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COORDINATE ANION EXCHANGE ASSAYS FOR HMG CoA REDUCTASE AND HMG CoA LYASE AND A CHARACTERIZATION OF THE PHOSPHATE ION SENSITIVITY OF HMG CoA LYASE

by

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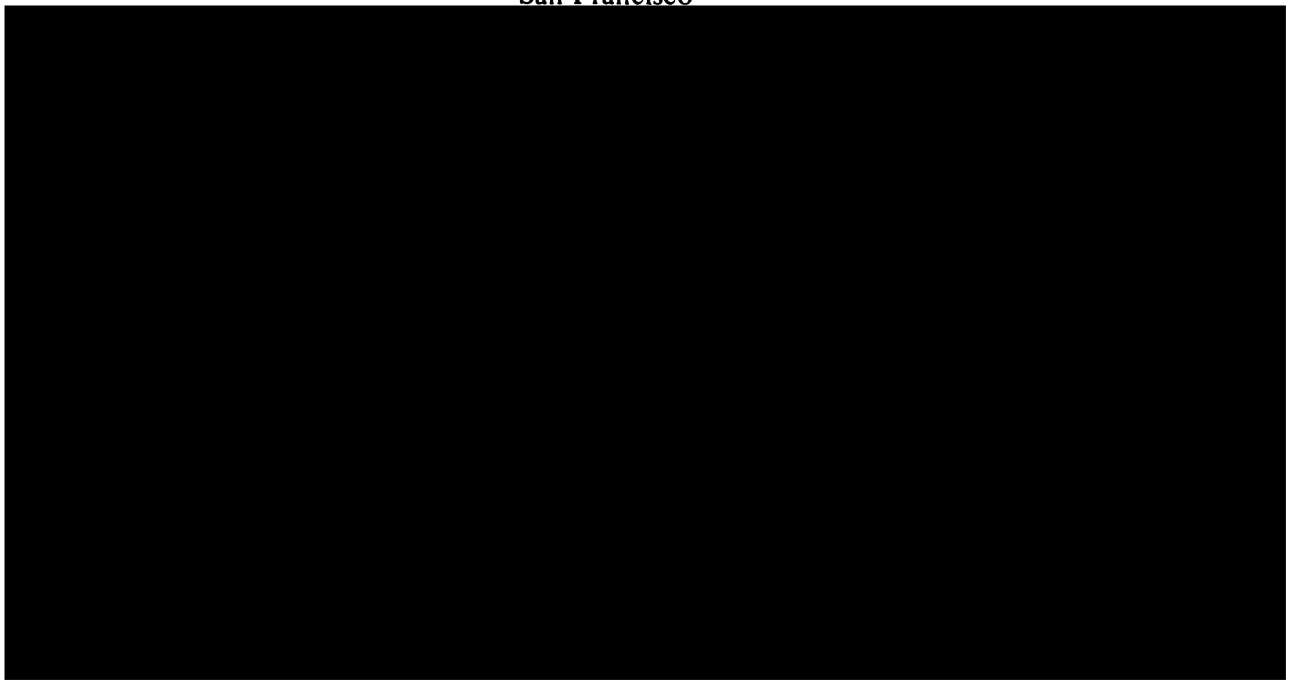

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ABSTRACT

The investigation presented in this dissertation was directed toward a characterization of β-hydroxy-β-methylglutaryl coenzyme A (HMG CoA) lyase (EC 4.1.3.4), the enzyme responsible for the synthesis of acetoacetic acid. The first step in these studies was the development of a rapid, sensitive assay for HMG CoA lyase, which actually was done in close concert with the development of an assay for HMG CoA reductase (EC 1.1.1.34), the rate-limiting enzyme of cholesterogenesis.

Thus, a simple anion exchange method for the assay of the enzymes, HMG CoA lyase and HMG CoA reductase, is described. In the HMG CoA reductase assay, the enzyme product, [3-^{14}C]mevalonic acid, is cyclized to [3-^{14}C]mevalonolactone, whereas the product of the HMG CoA lyase assay, [3-^{14}C]acetoacetate, is heat-decarboxylated to yield [2-^{14}C]acetone. In both the reductase and lyase assays, the anion exchange resin traps the [3-^{14}C]HMG CoA substrate and allows the derivatives of the respective enzyme products to elute. Although both mevalonolactone and acetone elute through the column, the presence of one enzyme does not interfere with the assay of the other. Both assays are rapid, sensitive and specific, and together they offer the means to investigate the two enzymes in a coordinated manner.

The second part of these studies involved an investigation of the nature of the lability of HMG CoA lyase activity. Phosphate ion was found to be responsible for the enzyme instability. In non-phosphate, zwitterionic buffers, the specific activity of HMG CoA lyase was considerably higher than in phosphate buffer, and in marked contrast, stable

at 37°C. Phosphate was found to have an inhibitory effect at a concentration as low as 5 mM. The inactivating process was generally found to be rapid and profound, though subject to influence by several factors. More concentrated enzyme samples were more stable against the inhibitory effects of phosphate than dilute samples. Dithiothreitol and $MgCl_2$ were found to increase the specific activity of the enzyme, and to confer some protection against the inhibitory effects of phosphate. These data suggest that HMG CoA lyase exists in two forms; the more active form exists in the absence of phosphate and the less active form in its presence. Little is known of the nature of the regulation of the activity of the ketogenic enzymes. These data suggest minimally that the HMG CoA lyase may be subject to some form of acute, allosteric or subunit-interaction type of regulation.

The rationale for an interest in the mechanisms controlling the activity of the hepatic ketogenic pathway is discussed, as well as the possibility of applying the principle of these assays to the study of substrate flux within intact mitochondria. Additionally, the concept of a functional cytosolic HMG CoA lyase is discussed in terms of its possible role in regulating substrate flux through the cholesterogenic pathway, along with the applicability of these HMG CoA reductase and lyase assays to clarifying this issue.

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INTRODUCTION

The investigation presented in this dissertation was directed toward a characterization of the hepatic enzyme, β-hydroxy-β-methylglutaryl coenzyme A (HMG CoA) lyase (EC 4.1.3.4). The first step in these studies was to develop a rapid and sensitive enzyme assay. This assay was actually developed in concert with an assay for another enzyme, hepatic HMG CoA reductase (EC 1.1.1.34). These two closely allied procedures are presented in subsection I of Experimental Results and Discussion. In subsection II are found the results and discussion of an investigation into factors regulating the in vitro activity of HMG CoA lyase, with an emphasis on the sensitivity of the enzyme to phosphate ion.

HMG CoA is the substrate for both HMG CoA reductase and HMG CoA lyase. HMG CoA reductase reduces HMG CoA to mevalonic acid, which is destined either for sterol synthesis or alternatively in some tissues, for metabolism to CO_2 via the mevalonic acid shunt pathway (16,38). HMG CoA lyase, however, plays a central role in the synthesis of ketone bodies via the Lynen cycle (24), as it cleaves HMG CoA into acetyl-CoA and acetoacetate. Hepatic HMG CoA lyase is considered the major source of blood ketones, because the rate of production of acetoacetate by direct deacylation of acetoacetyl-CoA is quite low (52).

HMG CoA reductase, the rate-limiting and feedback-regulated enzyme of cholesterol synthesis (42,43), has been extensively investigated. Several methods for the assay of HMG CoA reductase have been developed, all utilizing the double-label principle introduced by Goldfarb and Pitot (18). In these assays, ^{14}C-mevalonic acid production from

^{14}C-HMG CoA is measured, and the efficiency of ^{14}C-mevalonic acid recovery is monitored by using an internal standard of ^{3}H-mevalonic acid. Separation of the ^{14}C-mevalonic acid from the ^{14}C-HMG CoA is begun by acidification, which cyclizes the mevalonic acid to mevalonic acid lactone. Mevalonic acid lactone, being less polar than HMG CoA, can then be separated from the polar HMG CoA by extraction into organic solvents and thin-layer chromatography (1,19,41). Anion exchange chromatography also has been used as a separatory technique in this assay (3,20). This method exploits the fact that whereas the ^{14}C-HMG CoA is adsorbed by the anion exchange resin, the neutral ^{14}C-mevalonic acid lactone elutes freely through it.

The acetoacetic acid synthetic pathway, as originally described by Lynen and co-workers (24) consists of three enzymatic steps. Acetoacetyl CoA thiolase catalyzes the first step, the condensation of two molecules of acetyl CoA to yield acetoacetyl CoA; HMG CoA synthase catalyzes the second, the condensation of acetoacetyl CoA with another molecule of acetyl CoA to yield HMG CoA; and HMG CoA lyase catalyzes the final step, the cleavage of HMG CoA to yield acetoacetate and acetyl CoA. An alternative pathway of acetoacetate synthesis, described by Stern and Miller (47), which involves the direct deacylation of acetoacetyl CoA, was shown by Williamson et al. (52) to be of relatively minor quantitative importance. HMG CoA lyase metabolizes the HMG CoA that arises from other pathways as well, i.e., from the metabolism of leucine (11) and probably from the metabolism of mevalonate via the proposed transmethyl-glutocaconate shunt (16).

The relative importance of acetoacetyl CoA thiolase, HMG CoA synthase, and HMG CoA lyase with regard to limiting and regulating the

synthesis of acetoacetate is not fully understood. HMG CoA synthase is considered by Williamson et al. (52) to be the rate-limiting enzyme of ketone synthesis on the basis of the low activity of this enzyme as compared to the other two. The importance of HMG CoA synthase is also suggested by the observation that while all three enzymes do occur extrahepatically, the HMG COA synthase activity of the liver is notably higher than in other tissues (28). On the other hand, Huth and co-workers (21-23) make a strong case for the role of acetoacetyl CoA thiolase in controlling the rate of ketone synthesis. Of specific interest is their finding that the activity of acetoacetyl CoA thiolase in the condensation (forward) direction is lower than the activity of HMG CoA synthase.

Both Williamson et al. (52) and Clinkenbeard et al. (17) found the specific activity of HMG CoA synthase to be one-third to one-fourth that of HMG CoA lyase. This observation encourages the view that HMG CoA lyase may be a constitutive enzyme of the ketone synthetic pathway, and that metabolic flux is regulated at a site prior to the cleavage of HMG CoA.

Nonetheless, several factors stimulated an interest in HMG CoA lyase. The first was an opinion that while maximum enzyme velocities in in vitro systems are of critical interest in characterizing an enzyme and defining its optimal operating conditions, these kinds of data may not reflect rates of substrate flux within the in vivo metabolic pathway (44). The second factor was a view that HMG CoA and its lyase may play roles in several more metabolic pathways than have been generally recognized. The role of HMG CoA lyase in the transmethylglutaconate shunt is as yet uninvestigated, and there is also the

possibility that HMG CoA lyase plays a role in the recycling and diversion of substrate within the cholesterogenic pathway of the cytoplasm. A third factor encouraging an investigation of HMG CoA lyase was simply the fact that it has been investigated relatively little. The most thorough investigation of HMG CoA lyase has been that of Stegink and Coon (46), and in that report the authors observed, but did not investigate, the instability of the purified HMG CoA lyase preparation.

Three major methods to assay this enzyme can be found in the literature, the principles of which are as follows: 1) the generation of one of the enzyme products, acetoacetate, can be measured spectrophotometrically by the method of Walker (49); 2) the other product, acetyl-CoA, can be linked enzymatically to the reduction of NAD to NADH, which can be measured spectrophotometrically (46); and 3) the generation of ^{14}C-acetoacetate from ^{14}C-HMG CoA can be measured by inference as the evaporative disappearance of ^{14}C counts from a background of the non-volatile ^{14}C-HMG CoA (10).

The HMG CoA reductase assay described in this thesis is, in part, an adaptation of the methods described earlier; however, the HMG CoA lyase assay is novel and its principle is outlined in Fig. 1. In this assay, the ^{14}C-acetoacetate product is decarboxylated by heat to yield ^{14}C-acetone. When the HMG CoA lyase assay is applied to an anion exchange column, the ^{14}C-HMG CoA is adsorbed whereas the ^{14}C-acetone freely elutes. The resulting methods provide a rapid and sensitive means of simultaneously assaying HMG CoA reductase and HMG CoA lyase.

Figure 1. Schematic outline of anion exchange HMG CoA lyase assay. HMG CoA lyase catalyzes the cleavage of [3-^{14}C]HMG CoA to [3-^{14}C]acetoacetate and acetyl CoA. Heat decarboxylates the [3-^{14}C]acetoacetate to [2-^{14}C]acetone. The assay mixture is applied to an anion exchange resin column which traps the [3-^{14}C]HMG CoA and permits the elution of the [2-^{14}C]acetone.

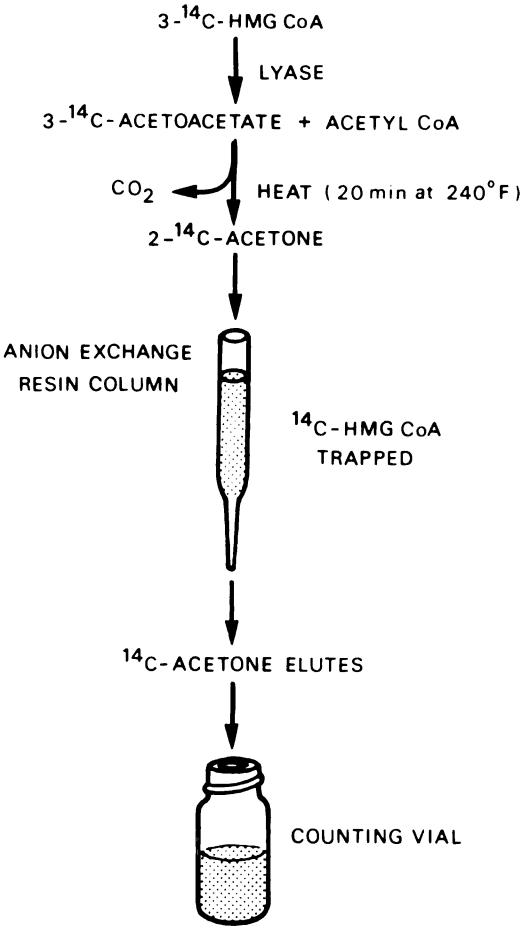

Figure 1. Schematic outline of anion exchange HMG CoA lyase assay.

MATERIALS AND METHODS

Chemicals

D-Glucose-6-phosphate (monosodium salt) and NADP were obtained from Sigma Chemical Co.; Dithiothreitol (A grade) and a series of zwitterionic buffers (MOPS, HEPES, TRICINE, BICINE, glyclyglicine, CHES, and CAPS), from Cal Biochem; glucose-6-phosphate dehydrogenase, from Boehringer Mannheim; unlabeled HMG CoA, from P-L Biochemicals; [3-^{14}C]HMG CoA, D,L-[5-^{3}H]mevalonic acid (dibenzylethylenediamine salt), D,L-[3-^{14}C]-hydroxybutyrate, and Aquasol, from New England Nuclear; ethyl [3-^{14}C]acetoacetate and [2-^{14}C]acetone, from Amersham/ Searle.

The anion exchange resin, AG 1-X8, 200-400 mesh, formate form, was obtained from BioRad Laboratories. Flame-sealable, 2-ml ampules were purchased from Wheaton Glass Co. Inorganic reagents were of analytical reagent grade, from J. T. Baker Chemical Co.

Cell fraction preparation

Rat liver cell fractions were prepared from the livers of 200-250-g Sprague-Dawley rats by standard techniques. Livers were homogenized in a Dounce homogenizer with several strokes of a loose pestle, followed by a single stroke with a tight pestle.

A general description of the homogenization buffers is as follows: 0.3 M sucrose, 50 mM buffer, 5 mM DTT, and 5 mM $MgCl_2$. When potassium phosphate buffer was used, the pH was adjusted 7.5 except when noted otherwise. When the zwitterionic buffers were used, the pH was adjusted to a point near their respective pKa's (detailed in text). Generally, the constitution of the assay medium was identical to the homogenization medium except for the absence of sucrose. In experiments

using phosphate buffer, however, potassium phosphate concentration of the assay medium was 100 mM rather than 50 mM.

The homogenates were kept ice-cold at all times; centrifugation steps were carried out at 4°C. Two "mitochondrial" fractions were utilized: a relatively crude fraction consisting of the entire 10,000 G pellet, and a more purified fraction collected between 300 G and 10,000 G, thus excluding much cellular debris and nuclei.

HMG CoA lyase assays were performed both on these particulate fractions and on soluble preparations derived therefrom. Solubilization of HMG CoA lyase from mitochondrial fractions was effected as follows: the centrifugal pellet was dispersed in a buffer identical to the homogenization buffer except for the absence of sucrose, rapidly frozen in a dry ice-alcohol bath, slowly thawed, and then homogenized. This freeze-thaw-homogenization procedure was repeated, and the cell fraction was then centrifuged at 100,000 G for 1 h. The supernatant of this procedure was used as a source of soluble enzyme.

Post-mitochondrial supernatants were prepared by centrifuging the homogenates at 10,000 G until a pellet no longer appeared (usually three 10-min centrifugations). Centrifugation of this supernatant at 100,000 G for 1 h yielded a cytosolic supernatant and a microsomal pellet. The microsomes, used as a source of HMG CoA reductase activity, were washed by resuspension in the homogenization medium and repelleted at 100,000 G.

Anion exchange resin columns

Standard disposable 5-1/2-in pipettes, Pasteur type, were plugged with #4 dental cotton. The pipettes were set in a styrofoam board with perforations corresponding to the position of scintillation-counting

vials held in a vial tray. The resin was dispersed in water and applied to the pipettes until a final height of 5.5 cm of packed resin was achieved. The resin columns were rinsed three times with 1 ml of water and were then ready for use. These columns elute only the volume that is applied to the resin and therefore do not go dry.

HMG CoA reductase assay

The HMG CoA reductase incubation procedure of Brown et al. (5) was used. Briefly, a 200 µl-volume assay contained 100 mM potassium phosphate buffer, 20 mM glucose-6-phosphate, 2.5 mM NADP, 0.7 units of glucose-6-phosphate dehydrogenase, 5 mM DTT, (D,L)-HMG CoA (standardly, 160 µM), and the enzyme sample, at pH 7.5. The assay was incubated at 37°C for 30 min and was terminated by acidification with 10 µl of 10 N HCl, at which time the ^{3}H-mevalonic acid internal standard was added. A further 20-min, 37°C incubation assured lactonization of the mevalonic acid.

To separate the ^{14}C-mevalonic acid lactone from the ^{14}C-HMG CoA, the entire assay mixture was applied to the top of an anion exchange column. After this volume had been absorbed by the column, a 200-µl water rinse of the assay tube was applied; this volume was absorbed, and an additional 200 µl of water was applied and allowed to be absorbed by the column. Until this point, the column eluate had been discarded. Next, the columns were positioned over counting vials, 1 ml of water was applied to the column, and the resulting 1 ml of eluate was collected in the counting vials. Aquasol (10 ml)was added to the vials, which were then counted in a liquid scintillation counter.

For the purposes of comparing the assay methods, HMG CoA reductase was assayed also by ether extraction, followed by thin-layer chromatography as described by Brown et al. (5).

HMG CoA lyase assay

The principle of the HMG CoA lyase assay is outlined in Fig. 1. HMG CoA lyase cleaves the 3-^{14}C-HMG CoA to 3-^{14}C-acetoacetate and acetyl-CoA. Heat causes the decarboxylation of the 3-^{14}C-acetoacetate to yield 2-^{14}C-acetone and CO_2. The 2-^{14}C-acetone then is separated from the 3-^{14}C-HMG CoA by anion exchange chromatography.

The HMG CoA lyase was assayed in a 2-ml flame-sealable ampule containing 100 mM potassium phosphate buffer (pH 7.5), 5 mM DTT, 5 mM $MgCl_2$, 1.5 mM (D,L)-HMG CoA and the enzyme sample, in a total volume of 200 µl. Incubation took place at 37°C; a standard assay time was 2 min. Prior to incubation the assay and the enzyme were kept in an ice-water or dry ice-alcohol bath; a return to the cold bath after the 37°C incubation terminated the enzyme reaction. Next, assay ampules were removed individually from the ice bath, rapidly sealed with an oxygen torch, and returned to the bath. The sealed assay ampules were then placed into a preheated autoclave and heated at 240°F for 20 min.

After removal from the autoclave and cooling in an ice bath to condense the acetone, the ampule seals were broken and the assay contents were applied directly to anion exchange columns. The steps at this stage of the procedure were identical with those of the reductase assay; i.e., the assay volume, a 200-µl rinse and an additional 200 µl of water were applied to the column, and the eluate was discarded. The eluate from the next 1 ml of water applied to the column then was collected in counting vials, to which 10 ml of aquasol was then added.

The vials were counted in a liquid scintillation counter using the full ^{14}C-window.

Were it not for a keto-enol tautomerism that allows the hydrogen atoms of acetoacetate to exchange, an internal standard of ^{3}H-acetoacetate might have been desirable to monitor the efficiency of ^{14}C-acetone recovery. As will be seen in the Results section, however, the recovery of ^{14}C-acetone in this assay was sufficiently high and consistent that an internal standard was not necessary.

An advantage of the absence of a ^{3}H-internal standard is that the full ^{14}C-window was available for counting, thereby permitting the use of a relatively low specific activity ^{14}C-HMG CoA substrate. An HMG CoA specific activity that yields 600 cpm/nmol in the full ^{14}C-window (85% counting efficiency in aquasol with 1 ml of water) was convenient for the HMG CoA lyase assay. By comparison, for an HMG CoA reductase assay a specific activity that yields in excess of 2,000 cpm/nmol in the partial ^{14}C-window (45% counting efficiency) was desirable.

EXPERIMENTAL RESULTS AND DISCUSSION

I. HMG CoA Reductase and HMG CoA Lyase Assays

HMG CoA reductase assay

To establish the validity of this assay method, initially the ^{14}C product of ^{14}C-HMG CoA that eluted through the anion exchange column had to be identified as ^{14}C-mevalonic acid lactone. Fig. 2 shows an autoradiographic comparison of HMG CoA reductase assays and assay blanks (no enzyme present) which were each handled in two ways. In one case, the assay and blank samples were applied to anion exchange columns. The 1-ml eluates, which normally would have been counted directly, were extracted instead into ether, evaporated, reconstituted in a small

Figure 2. An autoradiograph of a TLC plate comparing two methods of HMG CoA reductase assay. An HMG CoA reductase assay (B) and assay blank (A) were extracted directly into ether, evaporated to dryness, reconstituted with a small volume of acetone, and spotted on the TLC plate. Another HMG CoA reductase assay (C) and assay blank (D) were applied to anion exchange columns. The 1-ml eluate from each was extracted into ether and then treated as were A and B. Lane E was spotted with standard ^{14}C-mevalonolactone. The TLC plate was developed in acetone: benzene (1:1).

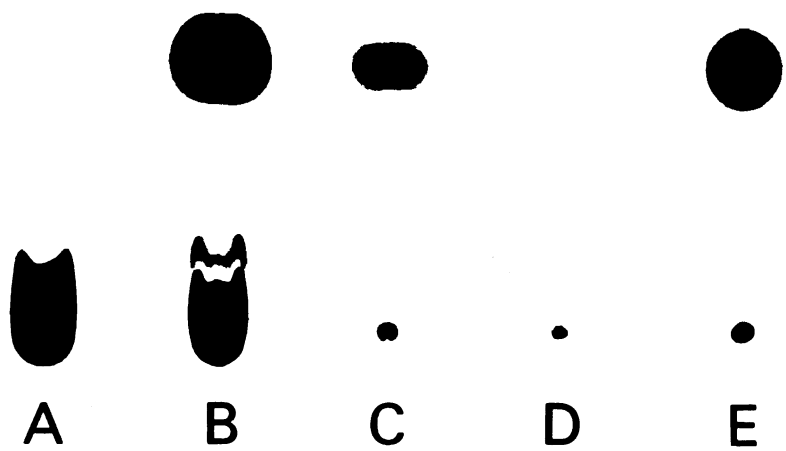

Figure 2. An autoradiograph of a TLC plate comparing two methods of HMG CoA reductase assay.

volume of acetone, spotted on a TLC plate, and developed in acetone: benzene (1:1). Alternatively, the assay and assay blank were extracted directly into ether and then handled exactly as the column eluates were. The autoradiograph shows that the ^{14}C-HMG CoA product obtained by direct ether extraction (B) and by anion exchange chromatography (C) are both identical with the standard ^{14}C-mevalonolactone (E). (The ^{14}C-mevalonolactone of "C" is lighter than that of "B" for three reasons: product loss occurred during the column step, conversion of mevalonolactone to mevalonic acid may have occurred within the neutral eluate, and ether extraction of the 1-ml volume of eluate was less efficient than that of the 200-µl assay volume.) Comparison of the blanks reveals a substantial amount of radioactivity at and near the origin of the ether-extracted blank (A) that is absent in the column eluate blank (D).

The identity of the column product as mevalonic acid lactone was also demonstrated by anion exchange chromatography. In this experiment an HMG CoA reductase assay, with ^{3}H-mevalonic acid lactone standard also present, was applied to a 12-ml column of the anion exchange resin. The water eluate was collected in sequential 10-drop volumes and counted. Fig. 3 shows these results, in which the ^{3}H and ^{14}C counts both have been normalized by adjusting the total recovered counts to 100%. The figure emphasizes the identity of the elution pattern of the ^{3}H-mevalonic acid lactone standard and the ^{14}C enzyme product of ^{14}C-HMG CoA.

To compare this assay method directly with that of Brown et al. (5), washed liver microsomes from a fed rat were isolated and duplicate assays of HMG CoA reductase activity at five different microsomal

Figure 3. Anion exchange chromatographic comparison of ^{3}H- mevalonolactone and the microsomal enzyme product of [3-^{14}C]HMG CoA. [5-^{3}H]mevalonic acid was added to a completed HMG CoA reductase assay; the assay mixture was acidified and then applied to a 12-ml-volume anion exchange column. The column was eluted with water and the eluate was collected in sequential 10-drop volumes and counted. The ^{3}H and ^{14}C counts have been normalized by adjusting the total recovery of each to 100%.

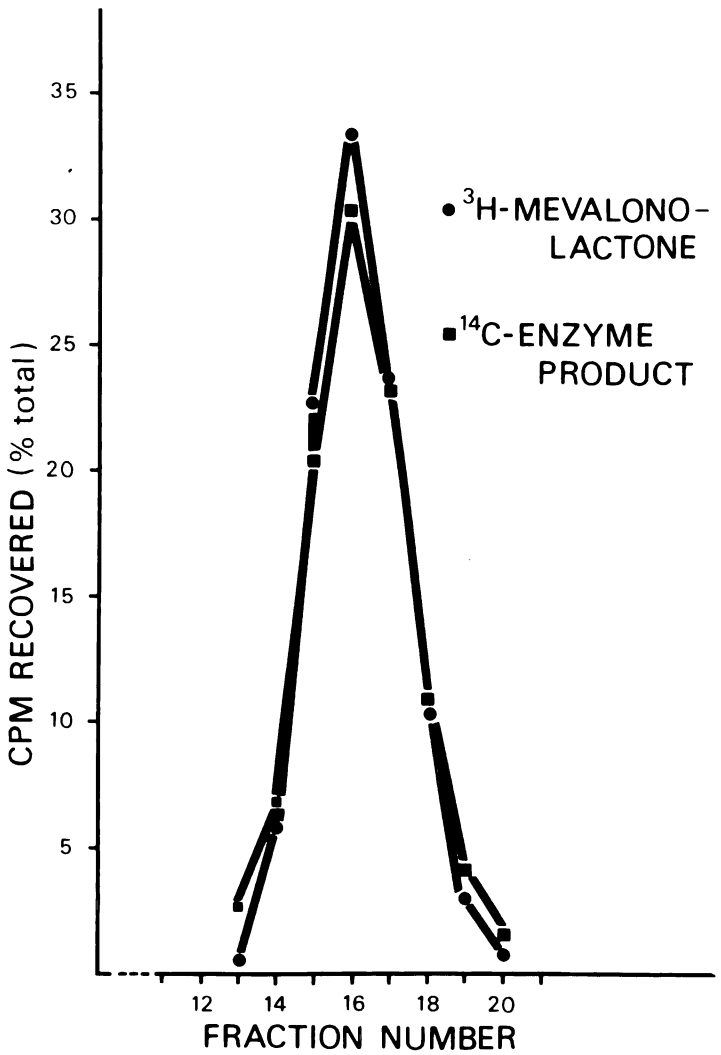

Figure 3. Anion exchange chromatographic comparison of ^{3}H-mevalono-lactone and the microsomal enzyme product of [3-^{14}C]HMG CoA.

protein concentrations were compared. Fig. 4 shows that the results yielded by the two methods are identical.

In general, the column method was found to be preferable to the ether extraction-TLC procedure, because it was more rapid, the internal standard recovery was higher (generally 75%, as compared to approximately 50% by the method of Brown et al.), and the variation between duplicate assays was less.

HMG CoA lyase assay

Identity of the product. To establish the validity of this assay, initially it was necessary to identify the cytosolic product of ^{14}C-HMG CoA that eluted through the column as ^{14}C-acetone. In one experiment the product in question, along with added unlabeled acetone, was precipitated with 2:4 dinitrophenylhydrazine. The bright yellow 2:4 dinitrophenylhydrazone derivative of acetone was dissolved in benzene, spotted on a thin layer chromatography plate, and developed in two dimensions with toluene and chloroform:diethyl ether (1:1) (45). The spot on the autoradiographic film of this plate, which reflected the position of the ^{14}C product, corresponded exactly in size and configuration with the yellow spot on the original thin layer plate. Thus, by these criteria--co-precipitation and chromatographic properties--the eluted ^{14}C product was identical with acetone.

Identity of the ^{14}C product as acetone was also shown by anion exchange chromatography. The ^{14}C product of a lyase assay, along with ^{3}H-mevalonic acid lactone, was applied to a 12-ml anion exchange column. Fig. 5B shows the water elution pattern of these two compounds; Fig. 5A shows the elution pattern of ^{3}H-mevalonic acid lactone and ^{14}C-acetone

Figure 4. Comparison of HMG CoA reductase activity as assayed by extraction and TLC (open symbol) and by anion exchange column (solid symbol). Quadruplicate assays were run at five different concentrations of microsomal protein. Duplicates of each protein concentration then were subjected either to the ether extraction, TLC procedure of Brown et al. (5), or to anion exchange column procedure described in Methods.

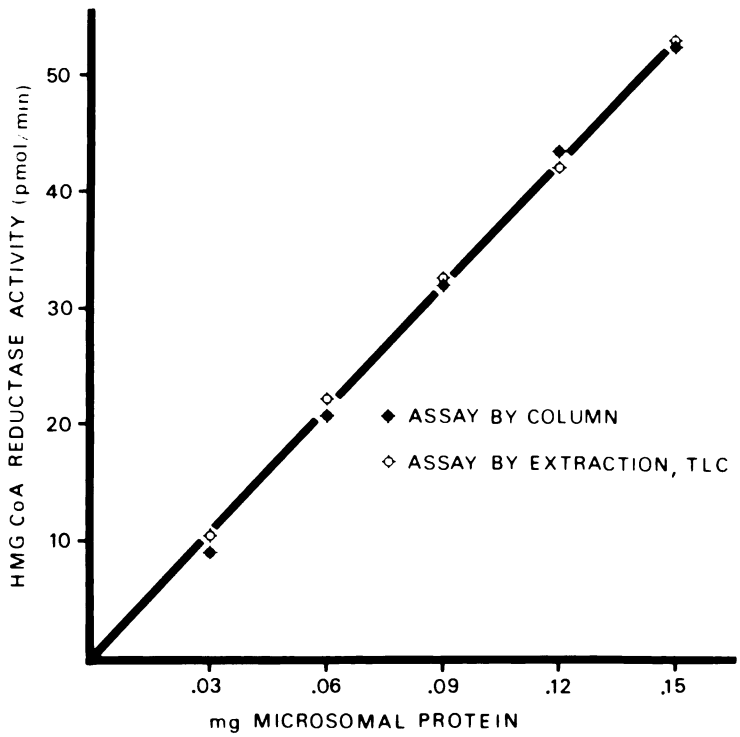

Figure 4. Comparison of HMG CoA reductase activity as assayed by extraction and TLC (open symbol) and by anion exchange column (solid symbol).

Figure 5. Anion exchange chromatographic comparison of [2-^{14}C]acetone and the heated, cytosolic enzyme product of [3-^{14}C]HMG CoA. Two 12-ml-volume anion exchange resin columns were constructed. Column A was loaded with standard [2-^{14}C]acetone and standard [5-^{3}H]mevalonolactone. Column B was loaded with the contents of an HMG CoA lyase assay that had been sealed and heated (as described in Methods) and standard [5-^{3}H]mevalonolactone. The columns were eluted with water, and sequential 10-drop volumes were collected and counted. The ^{3}H and ^{14}C counts have been normalized by adjusting the total recovery of each to 100%.

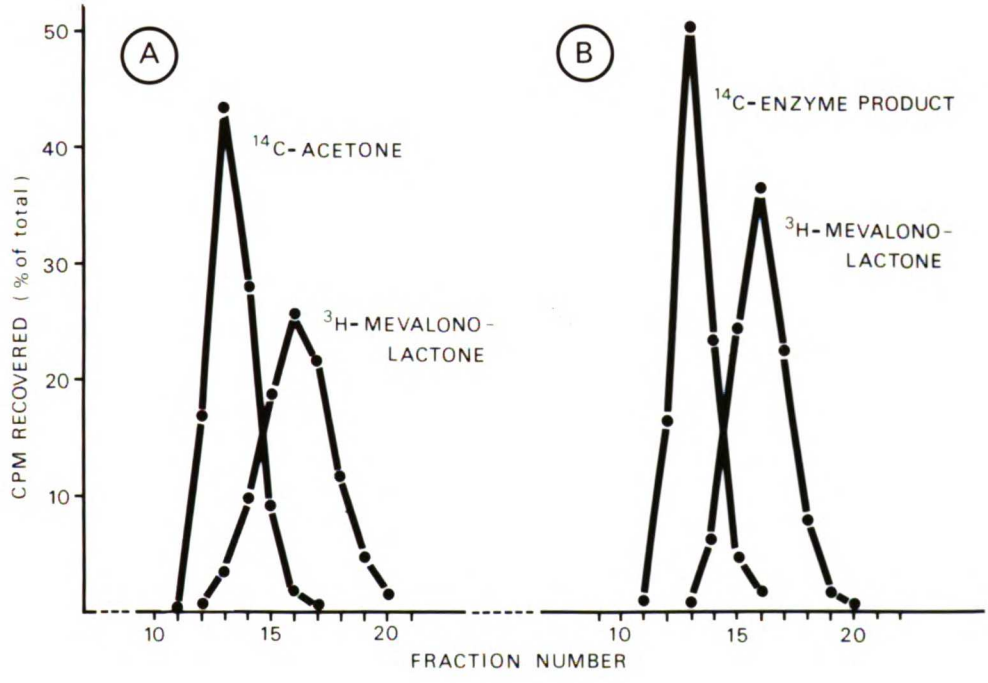

Figure 5. Anion exchange chromatcgraphic comparison of [2-^{14}C]acetone and the heated, cytosolic enzyme product of [3-^{14}C]HMG CoA.

standard. The identity of the ^{14}C product as ^{14}C-acetone is strongly suggested by their identical elution patterns.

The possibility that the ^{14}C product of the HMG CoA lyase assay was either ^{14}C-acetoacetate or ^{14}C-β-hydroxybutyrate was specifically eliminated by the finding that only trace amounts of ^{14}C eluted from the standard anion exchange column when either of these two "ketone bodies" was applied. The elution patterns of these few, and probably contaminant, ^{14}C counts were found also to be distinct from the standard ^{14}C-acetone.

Efficiency of recovery of ^{14}C-acetone from ^{14}C-acetoacetate. To determine the recovery of ^{14}C-acetone from ^{14}C-acetoacetate by this assay method, 3-^{14}C-acetoacetate was prepared in the following manner: ethyl-3-^{14}C-acetoacetate, at a concentration of 5 mM (9.2 mCi/mmol), was heated in the presence of 2 eq of NaOH at 50°C for 4 h and then neutralized by adding 1 eq of HCl. Whereas ^{14}C-acetoacetate was adsorbed by the anion exchange column, ethyl-^{14}C-acetoacetate elutes through the column (with a pattern distinct from that of ^{14}C-acetone). By this criterion of column elution, the efficiency of ethyl acetoacetate conversion to acetoacetate was 90%.

This ^{14}C-acetoacetate preparation was used immediately to determine the efficiency of recovery of ^{14}C-acetone. A known amount of ^{14}C-acetoacetate was added to assay ampules; the ampules were then handled in the manner of an HMG CoA lyase assay. By this procedure 91% of the added ^{14}C-acetoacetate was recovered as ^{14}C-acetone. The high efficiency and uniformity of product recovery made an internal standard within the assay unnecessary.

The stability of ^{14}C-acetoacetate in the cytosol. The validity of this assay for HMG CoA lyase also depends upon the stability of newly synthesized ^{14}C-acetoacetate within the cytosol under the standard assay conditions. The possibility of depletion of the ^{14}C-acetoacetate (e.g., by reduction to β-hydroxybutyrate or activation to acetoacetyl-CoA) was investigated by comparing the recovery of ^{14}C-acetoacetate from assay vials containing either cytosol or assay media only (blanks). A range of acetoacetate concentrations appropriate to those generated in HMG CoA lyase assays (0.32-9.6 μM) was employed. The duration of incubation was 2 min, and thereafter the assays were handled in the same manner as in the HMG CoA lyase procedure. At all ^{14}C-acetoacetate concentrations the recoveries of ^{14}C-acetone from blank and cytosol-containing assays were identical. It was therefore concluded that ^{14}C-acetoacetate is, in fact, stable in the presence of cytosol under the conditions of this assay.

HMG CoA lyase: preliminary studies. This method of HMG CoA lyase assay offered a means of characterizing the kinetic features of the enzyme found in both the cytosol and the mitochondria. Fig. 6 shows the results of an experiment in which HMG CoA lyase was assayed over a 20-fold range of cytosolic protein concentration. The linearity of activity with protein concentration was generally excellent provided the assay time was short (here, 2 min) and there was not a major substrate depletion (here, a maximum of 14%).

HMG CoA lyase was been found to be relatively unstable at 37°C in phosphate buffer, with substantial activity lost during a 30-min incubation. Fig. 7 shows the results of an experiment in which HMG CoA lyase was assayed at a range of protein concentrations under two conditions:

Figure 6. HMG CoA lyase activity as a function of cytosolic protein concentration. HMG CoA lyase was assayed in duplicate by the anion exchange method over a 20-fold range of cytosolic protein. See Experimental Methods for details of assay procedure.

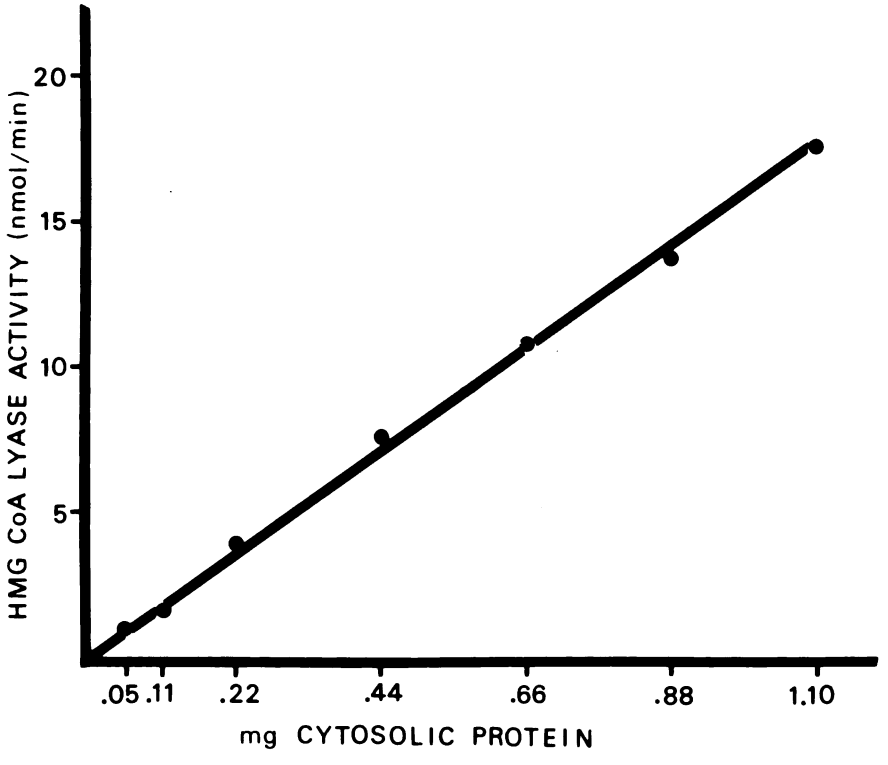

Figure 6. HMG CoA lyase activity as a function of cytosolic protein concentration.

Figure 7. The effect of preincubation on HMG CoA lyase activity. Quadruplicate samples of cytosolic protein at five different concentrations were added to ampules containing lyase assay media. Two vials of each protein concentration were preincubated at 37°C for 30 min prior to the addition of [3-^{14}C]HMG CoA. The other two assay ampules (without preincubation) received the [3-^{14}C]HMG CoA immediately before the initial warming to 37°C. The duration of the assay was 2 min. See Experimental Methods for further details.

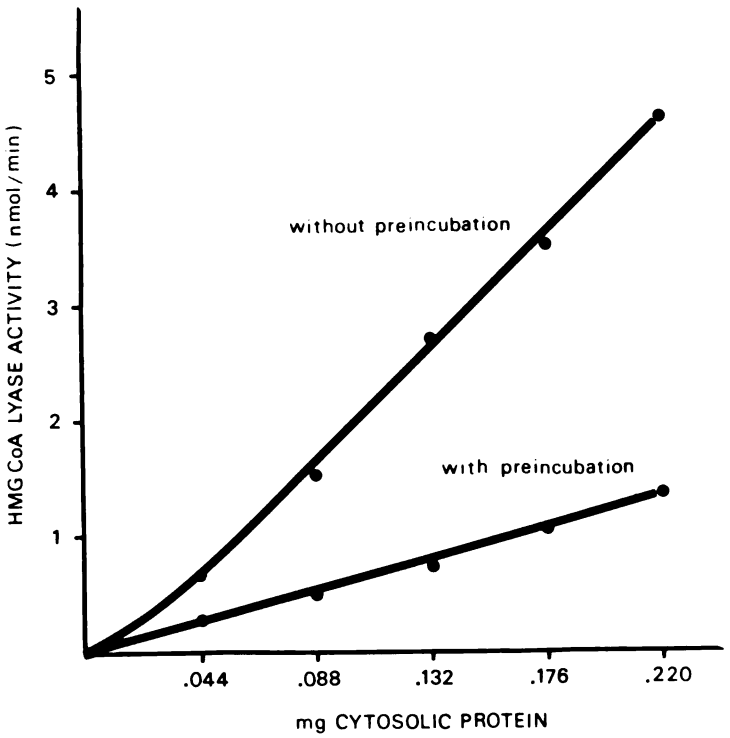

Figure 7. The effect of preincubation on HMG CoA lyase activity.

immediately after warming to 37°C without preincubation, and after a 30-min preincubation at 37°C. In both cases the enzyme activity was a linear function of protein concentration, but the 30 min of warming caused a 70% loss of the enzyme activity. This characteristic of the enzyme, which is dealt with in subsection II, made it advisable to keep the enzyme preparation cold prior to assay. This feature, however, can be exploited when HMG CoA reductase is being assayed in the presence of "contaminating" HMG CoA lyase (see below).

Distinguishing the products of HMG CoA lyase and HMG CoA reductase

Under these assay conditions a small amount of ^{14}C-acetoacetate generated by HMG CoA lyase spontaneously decarboxylates to ^{14}C-acetone, even in the absence of the heating step in the lyase assay procedure. In an HMG CoA reductase assay, therefore, the presence of HMG CoA lyase will cause an "artifactual" increase in the ^{14}C eluting through the anion exchange column. Conversely, it may also seem possible that HMG CoA reductase-generated ^{14}C-mevalonic acid could "artifactually" affect the results of an HMG CoA lyase assay.

With the assay conditions described here, the reductase contamination of a lyase assay is not an actual problem in assaying lyase activity. HMG CoA reductase requires NADPH as a substrate, and the lyase assay does not contain the NADP, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase components of the HMG CoA reductase assay. In a standard HMG CoA lyase assay, therefore, HMG CoA reductase activity is undetectable. A second factor eliminating this type of assay product confusion is that mevalonic acid must be cyclized to the lactone form by a low pH in order to be eluted from the resin column; this acidification step is not included in the lyase assay.

The contamination of an HMG CoA reductase assay with HMG CoA lyase is a more serious potential problem, because the lyase is a very active enzyme in the liver and a small fraction of the lyase product (i.e., ^{14}C-acetone) will elute with the reductase assay procedure. HMG CoA lyase is a soluble enzyme whereas HMG CoA reductase, with standard preparative methods, is primarily microsomal. When possible, therefore, washing microsomes free of HMG CoA lyase activity is the best way to prevent HMG CoA lyase contamination of an HMG CoA reductase assay. When the preparation of washed microsomes from tissue or cells is not feasible, the following acetone-evaporation step may be added to the HMG CoA reductase assay.

Fig. 8 demonstrates the feasibility of removing "contaminating" ^{14}C-acetone by evaporation. In this experiment, known amounts of standard ^{3}H-mevalonic acid lactone and ^{14}C-acetone were added to 1 ml of water in counting vials. The vials then were placed on a warming plate (45°C), removed at various time intervals, and after the addition of aquasol the ^{14}C was assayed. Although the ^{3}H-mevalonic acid lactone was non-volatile under these conditions, it can be seen that the ^{14}C-acetone rapidly disappeared from the vials and that after an hour none was present. The ^{14}C-acetone standard is commercially prepared from lithium ^{14}C-acetate and is 97-99% pure. This 1-3% impurity undoubtedly accounts for the remaining 0.7% of the added ^{14}C counts.

HMG CoA lyase also can interfere with an HMG CoA reductase assay by depleting the HMG CoA substrate. (This problem, of course, exists regardless of the assay technique.) The problem can be minimized by preincubating the assay for 30 min prior to adding ^{14}C-HMG CoA. This preincubation does not affect microsomal HMG CoA reductase activity,

Figure 8. Time course of [2-^{14}C]acetone evaporation and non-volatility of [5-^{14}C]mevalonolactone. A series of counting vials containing 1 ml of water and equivalent amounts of [2-^{14}C]acetone and [5-^{14}C]mevalonolactone were placed on a 45°C warming plate and removed after 5, 10, 20, 40 and 60 min (the 0-time vials were not placed on the warming plate). Upon removal, each counting vial immediately received 10 ml of aquasol and was counted.

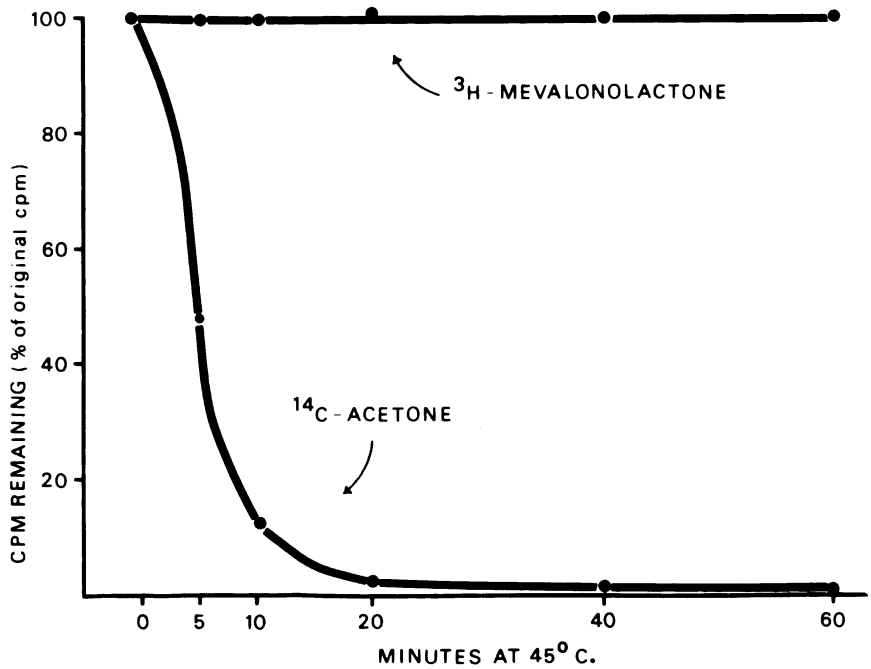

Figure 8. Time course of [2-^{14}C]acetone evaporation and non-volatility of [5-^{14}C]mevalonolactone.

but it does eliminate approximately 70% of the HMG CoA lyase activity present (Fig. 7).

To demonstrate the feasibility of accurately assaying HMG CoA reductase in the presence of HMG CoA lyase, an experiment was carried out in which liver HMG CoA reductase was assayed in the presence of liver cytosol. This situation was the "worst possible" for this assay for two reasons. First, the HMG CoA lyase activity is greater in liver than in other tissues; second, the liver was selected (at midday) so as to have a low microsomal HMG CoA reductase activity. In this experiment a rat liver was homogenized in the presence of $MgCl_2$ (to maximize the HMG CoA lyase activity), and the microsomes were washed in homogenization buffer without $MgCl_2$. The microsome and cytosol preparations were adjusted to the same final concentration of 1 g liver/4 ml. All assays that contributed to the data in Tables 1 and 2 were carried out with equivalent amounts of liver (i.e., microsomes and/or cytosol from 5 mg of liver). The enzyme specific activities shown in Tables 1 and 2 are expressed both in terms of milligrams of protein and milligrams of liver. The activity that is normalized to liver weight is the more useful when comparing the relative activities of the two enzymes in the two cell fractions.

Table 1 shows that after a 30-min preincubation, the cytosolic HMG CoA lyase activity of 917 pmol/min per milligram of liver was reduced to 272 pmol/min per milligram of liver. These assays, done under optimal conditions, show initial rates of activity. Table 1 also shows that when the equivalent amount of lyase was assayed in a reductase-assay medium for 45 min, the lyase activity decreased to an apparent 138 pmol/min per milligram of liver. When the assay was

TABLE 1. Actual and apparent HMG CoA lyase activity under different assay conditions

Assay conditions	pmol/min/ mg protein	pmol/min/ mg liver
Actual activity*		
Lyase media,† no preincubation, 2-min assay; tube sealed, heated	20,860	917
Lyase media, 30-min preincubation, 2-min assay; tube sealed, heated	6,177	272
Apparent activity‡		
Reductase media,§ 30-min preincubation, 45-min assay; tube sealed and heated	3,132	138
Reductase media, 30-min preincubation, 45-min assay, tube open, acidified, not heated (reductase activity subtracted)	427	19
Any assay condition, followed by evaporation of column eluate	undetectable	

*Actual activity data reflect initial rates of activity under conditions that assure efficient conversion of ^{14}C-acetoacetate to ^{14}C-acetone.

†See Materials and Methods.

‡Apparent activity data reflect rates of activity that are low due to time-dependent loss of activity, inefficient conversion of ^{14}C-acetoacetate to ^{14}C-acetone, or evaporation of the ^{14}C-acetone product.

§See Materials and Methods.

TABLE 2. HMG CoA reductase activity assayed on liver cell fractions in the absence and presence of HMG CoA lyase

Cell fraction	pmol/min/mg protein	pmol/min/mg liver
Microsomes (lyase absent)	245	2.94
Cytosol (lyase present)	20	0.89
Microsomes plus cytosol (lyase present)	69	3.86

All assays were preincubated without substrate for 30 min and then assayed with substrate for 45 min. Column eluates in counting vials were warmed to 45°C and allowed to evaporate for 1 h.

treated as a reductase assay (i.e., acidified, no heating), an apparent activity of 19 pmol/min per milligram of liver was seen. Regardless of the assay conditions, when the lyase assay-column eluate was evaporated on the warming plate this apparent activity dropped to undetectable levels.

Table 2 shows the results of HMG CoA reductase assays (that included the evaporation step) of the microsomes, cytosol, and the combined microsomes and cytosol. The cytosol, itself, had significant HMG CoA reductase activity. The identity of this non-evaporative, cytosolic metabolite as mevalonic acid lactone was confirmed by thin-layer chromatography. It is of interest here that the HMG CoA reductase activity of the microsomes and cytosol, when mixed and assayed together, is exactly equal to the sum of the two when assayed separately. This result was made possible by the use of a high concentration (750 μM) of HMG CoA. At lower concentrations of HMG CoA, such as the 160 μM used in the assay of washed microsomes, the substrate is easily depleted by the lyase, causing a partial, or at times, a total apparent inhibition of HMG CoA reductase activity.

To summarize, the presence of HMG CoA lyase poses two potential complications for the assay of HMG CoA reductase. The first is substrate depletion, which can be overcome by 1) preincubation in phosphate buffer, which results in a marked loss of HMG CoA lyase activity; and 2) the use of relatively high substrate concentrations. The second potential problem of product separation, one peculiar to this assay, is also readily resolved. In this case, the lyase-generated acetone can be evaporated, leaving behind only the reductase-generated mevalonolactone.

Discussion

These studies describe procedures for the rapid, ion exchange assay of both HMG CoA reductase and HMG CoA lyase. The HMG CoA reductase method is a simplification of an anion exchange procedure described earlier by Huber *et al.* (20) and Avigan *et al.* (3). Neither of these reports, however, verified the technique by identifying the eluting product as mevalonolactone. Moreover, the recent report of Ness and Moffler (36) casts some doubt on the accuracy of this method because of the elution of "a product which is similar to but not identical with mevalonate." The data shown here, however, by drawing from two approaches, affirm the validity of the HMG CoA reductase assay. First, by both thin-layer chromatography (Fig. 2) and anion exchange chromatography (Fig. 3), the microsomal enzymatic product of ^{14}C-HMG CoA appears to be exclusively ^{14}C-mevalonate. Second, identical results are obtained when HMG CoA reductase is assayed by the extraction-TLC method of Brown *et al.* (5) or by this method of anion exchange chromatography (Fig. 4). It is difficult to reconcile or compare these results with those of Ness and Moffler (36), because these authors did not report any features of their non-mevalonate product.

While the high and consistent recoveries of mevalonolactone (as judged by the recovery of ^{3}H-mevalonolactone internal standard and by the closeness of duplicate assay values) are positive features of this assay method, its chief advantage lies in its rapidity. The anion exchange columns can be prepared during the assay incubation period. Upon completion of the assay, and the conversion of ^{14}C-mevalonic acid to ^{14}C-mevalonolactone, assays can be applied to anion exchange columns and be ready for scintillation counting within minutes.

The method of assaying HMG CoA lyase (outlined in Fig. 1) differs markedly from previously published procedures (10,45,49). The validity of this method was shown in several ways. First, the identity of the ultimate ^{14}C-product as ^{14}C-acetone was confirmed by thin-layer chromatography (i.e., co-identity of 2,4-dinitrophenylhydrazone derivatives of the labeled product and unlabeled acetone) and by anion exchange chromatography (Fig. 4). The recovery of 91% of a preparation of ^{14}C-acetoacetate as ^{14}C-acetone was also shown. Finally, the stability of the ^{14}C-acetoacetate in the assay mixture was demonstrated, thereby eliminating the possibility of product depletion causing an underestimate of HMG CoA lyase activity.

This method of HMG CoA lyase assay is clearly less tedious, faster, and more specific than assay procedures that utilize spectrophotometric measurement of either acetoacetate (49) or NADH (46). The assay method of Clinkenbeard *et al.* (10), in which the synthesis of ^{14}C-acetate from ^{14}C-HMG CoA is inferred by the evaporative disappearance of counts from the background of non-volatile ^{14}C-HMG CoA, may be as rapid as this anion exchange method. However, the anion exchange method is inherently more sensitive in that it does not include the error introduced by the assumption that a uniform amount of ^{14}C-HMG CoA is added to each assay tube. Further, it offers the advantage of measuring the appearance of a product, rather than the disappearance of substrate.

The presence of HMG CoA lyase can complicate an HMG CoA reductase assay, because lyase-generated ^{14}C-acetone will elute along with ^{14}C-mevalonolactone (albeit with a distinct elution pattern). This potential complication is easily resolved, however, by evaporating the

^{14}C-acetone from the eluate, leaving behind only the ^{14}C-mevalonolactone (Fig. 8 and Tables 1 and 2).

HMG CoA lyase, simply by depleting available HMG CoA, can profoundly inhibit the apparent activity of HMG CoA reductase. It is therefore to the advantage of an investigator to be aware of the presence of HMG CoA lyase within an HMG CoA reductase assay. Thus, while the anion exchange method of HMG CoA reductase assay is subject to ^{14}C-acetone interference (whereas extraction-TLC methods are not), this easily resolved "problem" is accompanied by the advantage of permitting the detection of HMG CoA lyase. Finally, these rapid anion exchange assays for HMG CoA reductase and HMG CoA lyase together offer techniques that are so parallel that they facilitate investigation of the two enzymes in a coordinated manner.

II. Characterization of HMG CoA lyase and its optimal in vitro conditions

Intracellular distribution and solubility of HMG CoA lyase.

Table 3 shows the results of an experiment in which a rat liver was homogenized in potassium phosphate buffer and fractionated simply into a crude 10,000 G pellet and supernatant, additionally microsomes were isolated from the 10,000 G supernatant. The 10,000 G pellet was assayed immediately after its isolation, and later, following its freezing, thawing and homogenization. This solubilization procedure more than doubled the enzyme activity of this cell fraction. When the frozen-thawed homogenate was subjected to centrifugation at 100,000 G, 74% of the activity was soluble whereas 26% remained particulate. The activity of the 10,000 G supernatant was not affected by freezing, and

TABLE 3. Intracellular distribution of hepatic HMG CoA lyase in phosphate buffer

	nmol/min/mg liver	% total cellular
10,000 G pellet		
Assayed fresh	2.33	
Frozen, thawed 100,000 G supernatant	3.55	
100,000 G pellet	1.25	
Total (sum of supernatant and pellet)	4.80	89
10,000 G supernatant	0.77	11
100,000 G pellet (microsomes)	undetectable	0

*Liver was homogenized in 0.3 M sucrose, 50 mM potassium phosphate (pH 7.5), 5 mM DTT, and 5mM $MgCl_2$.

the activity of the enzyme in the microsomes was undetectable. In terms of the total cellular distribution of activity, 89% was found in the 10,000 G pellet and 11% was in the 10,000 G supernatant.

Table 4 shows the results of a similar experiment in which the liver was homogenized in BICINE buffer. In this case, the whole homogenate, the 300 G pellet, the 10,000 G pellet (collected after removal of the 300 G particulate fraction), and the 10,000 G supernatant were all frozen, thawed, and homogenized, and the resulting soluble and particulate fractions were separated by centrifugation at 100,000 G. It can be seen that the majority (70%) of the total cellular activity was associated with the crude 300 G pellet (containing whole cells, debris, nuclei, and mitochondria); 18% was associated with the 300-10,000 G pellet (predominantly mitochondria); and 7% was within the 10,000 G supernatant (i.e., the cytosol, since the microsomes are without activity. Approximately 70% of all particulate activity was found to be solubilized by the procedure described.

These two experiments together suggest that the majority of the hepatic HMG CoA lyase activity is mitochondrial, while a small percent is consistently found in the cytosol. The activation by freezing and thawing seen in Table 3 suggests that by breaking open the mitochondria, the enzyme originally contained within the organelle has greater access to the substrate.

In the studies that follow, the enzyme used has been derived from either the soluble portion of the 300-10,000 G (mitochondrial) pellet, or from the cytosol.

TABLE 4. Intracellular distribution and solubility of hepatic HMG CoA lyase activity in BICINE buffer*

	nmol/min/mg liver	Distribution within fraction	Distribution within liver
Whole homogenate			
Soluble	7.34	73	
Particulate	2.76	27	
Sum	10.10		100
300 G pellet			
Soluble	4.63	66	
Particulate	2.38	34	
Sum	7.01		70
10,000 G pellet			
Soluble	1.24	68	
Particulate	0.58	32	
Sum	1.82		18
10,000 G supernatant			
Soluble	0.69	100	7
Particulate	--		

*Liver was homogenized in 0.3 M sucrose, 50 mM BICINE buffer, 5 mM DTT, 5 mM $MgCl_2$. Following differential centrifugation, each fell fraction was frozen, thawed and homogenized, and centrifuged at 100,000 G. Both the supernatant (soluble) and the pellet (particulate) were then assayed.

Dependence of HMG CoA lyase on DTT and $MgCl_2$.

Table 5 shows the effects of added $MgCl_2$ and DTT upon HMG CoA lyase that was isolated in the absence of both these compounds. In this experiment, mitochondrial enzyme was not exposed to the added DTT or $MgCl_2$ until the time of the assay. This short period of exposure to DTT increased the enzyme activity to 178% of the control value, $MgCl_2$ exposure increased activity to 143% of the control value, and exposure of the enzyme to both compounds more than doubled the control rate of activity.

Figure 9 is another view of the importance of metal ion for the activity of HMG CoA lyase. In this case a cytosolic preparation of HMG CoA lyase, isolated in the absence of $MgCl_2$, was assayed in the presence of increasing concentrations of the chelating agent, EDTA. The enzyme is seen to be sharply sensitive to the presence of EDTA.

The thiol- and metal ion-dependence of HMG CoA lyase has been previously noted by Stegink and Coon (46). These data confirm that finding and justified the presence of DTT and $MgCl_2$ in the homogenate and assay media of the studies that follow. Further data on the effects of DTT and $MgCl_2$ on the stability of the enzyme are presented below.

HMG CoA lyase activity lability at 37°C

Figure 10 shows the results of an experiment in which mitochondrial HMG CoA lyase, isolated and assayed in potassium phosphate buffer, was found to lose activity very rapidly over a 20-min exposure to 37°C. The mitochondrial activity was assayed in its soluble form, as contained in intact mitochondria (maintained in 0.3 M sucrose), and as contained in osmotically damaged mitochondria (assayed in 0.15 M sucrose). The rates of loss of activity of all three preparations are similar, though

TABLE 5. The effects of DTT and $MgCl_2$ on mitosolic HMG CoA lyase*

Assay conditions	HMG CoA lyase activity	% of Control activity
	(nmol/min/mg liver)	
No additions (control)	2.02	100
10 mM DTT	3.60	178
10 mM $MgCl_2$	2.89	143
DTT + $MgCl_2$	4.11	203

*Enzyme was isolated in potassium phosphate buffer with no DTT or $MgCl_2$ present.

Figure 9. The effect of EDTA on HMG CoA lyase activity. Cytosol was prepared in a 100 mM phosphate buffer with DTT present and $MgCl_2$ absent. Assay samples were preincubated at 37°C for 5 min in the presence of varying concentrations of EDTA and then assayed for 10 min.

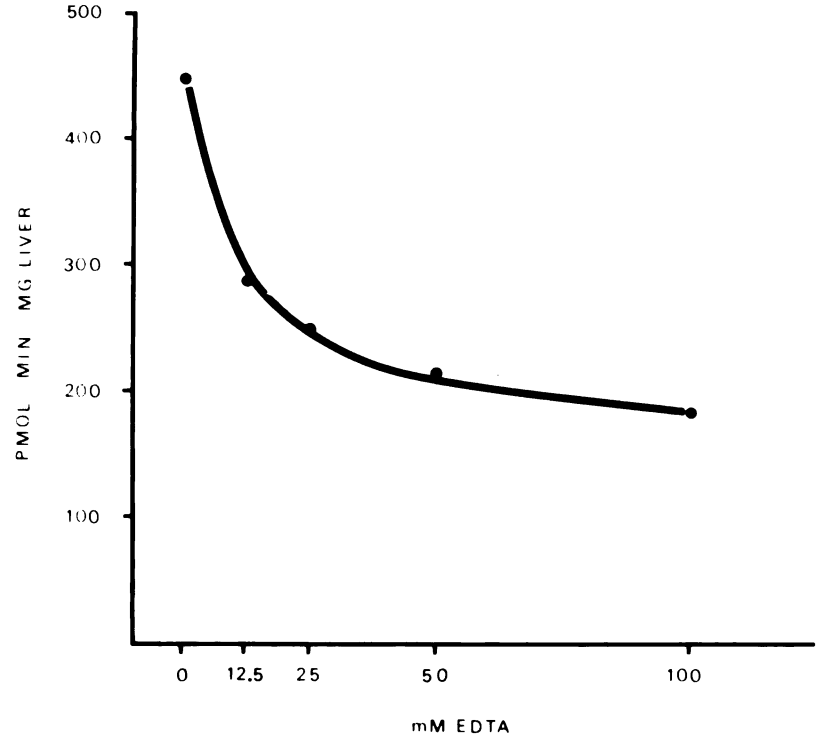

Figure 9. The effect of EDTA on HMG CoA lyase activity.

Figure 10. The effect of preincubation at 37°C on HMG CoA lyase activity within intact mitochondria and in soluble form. Mitochondria were isolated in the standard buffer. One portion of the preparation was used to prepare soluble enzyme. The other portion was divided again in two, one portion of which was maintained in 0.3 M sucrose-buffered solution and the sucrose concentration of the other was adjusted to 0.15 M. The respective sucrose concentrations of each preparation were maintained throughout the assay period.

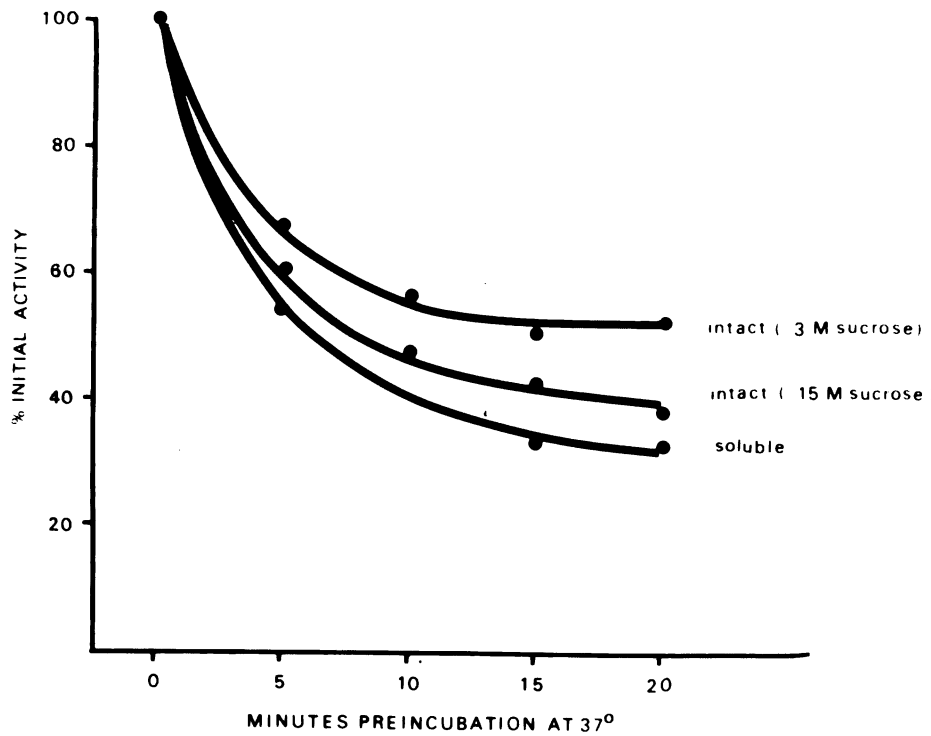

Figure 10. The effect of preincubation at 37°C on HMG CoA lyase activity within intact mitochondria and in soluble form.

the intact and "damaged" mitochondria appear to be somewhat more stable than the soluble enzyme preparation.

Another similar experiment was performed in which mitochondria were isolated in a 0.4 M sucrose buffer solution (in order to prevent osmotic breakage) and washed in the same solution (in order to remove any adhering soluble enzyme. This preparation also showed a profound time-dependent loss of activity, falling to a velocity 38% of the initial velocity within 20 min at 37°C.

Several aspects of this time- and temperature-dependent loss of enzyme activity within the potassium phosphate buffer are explored in the following sections.

Is the time- and temperature-dependent loss of activity reversed by cooling?

It seemed possible that the loss of activity observed over short periods of time at 37°C could be restored toward the initial observed activity by cooling or refreezing. This hypothesis was tested in the experiment depicted in Figure 11. Soluble mitochondrial enzyme was isolated in potassium phosphate buffer, assayed immediately and after 10 and 20 min exposure to 37°C. As expected, activity dropped quickly, to 44% of the original velocity within 20 min.

Following this exposure to 37°C, the enzyme preparation was divided into three portions and for the next 2 h either kept continuously at 37°C, cooled to 0° in an ice-water bath, or frozen. Each enzyme preparation then was assayed immediately and after a further 10- and 20-min exposure to 37°C. From Figure 11 it can be seen that all preparations continued to lose activity, though the rate of loss was greatest in that exposed continuously to 37°C. With reference to the question

Figure 11. The effects of preincubation, cooling, and dialysis on HMG CoA lyase activity. A standard phosphate buffered preparation of mitosolic lyase was divided into several portions and treated as follows: One portion was assayed over a 20-min period at 37°C; three portions were warmed over the same period and then either frozen, kept in an ice bath, or maintained at 37°C for the next 2 h; all three portions were then assayed over a 20-min period at 37°C. Two other portions were maintained in an ice bath for a 24-h period, during which one of the two was dialyzed against the same buffer of preparation while the other remained undialyzed; thereafter the two preparations were assayed for 20 min at 37°C.

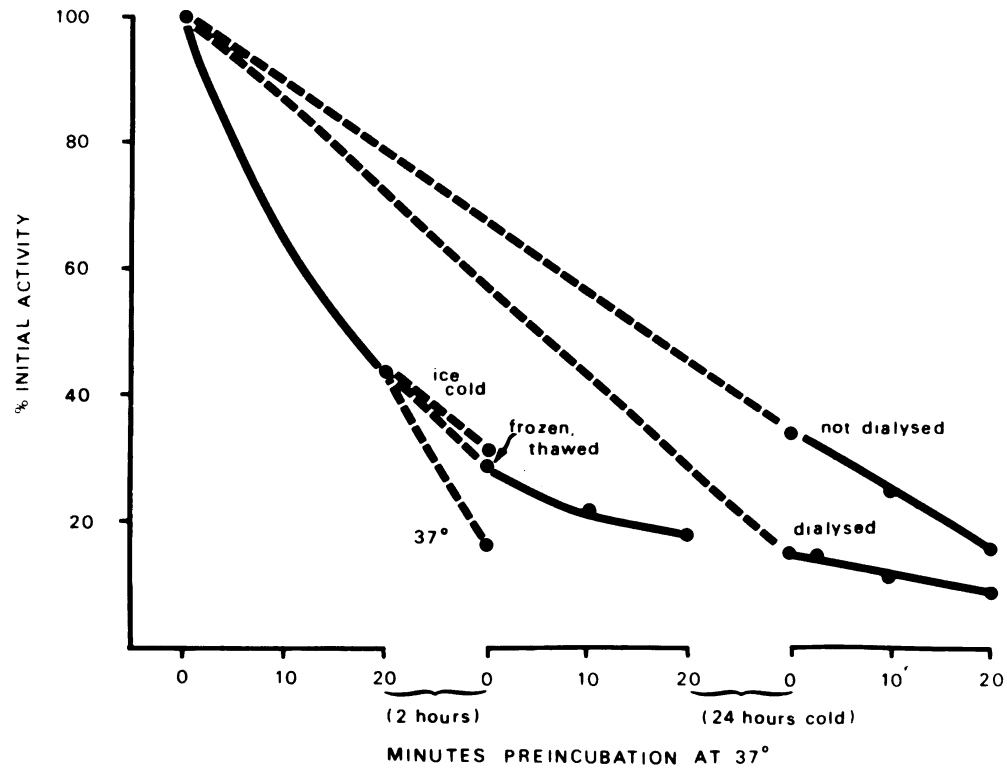

Figure 11. The effects of preincubation, cooling, and dialysis on HMG CoA lyase activity.

being tested by this experiment, however, it was noteworthy that neither cooling nor freezing the heat-exposed enzyme restored any activity.

The stability of the enzyme preparation over a longer period of time at 0° was also examined in this experiment, as was the possibility that dialysis might affect the stability of the enzyme. Accordingly, two portions of the original enzyme preparation were not exposed to 37°C but were kept continuously at 0° in an ice-water bath. One portion simply remained at this temperature for a 24-h period, while the other was dialyzed against the homogenization buffer over the same period of time. Following this 24-h period, the undialyzed and dialyzed preparations were assayed immediately after being brought to 37°C, and after 10- and 20-min exposure to this temperature. The undialyzed preparation was considered a reasonable "dialysis control" except for the fact that it was not subjected to the same physical agitation that the dialyzed preparation received. Examination of Figure 11 shows that both preparations lost considerable activity over the 24-h period, the undialyzed preparation falling to a rate of 34%, and the dialyzed preparation to a rate of 17% of that originally observed. Both preparations lost further activity upon exposure to 37°C.

According to these results, therefore, the loss of activity that occurs rapidly at 37°C also occurs at 0°, though at a slower rate. The loss of activity does not appear to be due to a dialyzable "inhibiting" factor, since it occurred despite dialysis.

Is the rate of enzyme activity loss affected by the enzyme concentration?

Figures 12, 13 and 14 show data from an experiment that examined the possibility (suggested, in part, by the data in Figure 7) that enzyme concentration could play a role in determining the rate of

Figure 12. The effect of preincubation on the activity of different enzyme concentrations. Two standard mitosolic enzyme preparations with a large difference in specific activity were utilized. Each preparation was used to make experimental samples with a fourfold difference in concentration (0.05 and 2.0 mg liver equivalents/assay). A and A' indicate the respective lower and higher concentrations of the lower specific activity preparation. B and B' represent the lower and higher concentrations of the higher specific activity preparation. All preparations were simultaneously assayed after varying periods of preincubation at 37°C. The data here are presented without normalization.

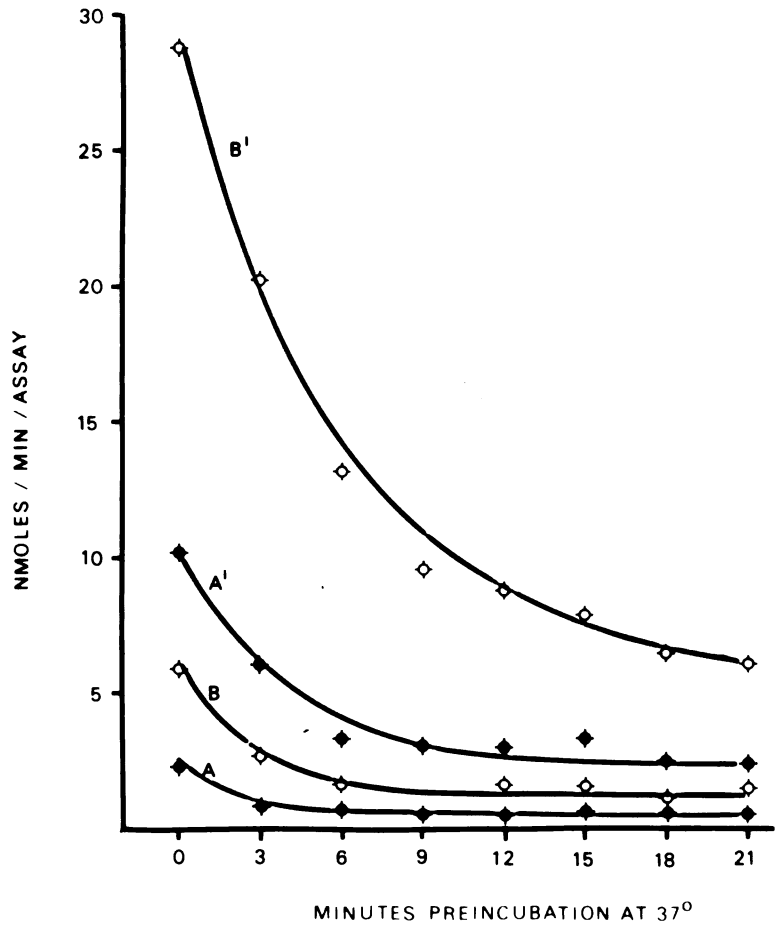

Figure 12. The effect of preincubation on the activity of different enzyme concentrations.

Figure 13. The effect of preincubation on the activity of different enzyme concentrations, with the data normalized. The data shown in Figure 12 have been normalized by adjusting the initial specific activity (no preincubation) of each sample to 100%.

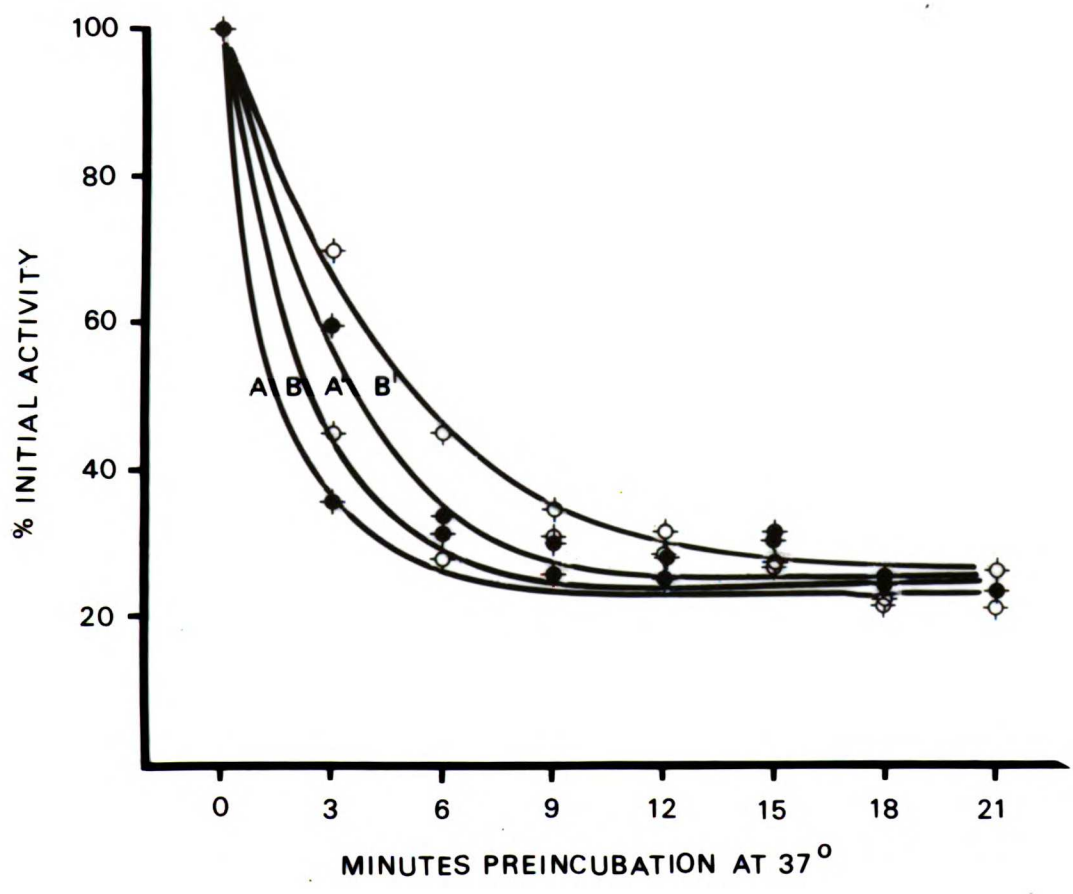

Figure 13. The effect of preincubation on the activity of different enzyme concentrations, with the data normalized.

Figure 14. The effect of preincubation of the $T_{\frac{1}{2}}$ of different enzyme concentrations. The time required for a 50% loss of the initial activity (see Figure 13) of each sample has been plotted as a function of its respective initial activity (see Figure 12).

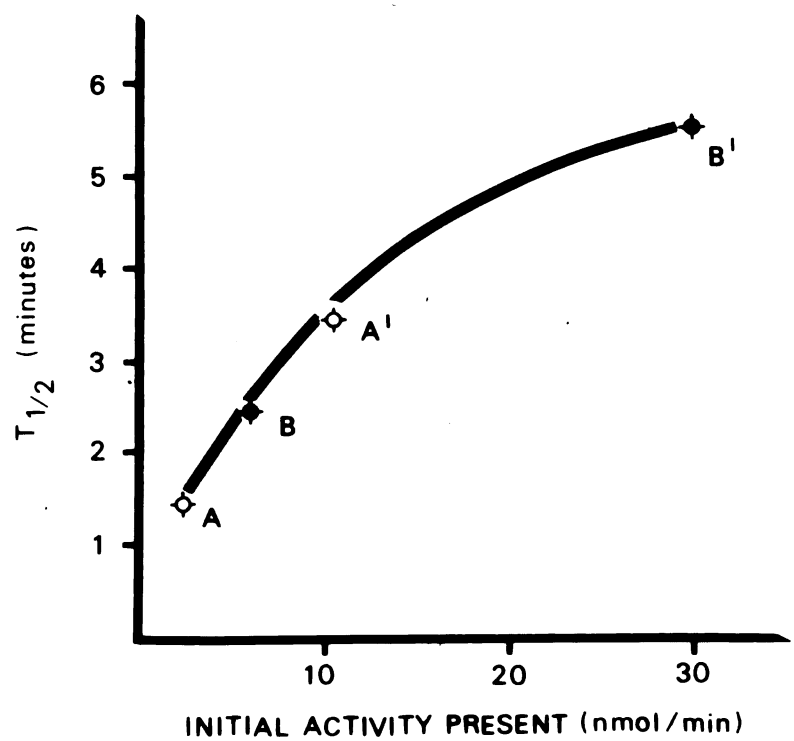

Figure 14. The effect of preincubation of the $T_{1/2}$ of different enzyme concentrations.

activity loss. Mitochondrial enzyme preparations from two animals (A and B) were used in this experiment. These two preparations were chosen because of the large disparity in their specific activities (A, 2.2 nmol/min/mg liver; B, 10.2 nmol/min/mg liver). In this experiment each preparation was assayed at two protein concentrations. In the figures A and B indicate assays that contained the mitochondrial protein of 0.5 mg of liver, and A' and B' indicate assays that contained the equivalent of 2 mg of liver.

In this experiment, the four experimental groups (A, A', B, B') were assayed immediately after being brought to 37°C and at 3-min intervals after exposure to this temperature over a total period of 21 min. Assays were of 2-min duration. Figure 12 shows these data expressed as nmol/min/assay tube. It can be seen that all experimental groups lost considerable activity, and that the greater the amount of activity originally present, the greater was the absolute amount of activity lost during the 21-min incubation period. The data in this form, however, do not show clearly the comparative rates of activity loss between the four groups.

In order to clarify this comparison, the data were normalized by adjusting the respective activity of each group at "0 time" to 100%. Figure 13 shows the same data as Figure 12 replotted in this manner. It can be seen that each group had a distinct rate of activity loss, although each arrived finally at a rate approximately 25% of the initial activity observed. It can also be seen that assay tubes with less activity originally present (A has the lowest activity, B' has the highest activity) lost their activity faster than tubes with more activity originally present.

This relationship is graphically represented in Figure 14 in which the time (in minutes) required for the four groups to fall to 50% of their original activity ($T_{\frac{1}{2}}$) is plotted against the original amount of activity present in the assay tube. It can be seen that the $T_{\frac{1}{2}}$ appears to be a smooth, hyperbolic function of the activity originally present. If the original activity is considered a reflection of HMG CoA lyase concentration, then it seems reasonable to conclude that the stability of HMG CoA lyase in phosphate buffer is a function of the enzyme concentration.

The relationship between enzyme concentration and activity stability was examined in a different manner in another experiment which employed cytosolic HMG CoA lyase (Figure 15). In this case, four protein concentrations (the cytosol of 0.25, 0.50, 0.75 and 1.0 mg of liver) were simply assayed for 2.5 min. The activity of these four enzyme concentrations is plotted both as a total activity (pmol/min/assay tube) and as a specific activity (pmol/min/mg liver). The total activity appears to be a linear function of enzyme concentration only after a "lag" phase. Over this low range of enzyme concentration, therefore, the specific activity does appear to increase with increasing enzyme concentration.

Since enzyme activity decreases so rapidly (Figures 12, 13, 14), undoubtedly significant activity is lost even during this short assay period of 2.5 minutes. Further, activity loss is more rapid in duluted enzyme preparations. Therefore, it seems reasonable to conclude that the S-shaped curve describing the specific activity as a function of enzyme concentration in Figure 15 is the result of the loss of activity that occurs during the incubation period.

Figure 15. HMG CoA lyase activity as a function of enzyme concentration. Four concentrations of a standard liver cytosolic preparation in phosphate buffer were assayed for 2.5 min. Both the specific activity (pmol/min/mg liver) and the total activity per assay vial (pmol/min/assay) are shown as a function of enzyme concentration.

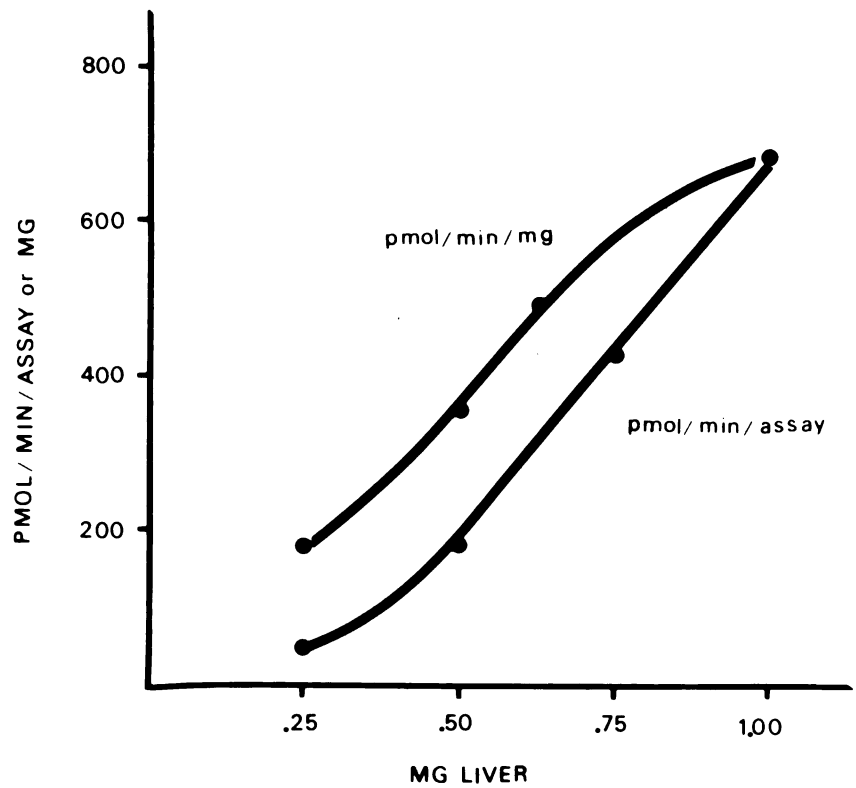

Figure 15. HMG CoA lyase activity as a function of enzyme concentration.

In the previous two experiments, the enzyme stability and enzyme specific activity increases observed as a function of protein concentration have been interpreted as the results of differences in enzyme concentration. There remained the possibility that the increases in activity were not due to enzyme concentration, but rather nonspecifically to protein concentration. This possibility was tested by determining the effect of added bovine serum albumin on the stability of mitochondrial HMG CoA lyase (Figure 16). There were three experimental sets of assay tubes; one contained 80 μg of mitochondrial protein, one contained 20 μg of mitochondrial protein, and the last contained 20 μg of mitochondrial protein plus 80 μg of bovine serum albumin. All groups were assayed immediately after exposure to 37°C and after 4 and 8 min of exposure to that temperature. Examination of Figure 16 shows that the tubes containing 20 μg of mitochondria were less stable than those containing 80 μg of protein and that the addition of 80 μg of albumin did not confer any increased stability upon the 20 μg of mitochondrial protein. It seems reasonable to conclude, therefore, that it is the increased concentration of enzyme per se, and not a non-specific protein effect, that is responsible for the stabilizing effect of increasing enzyme concentration described in this section.

Effects of $MgCl_2$ and DTT on the activity and stability of HMG CoA lyase in potassium buffer

Because $MgCl_2$ and DTT both have effects on the specific activity of HMG CoA lyase, it seemed possible that they might also influence the stability of the enzyme. To test this possibility, an experiment was conducted in which stability was examined both over a 24-h period at 0° and during acute exposure to 37°C. A rat liver was minced and divided

Figure 16. The lack of a stabilizing effect of bovine serum albumin on HMG CoA lyase activity. A standard mitosolic enzyme preparation was assayed after varying periods of preincubation at protein concentrations of 20 and 80 μg/assay. The lower enzyme concentration was assayed in the absence and presence of an additional 80 μg of bovine serum albumin.

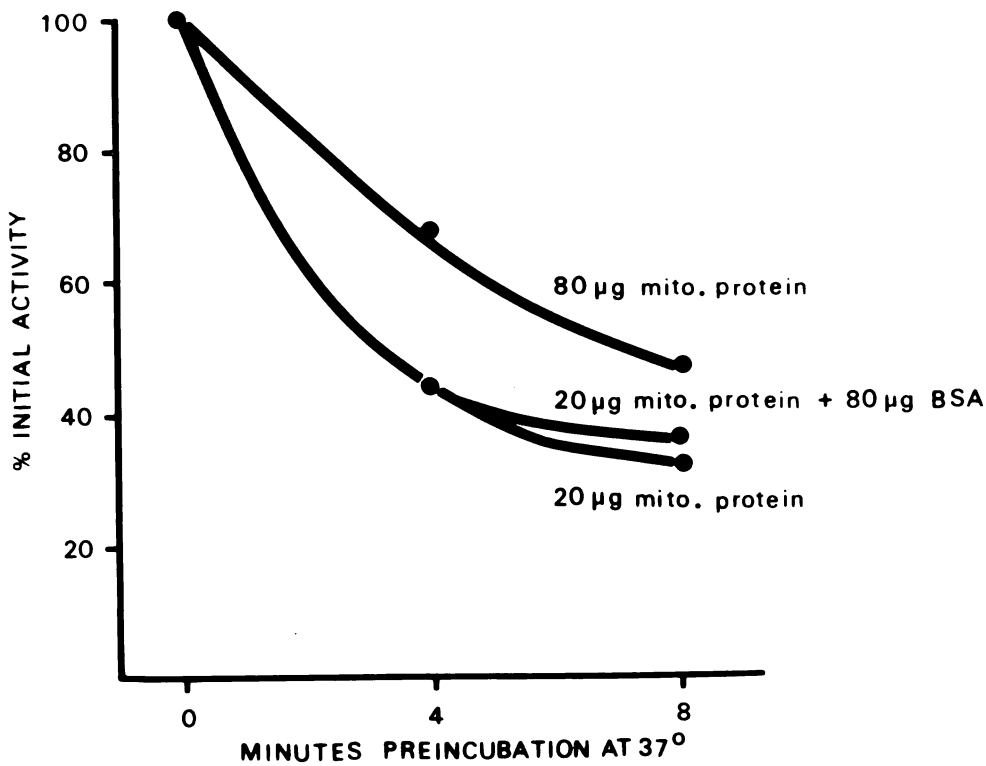

Figure 16. The lack of a stabilizing effect of bovine serum albumin on HMG CoA lyase activity.

into two portions. One portion was homogenized in potassium phosphate buffer in the absence of both $MgCl_2$ and DTT, the other in a buffer with both compounds present in 5 mM concentration. The resulting mitochondrial HMG CoA lyase preparations were assayed fresh, after 24 h at 0° and after 24 h of dialysis against phosphate buffer without DTT or $MgCl_2$.

In each of these situations, the enzyme preparations were assayed in the absence and presence of $MgCl_2$ and DTT, and both before and after a 10-min exposure to 37°C. See Table 6 for a description of these various experimental groups and assay conditions.

The numbers in parentheses in each section of Table 6 simply serve as a group "label" and are used as such in Figures 17 and 18. The numbers in the lower portion of each section represent the specific activity of HMG CoA lyase that has been normalized (for convenience) to the percent of the maximal specific activity observed (that of group 3). The conditions of group 3 were designed to be optimal for HMG CoA lyase activity, since the enzyme was both isolated and assayed (fresh) in the presence of $MgCl_2$ and DTT.

This table contains a number of points of interest. First, the comparison of group 2 (58.4) with group 3 (100) suggests that the simple presence of $MgCl_2$ and DTT within the assay does not immediately restore HMG CoA lyase activity to its maximal potential. An alternative interpretation is that $MgCl_2$ and DTT prevent the loss of activity that occurs prior to the time of the earliest possible assay.

The comparison of groups 4 and 6 with groups 5 and 7 is a dramatic demonstration of the effects of DTT and $MgCl_2$ in preventing the slow loss of activity over the 24-h period. Dialysis of the DTT and $MgCl_2$

TABLE 6. The effects of the presence of DTT and $MgCl_2$, during preparation and within the assay, on HMG CoA lyase activity

CONDITIONS OF HOMOGENATE	CONDITIONS OF ASSAY									
	Fresh assay				After 24 h at 0°		After 24 h dialysis at 0°			
	Control	DTT	$MgCl_2$	DTT + $MgCl_2$	Control	DTT + $MgCl_2$	Control	DTT	$MgCl_2$	DTT + $MgCl_2$
0 DTT	(1)[a]			(2)	(4)	(6)	(8)			(10)
0 $MgCl_2$	45.1[b]	54.3	42.5	58.4	13.6	25.2	7.3	18.6	14.6	20.9
+ DTT				(3)	(5)	(7)	(9)			(11)
+ $MgCl_2$				100	63.4	61.2	15.6	25.3	18.0	28.9

[a]Numbers in parentheses serve to identify these groups in Figures 17 and 18.

[b]Enzyme activity is expressed as a percentage of the velocity observed under the optimal conditions (group 3).

containing homogenates against DTT- and $MgCl_2$-free buffer results in a sharp loss of activity (compare 5 and 7 to 9 and 10). This removal of "protection" against activity loss is also demonstrated by the fact that groups 9 and 11 have activities quite similar to groups 8 and 10, while the difference between 4 and 6, and 5 and 7 were quite large.

The stability of each of the numbered groups was also tested by assaying them after 10-min exposure to 37°C (Figure 17). These data are expressed as specific activities and seem to suggest generally that groups showing greater initial activity suffer greater absolute loss in activity.

This relationship is examined more critically in Figure 18, in which the data have been normalized by adjusting the initial activity of each respective group to 100%. The activity of the various groups after a 10-min exposure to 37°C therefore ranges from 40% (group 3) to 88% (group 10) of their respective initial activities. Figure 18 plots these values (actually a measure of activity stability) as a function of the specific activity observed prior to exposure to 37°C. Figure 18 demonstrates that generally the greater the specific activity initially observed, the greater was the relative loss of activity over a 10-min acute exposure to 37°C. Additionally, most of the experimental groups appear to fall within a relatively well-defined band (shown darkened) while three groups stand out, in that they appear to be disproportionately unstable. These groups (1, 4 and 8) are all of the experimental groups that were isolated and assayed in the absence of both $MgCl_2$ and DTT. It can be concluded, therefore, that $MgCl_2$ and DTT together promote the stability of HMG CoA during acute exposure to 37°C in potassium phosphate buffer.

Figure 17. The activity and stability of HMG CoA lyase prepared and assayed under various conditions with regard to DTT and $MgCl_2$. Mitosolic enzyme from a single liver was prepared and assayed under conditions detailed in Table 6, which serves as a legend for the experimental group numbers of this figure. Each experimental group was assayed before and after a 10-min incubation at 37°C.

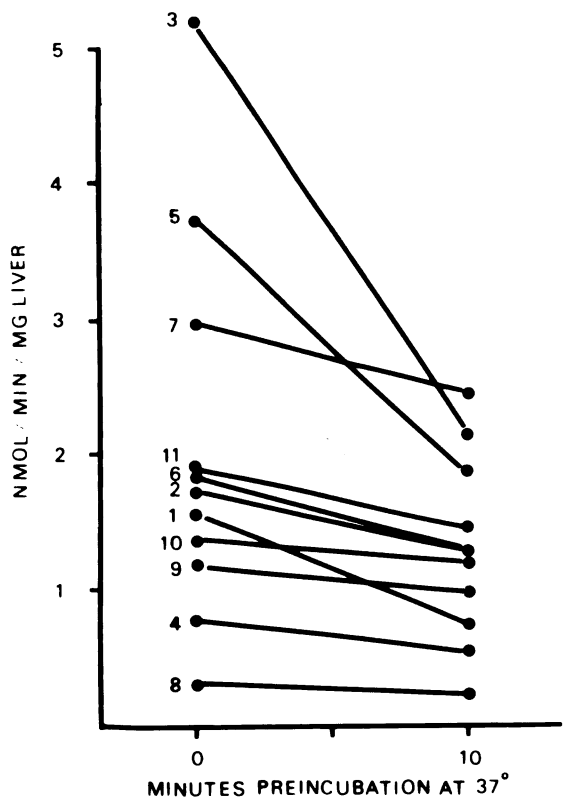

Figure 17. The activity and stability of HMG CoA lyase prepared and assayed under various conditions with regard to DTT and $MgCl_2$.

Figure 18. The stability of HMG CoA lyase prepared under various conditions as a function of initial specific activity. As in Figure 17, Table 6 serves as a legend to describe the experimental groups shown in this figure. The percentage of activity remaining in each sample after 10 min at 37°C, as compared to that seen initially (essentially an index of stability at 37°C), is shown as a function of the initial specific activity of the sample.

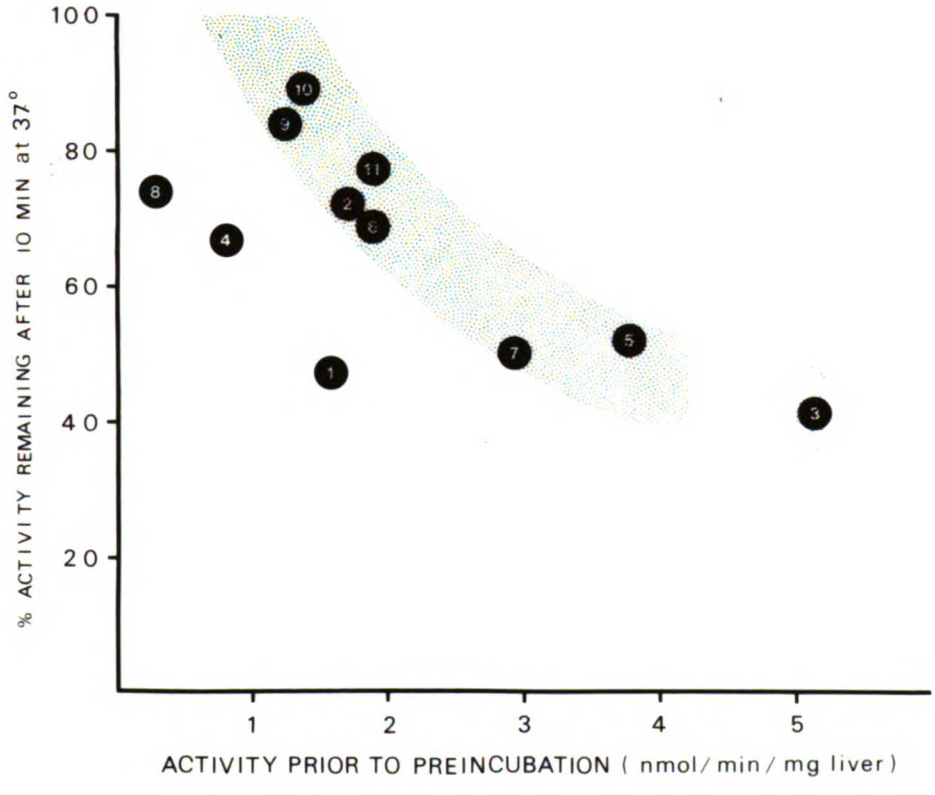

Figure 18. The stability of HMG CoA lyase prepared under various conditions as a function of initial specific activity.

It should be emphasized that the general conclusion reached here is not inconsistent with the conclusions reached earlier with regard to stability as a function of enzyme concentration. In this experiment the enzyme concentration is the same in all cases. The rates of initial activity observed in the different experimental groups are simply a manifestation of the total activity originally present in the sample. Thus, the relationship observed here could be described as follows: the greater the amount of potential activity observed under a given set of experimental conditions, the greater is the potential for a phosphate-induced loss of activity upon exposure to 37°.

HMG CoA lyase activity and stability as a function of pH

Figure 19 shows the activity of mitochondrial and cytosolic HMG CoA lyase as a function of pH in potassium phosphate buffer. A pH optimum of approximately 9 was observed, in agreement with the earlier finding of Stegink and Coon (46).

To test the possibility that the enzyme could have a greater stability at the more favorable pH of 9.1, cytosolic enzyme isolated in potassium phosphate buffer 7.5 was assayed in potassium phosphate buffer at pH 7.35 and pH 9.1. Figure 20 shows the stability of the enzyme over a 20-min exposure to 37°C. Though the activity at pH 9.1 is higher, it is lost at a greater rate, and the final specific activities of the two experimental groups appear to be approaching each other. In the pH 9.1 buffer, the activity at "0" time is actually lower than it is after 4 min of exposure to 37°C. This could be due to the fact that the enzyme was initially at pH 7.5, and its exposure to pH 9.1 was initiated at the same time as the exposure to 37°C (when the

Figure 19. The activity of cytosolic and mitosolic HMG CoA lyase as a function of pH in phosphate buffer. Mitosolic and cytosolic enzyme was prepared in the standard manner in phosphate buffer (pH 7.5). Enzyme samples were placed in vials of varying pH and assayed for 2 min.

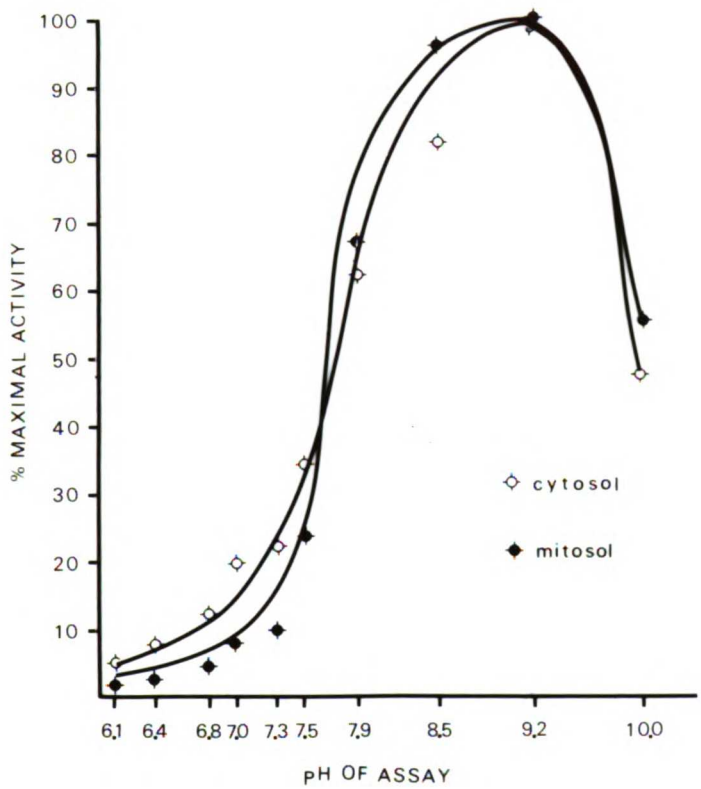

Figure 19. The activity of cytosolic and mitosolic HMG CoA lyase as a function of pH in phosphate buffer.

Figure 20. The stability of HMG CoA lyase in phosphate buffer at pH 7.35 and 9.10. A standard mitosolic enzyme preparation in phosphate buffer of pH 7.5 was put into two series of vials with the pH adjusted to 7.35 and 9.10, respectively, and assayed for 2 min after varying periods of preincubation at 37°C.

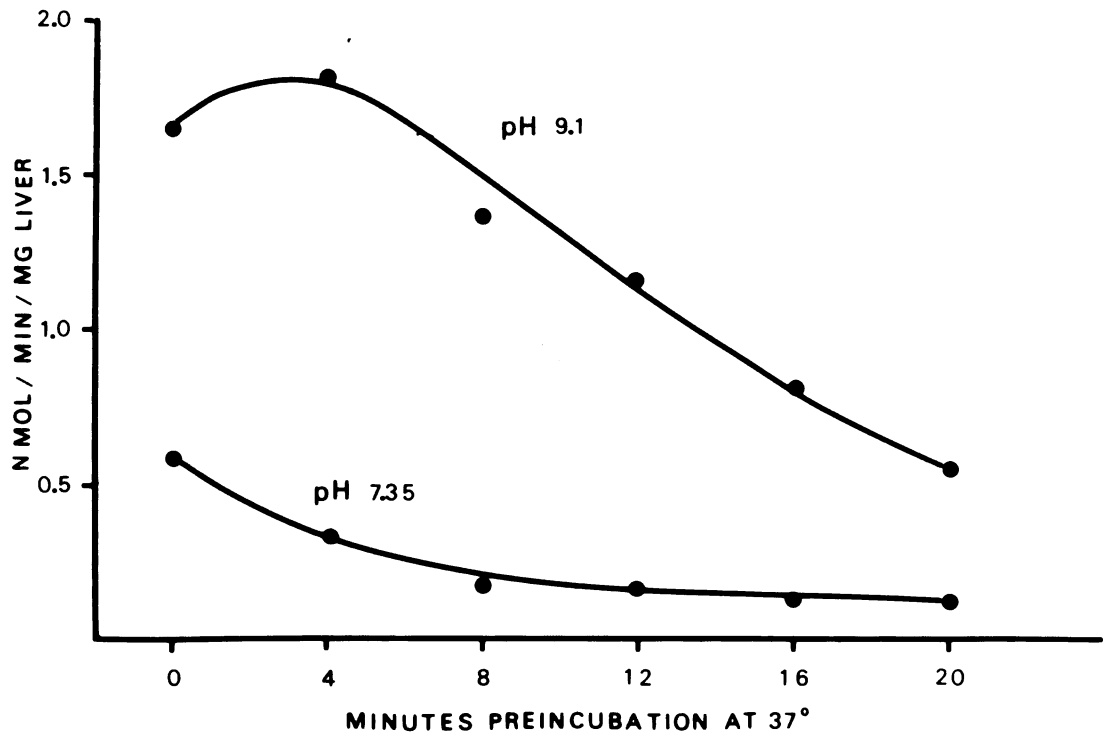

Figure 20. The stability of HMG CoA lyase in phosphate buffer at pH 7.35 and 9.10.

assay began). Therefore, a pH-dependent activation probably was occurring during the first few minutes of the incubation period.

HMG CoA lyase activity as a function of pH in zwitterionic buffers

To test the possibility that an alternative buffer system would provide more favorable conditions for the assay of HMG CoA lyase, a rat liver was homogenized in an unbuffered solution of 0.3 M sucrose, 5 mM DTT, and 5 mM $MgCl_2$. The mitochondrial fraction was collected in eight tubes and the pellets were dispersed in one of the following buffered solutions: MOPS (pH 6.7), HEPES (pH 7.1), TRICINE (pH 7.6), BICINE (pH 7.8), glycylglycine (pH 7.9), CHES (pH 8.9), CAPS (pH 9.6), and potassium phosphate (pH 7.5). These solutions had the general constitution of 100 mM buffer, 5 mM DTT, and 5 mM $MgCl_2$; the pH's were chosen so as to be near the respective pKa of the particular buffer. The soluble mitochondrial enzyme from these preparations was then assayed in the identical buffers. Figure 21 shows the results of this experiment, with the activities plotted as a function pH. A rough pH curve is evident from the distribution of enzyme activities; however, the exceptionally low activity of the potassium phosphate group stands out from the group of non-phosphate buffers.

BICINE was chosen as a buffer with which to pursue further studies because of its favorable pH range (a pKa of 8.35) and because blank values obtained from the enzyme assay were lower than with CHES. Figure 22 shows the results of an experiment in which mitochondrial HMG CoA lyase was isolated in BICINE buffer (pH 8.35) and then assayed at a range of pH values. As with phosphate buffer, a pH optimum of 9 is evident; however, the curve appears to be broader than that generated in phosphate buffer.

Figure 21. The activity of HMG CoA lyase in different buffers and as a function of pH. Mitosolic HMG CoA lyase was prepared in an unbuffered solution containing DTT and $MgCl_2$. After solubilization, samples were distributed into vials containing phosphate buffer and a series of zwitterionic buffers (detailed in figure) adjusted to pH's near their respective pKa's.

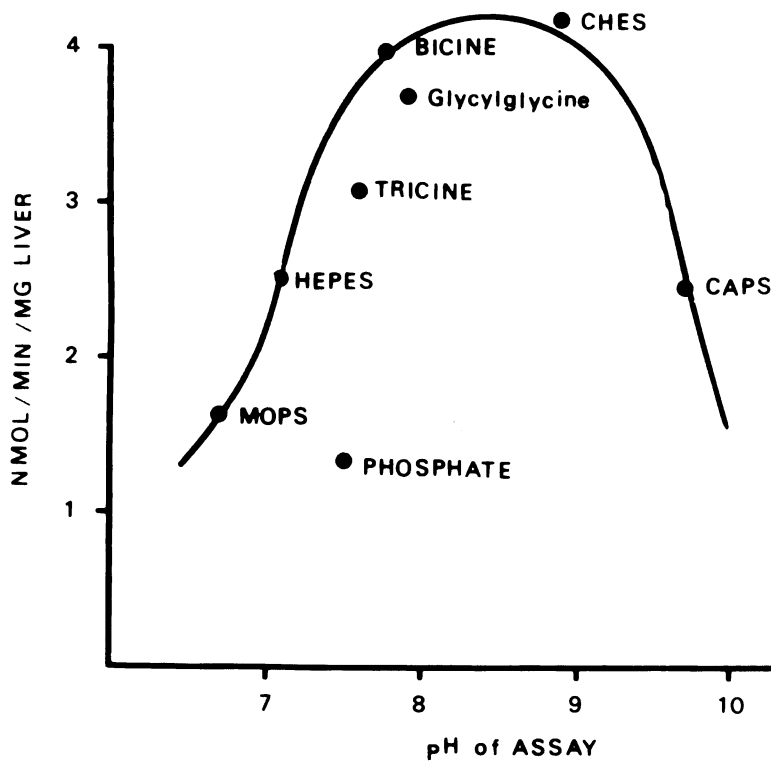

Figure 21. The activity of HMG CoA lyase in different buffers and as a function of pH.

Figure 22. The activity of HMG CoA lyase as a function of pH in BICINE buffer. Mitosolic HMG CoA lyase was prepared in a BICINE buffer solution, pH 8.35. The preparation was then distributed and assayed in vials of BICINE-buffered assay media of varying pH.

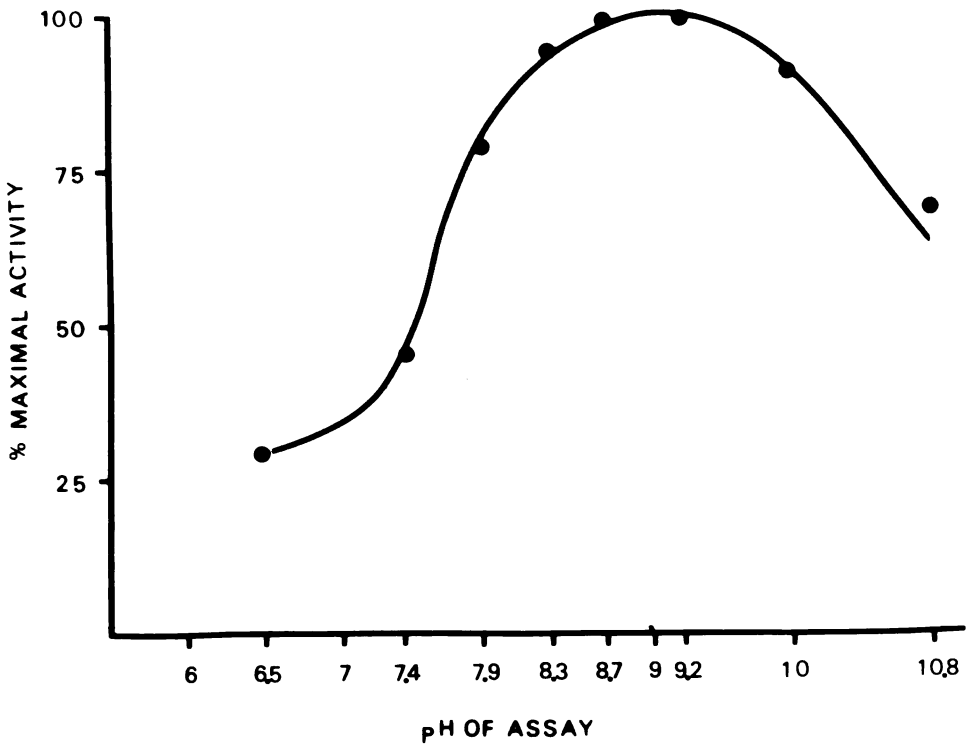

Figure 22. The activity of HMG CoA lyase as a function of pH in BICINE buffer.

HMG CoA lyase activity and stability in BICINE buffer as compared to phosphate buffer

To compare the activity and stability of HMG CoA lyase in BICINE and potassium phosphate buffer, a rat liver was minced, divided into two portions and homogenized either in 50 mM BICINE (pH 8.35) or 100 mM potassium phosphate (pH 7.4), in addition to the standard 0.3 M sucrose, 5 mM DTT, and 5 mM $MgCl_2$. The isolated cytosolic and mitochondrial enzymes were assayed in the same respective buffers. Table 7 shows the results of this experiment; the specific activity of the cytosol and mitochondrial fractions were 0.57 and 1.60 pmol/min/mg liver, respectively, in potassium phosphate, and 4.11 and 13.25 pmol/min/mg liver in BICINE. This seven- to eight-fold increase in specific activity was, of course, in part due to the pH difference between the two systems; however, Figure 23 illustrates another important difference.

These cell fractions were assayed immediately after being brought to 37°C, and after 4- and 8-min incubations at this temperature. Figure 23 shows the results of this experiment, in which the activity of each cell fraction at "0" time has been adjusted to 100%. It can be seen that the cell fractions in the potassium phosphate lost activity (although by comparison with previous experiments they appeared relatively stable). In contrast, however, cell fractions in BICINE were absolutely stable; in fact, the mitochondrial fraction gained activity, over the 8-min exposure to 37°C. It thus appeared that the time- and temperature-dependent loss of activity was due to the presence of phosphate ion.

In order to test for the possibility that the potassium ion, rather than the phosphate ion, was responsible for the enzyme inhibition,

TABLE 7. HMG CoA lyase activity in phosphate and BICINE buffers

Homogenization and assay buffer	HMG CoA lyase activity (pmol/min/mg liver)	
	Cytosol	Mitochondria
Potassium phosphate	0.57	1.60
BICINE	4.11	13.25

Figure 23. A comparison of the stability of HMG CoA lyase in phosphate and BICINE buffers. A liver was divided into two portions, from which mitosolic enzyme was prepared and assayed in either a phosphate- or BICINE-buffered solution. Table 7 gives the specific activities of these preparations before exposure to 37°C. This figure normalizes this initial activity to 100% and shows the activity response to exposure to 37°C.

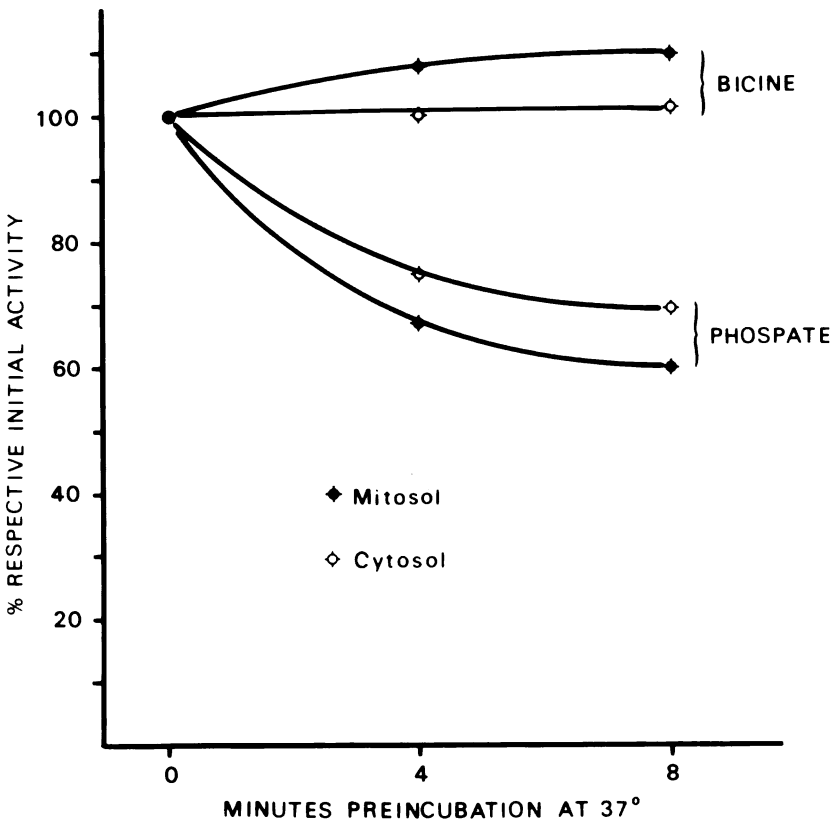

Figure 23. A comparison of the stability of HMG CoA lyase in phosphate and BICINE buffers.

a mitochondrial enzyme preparation, isolated in BICINE, was assayed in BICINE in the presence of various concentrations of sodium phosphate and potassium phosphate. Both phosphate buffers were adjusted to a pH of 8.35 (the pH of the BICINE) in order to keep the pH of the different assay media constant. Figure 24 shows that both sodium and potassium phosphate were equally effective in inducing a loss of activity. A sharp effect was obvious even at the lowest concentration of phosphate buffer tested (10 mM). On the basis of this experiment, it seems reasonable to conclude that the labile feature of the enzyme, which until this point had seemed to be inescapable, was in fact due solely to the presence of phosphate ion.

The sensitivity of HMG CoA lyase within intact mitochondria to phosphate ion in the surrounding media (suggested by the experiment illustrated in Figure 10) also was of interest. To examine this issue further, mitochondria were isolated in a solution of 0.4 mM sucrose, 50 mM BICINE (pH 8.35), 5 mM $MgCl_2$, and 5 mM DTT. The mitochondria were maintained in a hyperosmotic concentration of sucrose throughout the assay period in order to minimize osmotic lysis. The intact mitochondria were exposed to various phosphate concentrations for two periods of time. First, they were incubated 10 min at 20°C; following this they were brought to 37°C and either assayed immediately or after a 10-min exposure to this temperature. The results of this experiment are shown in Figure 25.

A relatively mild effect of the phosphate presence is apparent after the 10-min exposure to 20°C; however, the effect is profound after the 10 min at 37°C, and is sharply evident even at 5 mM phosphate concentration. Interestingly, the 10-min exposure to 37°C increased

Figure 24. A comparison of the effects of potassium and sodium phosphate upon the lability of HMG CoA lyase. Mitosolic enzyme was prepared in a BICINE-buffered solution (pH 8.35) and then distributed into two series of samples to which were added varying concentrations of either potassium phosphate or sodium phosphate (both pH 8.35).

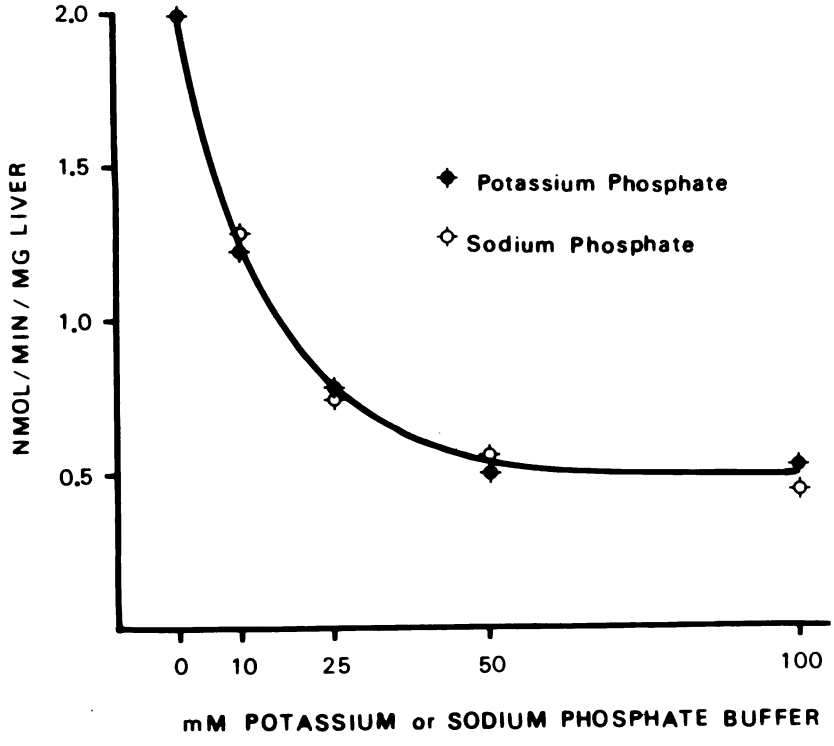

Figure 24. A comparison of the effects of potassium and sodium phosphate upon the lability of HMG CoA lyase.

Figure 25. The stability of HMG CoA lyase within intact mitochondria in BICINE buffer as a function of potassium phosphate concentration. Intact mitochondria were prepared in a BICINE-buffered solution and distributed into two series of samples containing varying concentrations of potassium phosphate. Both series were allowed to sit at 20°C for 20 min, after which one of the series was exposed for an additional 10 min to 37°C prior to the assay.

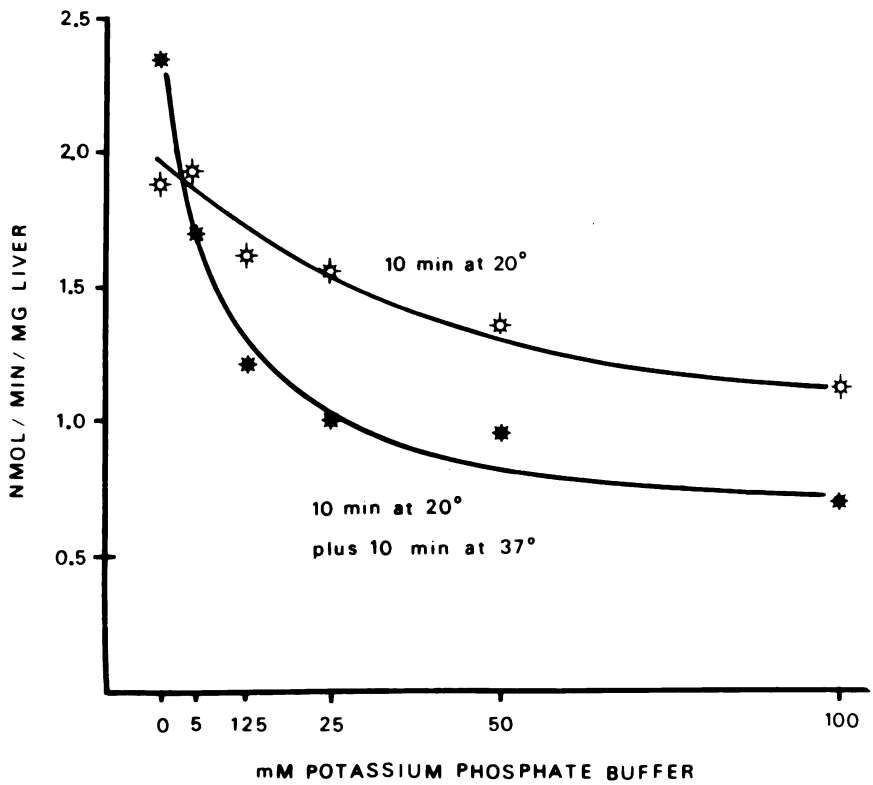

Figure 25. The stability of HMG CoA lyase within intact mitochondria in BICINE buffer as a function of potassium phosphate concentration.

the activity of the group containing BICINE buffer alone (without added phosphate), a result seen earlier in Figure 23.

Substrate kinetics of mitochondrial and cytosolic HMG CoA lyase

To determine the K_m of mitochondrial and cytosolic HMG CoA lyase isolated and assayed under optimal conditions, these cell fractions were isolated in a homogenization buffer of 0.3 M sucrose, 50 mM BICINE (pH 8.35), 5 mM DTT, and 5 mM $MgCl_2$. The difficulty in obtaining accurate kinetics involved primarily the problem of substrate depletion at low substrate concentrations. To overcome this difficulty, very small amounts of protein were used (the equivalent of 0.13 mg of liver for the cytosolic assays, and the equivalent of 0.05 mg of liver for the mitochondrial assays). Assay times were of 1-min duration. Because of the very low amount of enzyme activity at the low substrate and low protein concentrations, a very high specific activity of ^{14}C-HMG CoA was required (20,000 cpm/nmol, with the full ^{14}C scintillation counting window, as opposed to a more usual specific activity of 800 cpm/ nmol).

Figure 26 shows the activity of mitochondrial HMG CoA lyase as a function of increasing substrate concentration, while Figure 27 shows the Lineweaver-Burke plot of the same data. The Lineweaver-Burke-derived K_m is 13.9 μM.

Figure 28 shows the activity of cytosolic HMG CoA lyase as a function of increasing substrate concentration, while Figure 29 shows the Lineweaver-Burke plot of the same data. The Lineweaver-Burke-derived K_m of 16.7 μMis in close agreement with the mitochondrial K_m, and further agrees with the K_m determination of Stegink and Coon (46).

Figure 26. The activity of mitosolic HMG CoA lyase as a function of substrate concentration, a Michaelis-Menton plot. The mitosolic enzyme was prepared in BICINE buffer (pH 8.35). The amount of enzyme assayed was that from 0.05 mg of liver.

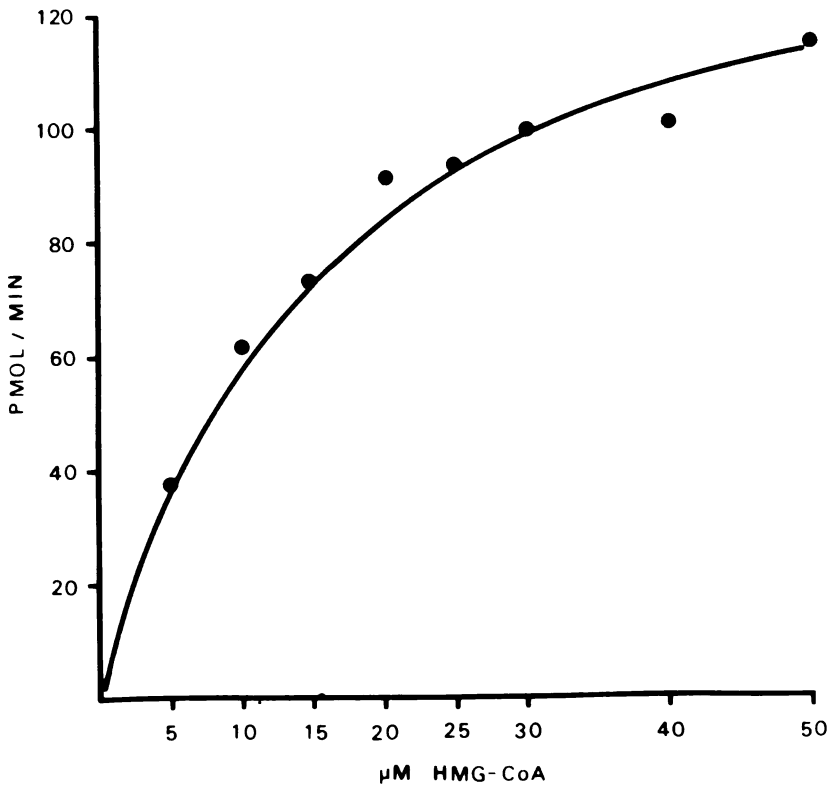

Figure 26. The activity of mitosolic HMG CoA lyase as a function of substrate concentration, a Michaelis-Menton plot.

Figure 29. The activity of cytosolic HMG CoA lyase as a function of substrate concentration, a Lineweaver-Burke plot. These are the same data as those seen in Figure 28, arranged in the double-reciprocal manner.

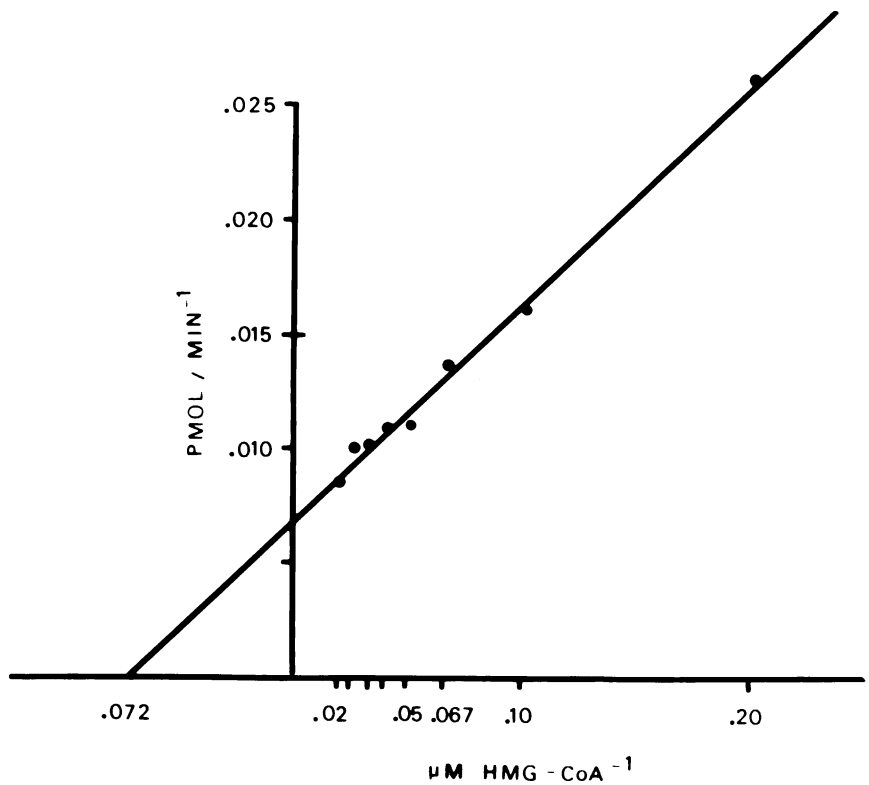

Figure 27. The activity of mitosolic HMG CoA lyase as a function of substrate concentration, a Lineweaver-Burke plot.

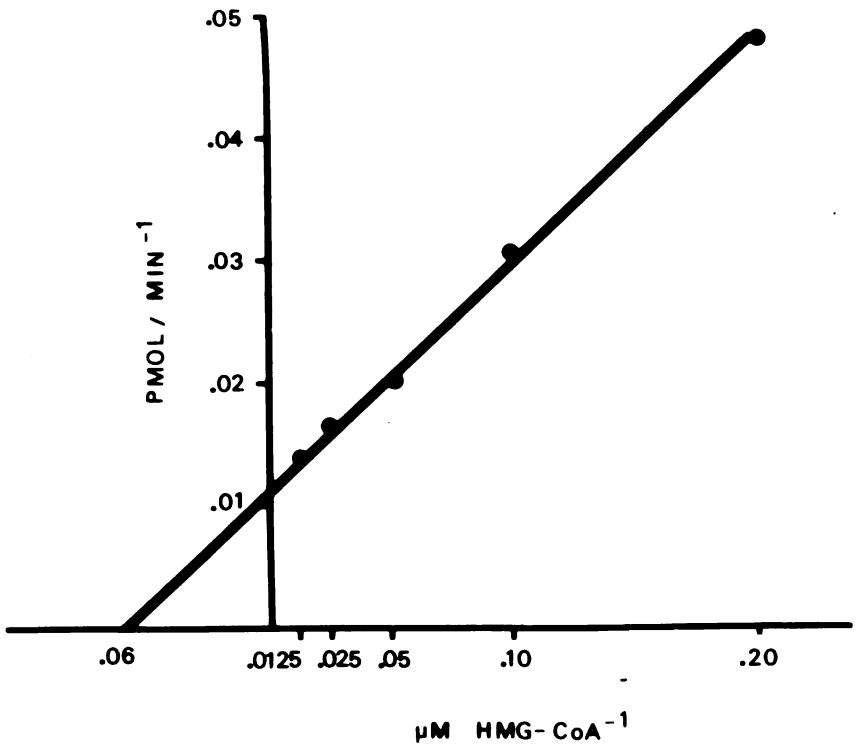

Figure 29. The activity of cytosolic HMG CoA lyase as a function of substrate concentration, a Lineweaver-Burke plot.

Discussion

This investigation of HMG CoA lyase was focused primarily on the nature of its instability in the presence of phosphate ion. That this observed inactivation was in fact due to phosphate was shown in several ways. In contrast to HMG CoA lyase prepared and assayed in phosphate buffer, the enzyme prepared and assayed in BICINE buffer was seen to have a considerably higher specific activity and also to be stable at 37°C (Figure 23). This enhanced activity and stability in BICINE buffer was not due to the BICINE buffer per se, but rather to the simple absence of phosphate. This was most clearly demonstrated in the experiment shown in Figure 21, in which HMG CoA lyase was assayed in a number of non-phosphate, zwitterionic buffers adjusted to pH's near their respective pKa's. The range of activities shown formed a smooth curve, suggesting that the activity was solely a function of pH. Quite below this activity curve was the enzyme velocity observed in phosphate buffer. The possibility that the potassium ion, rather than phosphate, was the agent responsible for enzyme inactivation was reasonably eliminated by the demonstration that the enzyme was equally inhibited by the presence of potassium phosphate and sodium phosphate (Figure 24). This particular experiment showed an enzyme sensitivity to a 10 mM phosphate concentration, while in the next experiment (Figure 25) an enzyme sensitivity to a 5 mM concentration was evident following a 10-min preincubation at 37°C.

HMG CoA lyase has the rather high optimal activity pH range of 8.9-9.1 (Figures 19, 20 and 22). The possibility that the higher activity observed at pH 9 (as compared to that at physiological pH) could be the result of a greater stability was tested experimentally and

found not to be the case. Figure 20 illustrates the experiment in which the enzyme in phosphate buffer at pH 9 was also found to be highly labile.

The rate and magnitude of the phosphate-induced inactivation were found generally to be rapid and profound, but nevertheless subject to influence by several factors. There is an enzyme-concentration effect in stabilizing activity against the effects of phosphate, and this feature is most clearly demonstrated in Figures 12, 13 and 14. This experiment utilized two enzyme concentrations (with a 4-fold difference between them) of two separate enzyme preparations with a 5-fold difference in specific activity (therefore a total of four experimental samples). The relationship described in Figures 12 and 13 is one in which assay samples containing greater enzyme activity (i.e., concentration) show a slower rate of activity loss, although they ultimately reach the same level of inactivation as samples of lower activity (in these cases, an ultimate loss of nearly 80% of the initial activity). Figure 14 expresses this relationship in terms of $T_{\frac{1}{2}}$ (the time required for loss of 50% of the initial rate of activity). The $T_{\frac{1}{2}}$ is seen to be a hyperbolic function of the initial activity within the sample.

This relationship inevitably leads to the extreme instability of dilute enzyme preparations, and is probably responsible for the non-linear relationship between enzyme concentration and activity seen initially in Figure 7, but more clearly in Figure 15. Here (at low concentrations), the enzyme specific activity was observed to increase as a function of enzyme concentration. It seems probable that the observed disproportionately low specific activity of the dilute samples was due to a loss of activity <u>during</u> the time of assay.

The instability of dilute enzyme preparations could have been due either to the decreased concentration of protein in general, or to a decreased concentration specifically of the enzyme itself (or of another cytosolic factor). The efficacy of a non-specific protein (bovine serum albumin) in stabilizing a dilute enzyme preparation was tested in the experiment depicted in Figure 16. Here, two enzyme concentrations were assayed, the lower concentration being assayed in the absence and presence of albumin. The lower enzyme concentration was indeed less stable than the more concentrated one, and its stability was not increased by the added albumin. Therefore, it seems likely that enzyme-enzyme interaction, or an enzyme interaction with another cytosolic factor, increases the stability (of activity) against the action of phosphate.

The phosphate-induced inactivation of lyase appeared to be dependent on both time and temperature. Whether warming per se contributed to the inactivation, or if it merely accelerated the phosphate inactivation process was unclear. The reversibility of the warming-inactivation of the enzyme was tested in the experiment shown in Figure 11, in which an enzyme preparation was warmed and then subjected either to a continuation of 37°C, cooling to 0°, or freezing for a 2-h period. An assay following this treatment showed no recovery by the cooled or frozen enzyme, although the loss of activity was less than in the enzyme maintained at 37°C. Upon warming the enzyme that had been cool, the activity continued its decline.

Two other samples were not exposed to the original preincubation, but rather were kept at 4°C for 24 h. Both samples (one dialyzed, the

other not) lost considerable activity over this period of time, comparable to that lost by the other samples during the 20 min at 37°C.

The metal ion and thiol dependence of HMG CoA lyase observed by others (46) and confirmed here (Figure 9 and Tables 5 and 6) was found to be manifest not only in the enzyme specific activity, but also as a partial protection against the progressive inhibitory effects of phosphate. The experiment tabulated in Table 5 demonstrated that DTT and $MgCl_2$ prevent a substantial amount of loss of activity that occurred over a 24-h period (at 4°C) of exposure to phosphate. This protection was lost when these compounds were removed by dialysis.

Experimental samples in this study were assayed immediately after warming to 37°C and after a 10-min incubation at this temperature. Figure 17 depicts the results in a direct manner, and Figure 18 presents the same data arranged in such a manner that the enzyme activity stability over the 10-min period can be seen as a function of the initial activity (i.e., that observed immediately upon warming to 37°C). In general, samples showing greater initial activity are proportionately less stable than preparations initially showing less activity. Further, enzyme samples that had no exposure to either DTT or $MgCl_2$ were distinctly less stable than all others.

It can thus be hypothesized that the enzyme has a wide range of activity, the upper limit of which is expressed in the absence of phosphate and the lower limit in its presence. The conversion from the active to less active form is consistently observed in the presence of phosphate (with sufficient time), but the process is accelerated by warming and dilution and slowed by the presence of DTT and $MgCl_2$.

GENERAL DISCUSSION AND PERSPECTIVE

The development of these enzyme assays for HMG CoA reductase and HMG CoA lyase, and the further studies on the phosphate inhibition of the latter enzyme, were undertaken because of a more general and continuing interest in the ketone and cholesterol biosynthetic pathways, and the possibility of interaction between them.

Acetoacetate, the initial product of the ketone synthetic pathway, and its reduced companion, β-hydroxybutyrate, are metabolic fuels of particular importance to the brain and during certain physiological states, such as pregnancy and starvation. The concentration of blood "ketones" varies over an extraordinarily large range, the upper limit of which occurs during diabetic ketosis and is life-threatening due to the accompanying acidosis. The biochemistry of ketone synthesis and utilization has thus received the attention of many investigators over the years (for reviews see 27,50,53).

Ketone bodies in blood arise as products of the hepatic fatty acid metabolism which courses through two potential branch points. The ketogenic liver, first described by Mayes and Felts (25), appears to regulate fatty acid metabolite flux at both sites. The studies of McGarry and Foster (for reviews see 27,32,33) have defined the role of the mitochondrial carnitine acyl-transferase system the first branch point) in directing fatty acyl-CoA away from esterification and into the β-oxidative pathway. However, the studies of McGarry and Foster (30,31) and the report of Dietschy and Brown (13) also indicate that the mitochondrial transport system is not the sole ketogenic regulatory mechanism. Octanoic acid, a medium-chain fatty acid that bypasses the mitochondrial transport system, supports a higher rate of ketone syn-

thesis in the fasted or diabetic liver than it does in the fed liver. The mechanisms that govern the fate of mitochondrial acetyl-CoA (the site of the second branch point) are poorly understood. A common explanation for the diversion of acetyl-CoA into ketone synthesis is that it follows as a direct result of an inhibition of the TCA cycle (51). Although a severely inhibited TCA cycle could assist in directing "overflow" acetyl-CoA into the ketogenic pathway, this situation remains to be demonstrated experimentally. On the contrary, the TCA cycle of the isolated perfused ketogenic liver is only modestly depressed below control activity (29,30), and the TCA cycle of ketogenic liver slices is equal to that of fed-liver slices (13). It is also worth noting that the ketogenic liver is coincidentally a gluconeogenic liver, and this state requires a functioning TCA cycle in order to accomodate the influx of gluconeogenic amino acids.

A corollary of the TCA-cycle-inhibition concept of ketogenic drive is that the activity of the ketogenic pathway would be determined solely by the availability of acetyl-CoA. An early study of Foster (17) addressed this issue and showed a distinct dissociation between the ketogenic rate of the liver and its acetyl-CoA content during fasting and recovery from fasting. The recent study of Brunengraber (6) shows an equivalent acetyl-CoA concentration in fed and 2-day fasted liver, despite a 2.5-fold difference in the rate of ketogenesis from endogenous substrate in a perfusion system. Thus, neither a depressed TCA cycle nor an acetyl-CoA excess appears to be a primary requirement of the ketogenic liver.

Another possible and reasonable explanation for the increased utilization of acetyl-CoA by the ketone synthesis pathway would involve

activation of enzymes within the ketogenic pathway itself. However, the enzymes of ketone synthesis have not been thoroughly investigated, and their possible role in regulating ketone synthesis in starvation or diabetes has been examined even less. Only one study appears to have specifically addressed this issue, that of Williamson et al. (52), in which the authors concluded that "variations in the concentrations of enzymes involved in acetoacetate synthesis play no major role in the regulation of ketone-body formation in starvation and alloxan-diabetes."

In a series of studies, I compared the activity of HMG CoA lyase in normal and streptozotocin-ketotic rats. The results were inconsistent, require further clarification, and for these reasons have not been reported here. Nevertheless, I believe that the question of the role of the ketogenic enzymes in regulating acetoacetate synthesis is an open one for several reasons. First of all, little is known about the activity of the enzymes within the mitochondria (in contrast to their activity in a soluble in vitro systems), and nothing is known of any possible acute allosteric regulation. Further, no studies have examined the issue in terms of the rate of substrate flux through the enzymes in series.

The studies described here emphasize that HMG CoA lyase is an elusive enzyme. Its activity in vitro easily varies fivefold simply as a function of its exposure to phosphate. This phenomenon itself may or not be of direct physiological signififance, although the sensitivity of the enzyme within intact mitochondria suggests that the concentration of intracellular phosphate could affect the activity of HMG CoA lyase. Further, the intracellular phosphate depletion that coincides with diabetic ketosis and the possibly consequent activation of HMG CoA lyase

would be consistent events. In any case, a conservative interpretation of these data permits the suggestion that the activity of the enzyme is subject to acute regulation, and the phenomenon that stability is dependent on enzyme concentration suggests a form of regulation involving subunit-subunit interaction.

In looking ahead to further studies, the HMG CoA lyase assay described herein seems to be very suitable for adaptation to measure substrate flux through the pathway as a whole. With the appropriate substrates it should be possible to compare flux through acetoacetyl-CoA thiolase, HMG CoA synthase and HMG CoA lyase in series, HMG CoA synthase and lyase in series, and HMG CoA lyase alone. Further, with permeable mitochondria (26) these studies can be conducted with the enzymes in a situation that physically approximates their physiological state more closely than does a cell-free, soluble system.

Another unsettled issue regarding the ketogenic enzymes concerns their intracellular location. The first two enzymes of the ketogenic pathway are indisputably present in the liver cytosol, and there they act as the first two enzymes of the cholesterogenic pathway (10,11). The presence of the third ketogenic enzyme, HMG CoA lyase, in the cytosol is a controversial issue. Clinkenbeard and co-workers (10) observed that only a relatively low percentage of total cellular lyase activity is cytosolic. On the basis of the apparent physiochemical identity of the mitochondrial and "cytosolic" lyase, they concluded that the "cytosolic" lyase was an artifact of mitochondrial leakage. Two groups (2,40), however, in addressing the same issue, controlled for mitochondrial leakage by following the activity of the mitochondrial "marker" enzyme, glutamate dehydrogenase. They found that the relative

percentage of its activity in cytosol was consistently less then that of HMG CoA lyase, and therefore concluded that the lyase was physiologically present in cytosol.

The study of Brunengraber (6) also bears on the issue of cytosolic lyase. By way of introduction, it is generally thought that cytosolic acetyl-CoA originates in the mitochondria and is transported to the cytosol in the form of citrate. In the cytosol, citrate-cleavage enzyme liberates acetyl-CoA and oxalacetate. Hydroxycitrate is an inhibitor of this enzyme and as such is an effective inhibitor of both fatty acid and sterol synthesis. The Brunengraber study shows that hydroxycitrate also blocks the relatively low level of ketogenesis of the fed, perfused liver (although it does not interfere with the higher ketogenic rates induced by starvation or by oleate infusion). This result, therefore, strongly suggests that significant amounts of HMG CoA lyase may well be functionally located in the cytosol.

Another group of observations which may be relevant to the presence of HMG CoA lyase in the cytosol stems from the generalization that HMG CoA reductase is the site of the feedback regulation of cholesterol synthesis. This concept, initially proposed by Siperstein (41,43), has been confirmed, extended, and reviewed extensively (4,14,39). Recently, however, several interesting exceptions to the proposition that HMG CoA reductase is the rate-limiting enzyme of cholesterol synthesis have been reported. Dugan et al. (15) found that an HMG CoA reductase diurnal rhythm entrained in rats would persist through a day of fasting, during which time the incorporation of acetate into cholesterol remained low. Nervi et al. (35) reported that the intravenous administration of triglyceride to rats caused a rise in HMG CoA reductase activity,

accompanied by a fall in the incorporation of acetate into cholesterol. Lastly, the Melnykovych group (7,8,34) has reported that, whereas glucocorticoids induce a rise in HMG CoA reductase activity in HeLa cells, cholesterol synthesis from either acetate or tritiated water is depressed and the incorporation of mevalonate into cholesterol is unaffected.

The common theme of these observations is the occurrence of high HMG CoA reductase activity coincident with a low rate of cholesterol synthesis. It is possible that all these situations could be explained by an activation of cytosolic HMG CoA lyase, which would divert HMG CoA from the cholesterol pathway. More specifically, cytosolic HMG CoA lyase would recycle one-third of the carbon-unit substrate (as the acetyl-CoA product) and direct two-thirds of the carbon-unit substrate (as the acetoacetate product) out of the liver. Thus, the potential effect of cytosolic HMG CoA lyase, by substrate recycling and diversion, would be to modulate the rate of hepatic cholesterogenesis.

Again, the assays described in the first section of the dissertation are eminently suited for investigating the relative roles of HMG CoA lyase and HMG CoA reductase in regulating substrate flux toward cholesterol. In cytosolic preparations it should be possible to measure flux from acetyl-CoA through the two common enzymes, acetoacetyl-CoA thiolase and HMG CoA synthase, and through the divergent reductase and lyase simultaneously. Of particular interest would be a comparison of the cytosols from animals in which HMG CoA reductase activity and cholesterol synthesis activity are in accord and from animals in states such as those described above in which high HMG CoA reductase activity coincides with a low rate of cholesterol synthesis.

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