBAP, we titrated TBAOH (tetrabutylammonium hydroxide) with orthophosphoric acid as described (17). To improve the recovery of the product, HMG-CoA was added to the incubation mixtures at a concentration of 250 μmol/l, and [^{3}H]HMG-CoA was included as an internal standard. Ac-CoA, AcAc-CoA, and HMG-CoA were separated by means of reversed-phase ion-pair chromatography using a Hewlett-Packard type 1050 system, essentially as described by Baker and Schooley (17, 18). Aliquots (20–50 μl) of the incubation mixtures were loaded to a LiChrosorb RP-8 column (25 · 0.6 cm, Merck, Darmstadt, Germany) and eluted with TBAP buffer. Fractions of 500 μl were collected, and the radioactivity in the HMG-CoA containing fractions was determined.

Partial purification of HMG-CoA synthase

HMG-CoA synthase was partially purified from chicken liver as described by Gil et al. (7), with the exception that the last step of their procedure (hydroxyapatite chromatography) was omitted. All purification steps were performed at 4°C.

Buffers. Buffer A: 300 mmol/l sucrose, 20 mmol/l sodium phosphate and 0.1 mmol/l EDTA, pH 7.0; buffer B: 20 mmol/l sodium phosphate, 0.1 mmol/l EDTA, and 0.5 mmol/l dithiothreitol, pH 7.0; buffer C: 10 mmol/l sodium phosphate, 15% (v/v) glycerol, 0.1 mmol/l EDTA, and 0.5 mmol/l dithiothreitol, pH 7.0; buffer D: 10 mmol/l sodium phosphate, 10% (v/v) glycerol, 0.1 mmol/l EDTA, 0.5 mmol/l dithiothreitol, pH 6.0. All buffers contained, in addition, 10,000 KIU/l aprotinin and 1 mmol/l phenylmethylsulfonylfluoride.

Preparation of a cytosolic fraction. One hundred g chicken liver was ground in buffer A with a motor-driven mixer and homogenized in a Potter-Elvehjem homogenizer with 10 strokes of a Teflon pestle. The cell homogenate was centrifuged at 30,000 *g* and 4°C for 20 min. The supernate was centrifuged at 100,000 *g* and 4°C for 90 min. The resulting cytosolic fraction was dialyzed against buffer B at 4°C.

Anion exchange chromatography. The cytosolic fraction was added to 200 ml of DEAE cellulose, previously equilibrated with buffer B. The slurry was carefully homogenized and packed into a 24 · 3.2 cm chromatography column. The column was washed with 900 ml buffer B and subsequently eluted with a linear gradient of NaCl (0 to 200 mmol/l) in buffer B. The gradient volume was 400 ml. Fractions containing HMG-CoA synthase activity (from 120 to 140 mmol/l NaCl) were pooled, concentrated by precipitation with ammonium sulphate at 50% saturation, and dialyzed against buffer C.

Affinity chromatography. Affinity chromatography was carried out using a 10 · 2.0 cm Blue Sepharose CL-6B column, equilibrated with buffer C. HMG-CoA synthase-containing material was loaded to the column, which was then washed with 500 ml buffer C and eluted with the same buffer, additionally containing 250 mmol/l NaCl. The eluate was dialyzed against buffer D.

Cation exchange chromatography. Affinity chromatography-derived fractions were loaded to a cellulose phosphate column (9 · 2.3 cm), previously equilibrated with buffer D. After washing with buffer D, HMG-CoA synthase activity was eluted with 80 ml of 0.2 mmol/l AcAc-CoA in buffer D. Fractions containing enzymatic activity were pooled, dialyzed against buffer C, and concentrated by ultrafiltration using a Diaflo PM-30 membrane and a stirred Amicon cell.

TABLE 1. Purification of cytosolic HMG-CoA synthase from chicken liver

Step	Protein	Specific Activity	Purification	Yield
	mg	*nmol · min⁻¹ · mg⁻¹*	*-fold*	*%*
1 Cytosolic fraction	6900	0.0145	(1)	100
2 DEAE-cellulose chromatography	405	0.128	8.8	52
3 Blue Sepharose chromatography	6.5	3.9	213	25
4 Cellulose phosphate chromatography	1.8	10.1	696	18

Other methods. Protein was determined as described (19). SDS-polyacrylamide gel electrophoresis was carried out in a discontinuous buffer system (20). Reaction rates were fitted to a one-substrate Michalis and Menten kinetics using P.FIT, a non-linear curve-fitting program from FIG.P Software (Durham, NC).

RESULTS

HMG-CoA synthase was partially purified from chicken liver; approximate yields and recoveries are summarized in **Table 1.** The products of the purification steps were analyzed by SDS polyacrylamide gel electrophoresis. The final product contained two major protein bands with apparent molecular masses of 65 kDa and 53 kDa, and one minor band of 47 kDa, respectively. The molecular mass previously determined for cytosolic HMG-CoA synthase from chicken liver (4) is 53 kDa, suggesting that our 53 kDa band was HMG-CoA synthase. The 47 kDa band probably corresponded to the proteolytic degradation product observed by Gil et al. (7), which lacks the carboxy terminus of the molecule.

We compared two methods for the determination of the HMG-CoA synthase activity. Method A was a modification of the procedure described by Clinkenbeard et al. (3). In this method, thioesters were hydrolyzed and the amount of HMG-CoA generated was determined from the radioactivity that remained after the unreacted acetic acid had been volatilized at 95°C for 2 h. In our hands, this did not quantitatively separate precursor and product of the reaction. About 30% of the initial amount of acetic acid was still present after 2 h of incubation at 95°C, while, at the same time, a significant proportion of HMG-associated radioactivity had disappeared (**Fig. 1**). Further

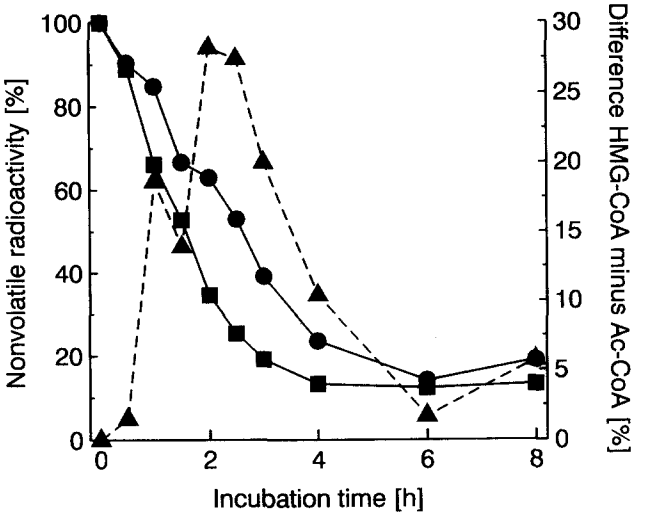

Fig. 1. Separation of acetate and HMG by incubation at 95°C. [^{14}C]HMG-CoA (specific activity 5000 cpm/nmol) or [^{14}C]Ac-CoA (specific activity 5400 cpm/nmol) were dissolved at concentrations of 400 μmol/l each in 0.1 mol/l Tris-HCl (pH 8.0), 0.1 mmol/l EDTA, 20 mmol/l $MgCl_2$. Two hundred μl of the HMG-CoA (circles) or Ac-CoA (squares) containing solutions were mixed with 100 μl 6 mol/l HCl and incubated separately at 95°C. After the time intervals indicated on the abscissa, the nonvolatile radioactivities were determined in a scintillation counter. The difference in percent (triangles) between the two nonvolatile radioactivities was greatest after 2 h of incubation.

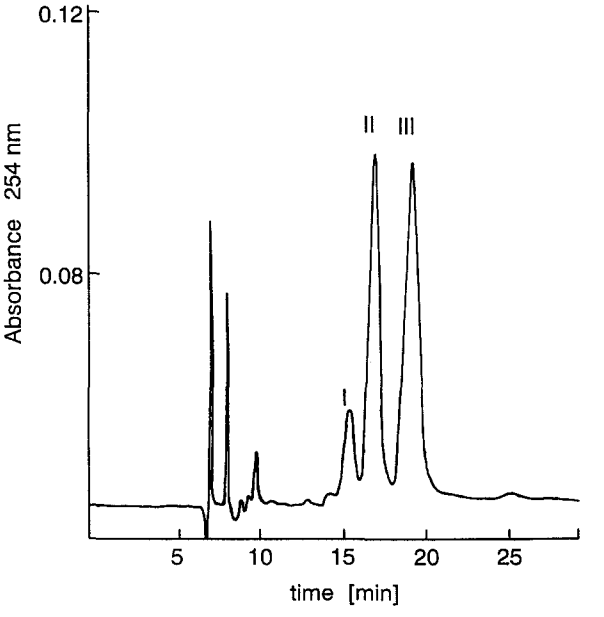

Fig. 2. Separation of AcAc-CoA (I), Ac-CoA (II), and HMG-CoA (III) by reversed-phase ion-pair chromatography. HMG-CoA synthase was prepared from chicken liver; the enzyme was incubated with [1-^{14}C]Ac-CoA and AcAc-CoA for 15 min as described in Methods. Before chromatography, HMG-CoA was added to the incubation mixture at a concentration of 250 μmol/l and [^{3}H]HMG-CoA was included as an internal standard. The separation was carried on a LiChrosorb RP-8 column (25 × 0.6 cm), eluted with 0.05 mol/l TBAP, pH 5.5, in methanol–water 47.5:52.5 at a flow rate of 350 μl/min.

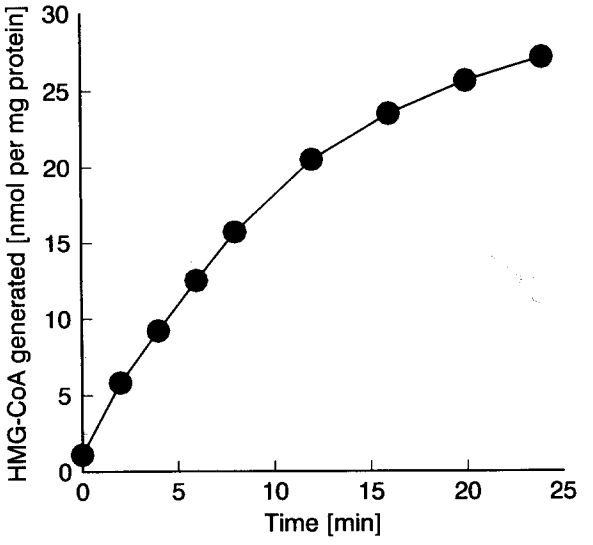

Fig. 3. Time course of the formation of [^{14}C]HMG-CoA from [^{14}C]Ac-CoA and AcAc-CoA by partially purified HMG-CoA synthase. HMG-CoA synthase was prepared from chicken liver as described in Methods. The enzyme was incubated with [1-^{14}C]Ac-CoA and AcAc-CoA; at the time points indicated on the abscissa the amount of [^{14}C]HMG-CoA was determined using reversed-phase ion-pair chromatography.

evaporation removed the acetic acid-bound radioactivity almost completely, but the same was true for HMG.

For this reason, we sought an alternative approach to recover HMG-CoA quantitatively from the incubation mixture. This was achieved by using RP-IPC, which was previously demonstrated to allow high-resolution separation of acyl-CoA thioesters (17, 18). **Figure 2** shows a typical chromatogram. AcAc-CoA, Ac-CoA, and HMG-CoA were resolved after 25 min. Compared to the original protocol, we increased the ratio of methanol to water in the mobile phase to 47.5:52.5. This resulted in shorter retention times without deteriorating resolution.

Using the RP-IPC based method (method B) we analyzed the time kinetics of the HMG-CoA synthase reaction. As shown in **Figure 3,** HMG-CoA production was linear with time for 15 min. After that period, the rate of HMG-CoA production gradually decreased.

When we determined the activity of partially purified HMG-CoA synthase, the RP-IPC based method (method B) produced approximately twofold higher activities than the conventional method (method A). On repeated measurements of the same preparation, the standard deviations were substantially lower in method B than in method A. These findings were confirmed in experiments in which we examined whether or not the suggested method would be able to demonstrate drug effects. As a model compound, we used Lifibrol (K12.148). Lifibrol was added to partially purified HMG-CoA synthase, and the enzyme activity was determined with the two methods. As shown in **Table 2,** method B produced approximately twofold the activities of method A. We calculated the standard deviations and coefficients of variation from four determinations. Average coefficients of variation were 40.5% and 5.1% for method A and B, respectively. Method A failed to demonstrate an effect of Lifibrol on HMG-CoA synthase (Table 2). In contrast, when we measured the activity of the enzyme with method B, we found that Lifibrol inhibited HMG-CoA synthase in a dose-dependent fashion. At 10^{-5} mol/l, Lifibrol reduced HMG-CoA synthase activity by approximately 20%.

Figure 4 shows the effect of Lifibrol on partially purified HMG-CoA synthase at varying concentrations of Ac-CoA and a constant concentration of 60 μmol/l AcAc-CoA. Under these conditions, the inhibitory effect of Lifibrol was greatest between 400 and 1200 μmol/l Ac-CoA. Further increases in the substrate concentrations partly reversed the inhibitory effect of Lifibrol; at 4000 μmol/l Ac-CoA, HMG-CoA synthase activity was inhibited by 10% only, even in the presence of 10^{-4} mol/l Lifibrol. To determine the type of inhibition, we calculated V_{max}, the maximum reaction rate, and K_m, the

TABLE 2. Comparison of two methods for the determination of HMG-CoA synthase

	Method A			Method B		
Lifibrol	Activity	Relative Activity[a]	CV	Activity	Relative Activity[a]	CV
	nmol · min^{-1} · mg^{-1}	%	%	*nmol · min^{-1} · mg^{-1}*	%	%
0 (Control)	4.2 ± 1.9	100.0	45.2	10.1 ± 0.4	100.0	4.0
10^{-7}	3.8 ± 1.2	90.4	31.6	10.7 ± 0.6	100.6	5.6
10^{-6}	5.1 ± 2.6	121.4	51.0	10.0 ± 0.4	98.9	4.0
10^{-5}	4.1 ± 1.6	97.6	39.0	8.0 ± 0.5	79.0	6.3
10^{-4}	3.9 ± 1.4	92.8	35.9	7.1 ± 0.4	70.2	5.6

Reaction mixtures containing 0.1 mol/l Tris-HCl, pH 8.0; 0.1 mmol/l EDTA; 20 mmol/l $MgCl_2$; 80 μg partially purified HMG-CoA synthase; 60 μmol/l AcAc-CoA; 417 μmol/l [1-^{14}C]Ac-CoA (specific activity 220 cpm/nmol) and the indicated concentrations of Lifibrol (K12.148) were incubated as described in Methods. In method A, precursor and product of the reaction were separated by hydrolysis of the CoA thioesters and evaporation at 95°C for 2 h. In method B, precursor and product were separated by RP-IPC. Enzyme activities are normalized to the amount of protein present in the reaction mixtures. Entries are mean values ± standard deviations calculated from quadruplicates; CV, coefficient of variation.

[a]Activity as percent of the control.

Michaelis constant, from the data shown in Figure 4. Formally, the results were compatible with the assumption that Lifibrol acted as a competitive inhibitor. As shown in **Table 3,** Lifibrol had no effect on V_{max}, but increased the apparent K_m. The estimates for K_i, the inhibitor constant, obtained at 10^{-5} mol/l and 10^{-4} mol/l were 23 and 93 μmol/l (average value 58 μmol/l), respectively.

TABLE 3. Effect of Lifibrol on HMG-CoA synthase

Lifibrol	Apparent K_m	V_{max}	K_i	r
mol/l	*μmol/l*	*nmol · min⁻¹ · mg⁻¹*	*μmol/l*	
0 (Control)	1760	86		0.988
10^{-6}	1560	84	nd	0.990
10^{-5}	2524	86	23	0.996
10^{-4}	3655	94	93	0.991

V_{max} (maximum reaction rate), K_m (Michaelis constant), and r (correlation coefficient) were determined by non-linear regression analysis from the data plotted in Figure 4; nd, not determined.

DISCUSSION

Cytoplasmic HMG-CoA synthase was partially purified from chicken liver using the method described by Gil et al. (7) for hamster liver, with the exception that we omitted the last step of the original protocol. We did not attempt to further purify the enzyme for two reasons. First, it has been shown that HMG-CoA synthase obtained by substrate elution from phosphocellulose is essentially free of AcAc-CoA thiolase (13, 21) which may deplete the AcAc-CoA substrate (13, 22). Second, in spite of the shorter purification procedure, our preparation had characteristics similar to those reported (7) for purified hamster liver HMG-CoA synthase when analyzed by SDS polyacrylamide gel electrophoresis.

Using the partially purified enzyme, we compared two methods to measure the activity. Both have in common that the incorporation of radioactively labeled Ac-CoA into HMG-CoA is determined. In the first, CoA-thioesters were cleaved and non-reacted acetate was evaporated at 95°C for 2 h (3). On the one hand, this removed only 65% of the acetate; on the other hand, it resulted in the loss of approximately one third of the HMG-associated radioactivity. Method A also suffered from poor precision. Thus, it may at best serve to follow the activity of the enzyme during purification, but it is not precise enough to perform kinetic studies.

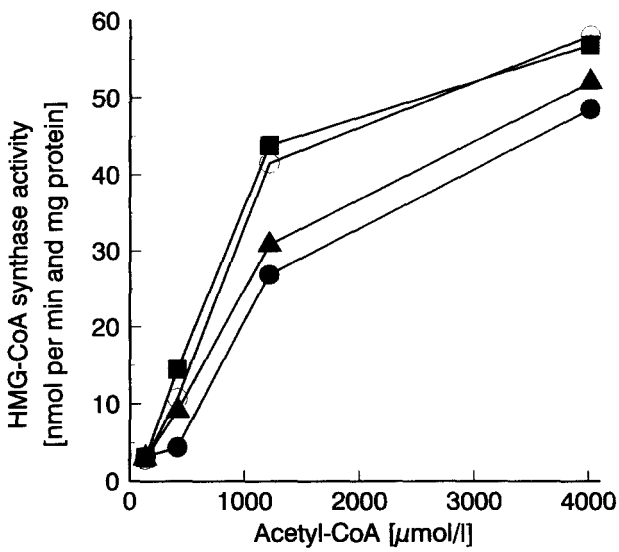

Fig. 4. Effect of Lifibrol on HMG-CoA synthase. HMG-CoA synthase was partially purified from chicken liver. Incubation mixtures contained 60 μmol/l AcAc-CoA and the indicated concentrations of Ac-CoA substrate. Lifibrol was added at final concentrations of 10^{-4} mol/l (closed circles), 10^{-5} mol/l (triangles), and 10^{-6} mol/l (squares); open circles: controls without Lifibrol. The enzyme activity was determined using the RP-IPC based method (method B).

These difficulties prompted us to examine whether the recovery of the reaction product could be improved. This was achieved by means of RP-IPC (method B). Recovery of HMG-CoA was more than 90% and the coefficient of variation for the determination of HMG-CoA synthase activity was only 5%.

Using the RP-IPC based method, we were able to show that Lifibrol (K12.148) inhibits cytosolic HMG-CoA synthase. At a concentration of 10^{-5} mol/l, Lifibrol reduced HMG-CoA synthase activity by 20 to 25%. We have recently shown that in normal human skin fibroblasts and in HepG2 cells, Lifibrol, at a concentration of 10^{-5} mol/l, reduced the incorporation of acetate into newly synthesized sterols by 30% (23), which is very close to the inhibitory effect of Lifibrol on HMG-CoA synthase. Lifibrol had no effect on the generation of sterols from mevalonate nor did it inhibit HMG-CoA reductase (23). We, therefore, hypothesized that Lifibrol affected cholesterol biosynthesis by influencing HMG-CoA synthase. The results presented here are in favor of this hypothesis. They furthermore suggest that the inhibition of cholesterol biosynthesis caused by Lifibrol is fully accounted for by its action on HMG-CoA synthase. Whether this sufficiently explains the marked and rapid cholesterol-lowering effect of Lifibrol in humans (16) remains to be elucidated.

In conclusion, we present an optimized procedure for the measurement of cytosolic HMG-CoA synthase activity which overcomes the major limitations of most techniques described so far. As this method is easily automated it may be of great potential value in future studies on the kinetics and regulation of the HMG-CoA synthase activity as well as in the search for new agents modulating the activity of this enzyme.

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