The effect of cholestyramine and Mevinolin on the diurnal cycle of rat hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase

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Abstract Rats were fed powdered rat chow or a rat chow diet containing 5% cholestyramine or 5% cholestyramine with Mevinolin (112 mg/100 g food, 200 mg/kg body weight per day). The specific activity of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase was determined at different times during the diurnal cycle of the enzyme. Animals fed cholestyramine had higher specific activities of HMG-CoA reductase at all time points tested when compared to controls. The specific activity at the peak in the diurnal cycle was approximately 8-fold higher in cholestyramine-treated animals. Rats administered the cholestyramine-Mevinolin diet had higher specific activities of the enzyme than either cholestyramine-treated or control animals. In the cholestyramine-Mevinolin-treated animals the peak in the diurnal cycle was shifted to D-12 (12th hour of the dark cycle) and the specific activity at this point was approximately 133-fold greater than the basal (L-6) activity in control animals. Optimal conditions for immunotitration studies were determined such that valid conclusions could be drawn from these data. Based on immunotitration experiments, the increased hepatic HMG-CoA reductase activity in cholestyramine-treated animals resulted in part from a 3-fold activation of the enzyme, while the increased specific activity in the cholestyramine-Mevinolin-treated animals was due solely to increased enzyme mass. Hence the administration of Mevinolin blocked the activation of the enzyme induced by feeding cholestyramine alone. This was confirmed by purifying the enzyme to apparent homogeneity from cholestyramine-Mevinolin-treated animals; the specific activity of this enzyme was 4,000–7,700 nmol NADPH oxidized/min per mg protein. Cholestyramine-Mevinolin treatment affords a novel system for generating milligram quantities of rat hepatic HMG-CoA reductase.—**Tanaka, R. D., P. A. Edwards, S. Lan, E. M. Knöppel, and A. M. Fogelman.** The effect of cholestyramine and Mevinolin on the diurnal cycle of rat hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase. *J. Lipid Res.* 1982. **23:** 1026–1031.

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The enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase catalyzes the rate-controlling step in the biosynthesis of cholesterol (1, 2). Chemical analyses of this enzyme are difficult because of the relatively low concentrations of HMG-CoA reductase present in rat and human liver (3, 4). However, several methods are utilized to increase the yield of the hepatic enzyme. In rat liver the activity of HMG-CoA reductase exhibits a diurnal cycle (2) and the enzyme can then be isolated at the peak. Administration of the bile acid sequestrant, cholestyramine, to rats also stimulates reductase activity throughout the diurnal cycle (5, 6).

To further facilitate the study of HMG-CoA reductase, we were interested in finding new methods for increasing the specific activity of the rat hepatic enzyme beyond those achieved by cholestyramine treatment. Compactin, a competitive inhibitor of HMG-CoA reductase, greatly elevates the activity of reductase in rats (7), isolated rat hepatocytes (3), and human fibroblasts (8). Mevinolin, an analog of Compactin with 4–5-fold greater potency (9), might be expected to have similar effects on reductase activities. To optimize the effect of Mevinolin, we combined this drug with cholestyramine treatment in rats and examined the specific activity of the hepatic enzyme during the diurnal cycle. The results were compared to either animals treated with cholestyramine alone or to untreated animals. Alterations in hepatic HMG-CoA reductase activity were also examined by immunotitration experiments to determine if the drug-induced changes in enzyme activity were due either to changes in enzyme mass or to activation of the enzyme. We further demonstrated that feeding rats the diet containing cholestyramine and Mevinolin allows isolation of milligram amounts of purified reductase.

MATERIALS AND METHODS

Male Sprague-Dawley rats (100–150 g) were housed under a reverse illumination cycle (3) and food and

Abbreviations: IgG, immunoglobulin G; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MVA, mevalonic acid.

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water were provided ab libitum. In this alternating 12 hr light–12 hr dark cycle we have indicated the times as previously described (3) (e.g., D-6 or D-9 refer to the sixth and ninth hour of the 12-hr dark period, respectively). Animals were maintained on the reverse light-dark cycle for at least 10 days before receiving powdered food supplemented with 5% cholestyramine (Questran) (3). Some animals received this diet for 4 days, while other rats received the diet for 2 days and then for the remaining 2 days they were switched to powdered food supplemented with 5% cholestyramine plus Mevinolin (112 mg/100 g food). The concentration of Mevinolin was calculated to deliver approximately 200 mg Mevinolin/kg body weight per day. Control animals were maintained under similar conditions but received only the normal diet.

Animals were killed at various times during the light-dark cycle, the livers were excised, and microsomal pellets were prepared (3). Solubilization of the reductase was as previously described (10). HMG-CoA reductase activity was measured either by radioassay or spectrophotometric assay (10). One unit of enzyme activity is defined as the amount of protein that converts 1 nmol of HMG-CoA to mevalonic acid/min. In cases where spectrophotometric assays were used, the enzyme activity was expressed as nmol of NADPH oxidized/min and one unit of activity was the amount of protein that oxidized 1 nmol NADPH/min. Protein concentrations were determined by a modification (3) of the method described by Bradford (11).

HMG-CoA reductase was purified from livers of rats fed a diet containing 5% cholestyramine for 2 days and then switched to a diet containing 5% cholestyramine plus Mevinolin (100 mg/100 g food) for 2 days. Twenty-one animals were killed at D-9, and the hepatic HMG-CoA reductase was purified from approximately 200 g of liver as previously stated (3).

Monospecific antibody to the purified rat liver HMG-CoA reductase obtained from cholestyramine-fed rats was prepared in rabbits (3). IgG was purified from the monospecific antisera using protein-A-agarose affinity chromatography (12). The protein concentration of the IgG solution was 1 μg/μl. Immunotitrations were conducted by adding increasing amounts of antibody to a constant amount of enzyme, and after a 30-min incubation at 37°C, the remaining enzyme activity was measured using either the radioassay or the spectrophotometric assay. The equivalence point is defined as the number of enzyme units inactivated/μl of antibody (3).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was as described by Laemmli (13). The thickness of the slab gels was 1.5 mm, and the percent acrylamide in the stacking and separating gels was 2.5% and 7.5%, respectively. Buffers and the protocol for electrophoresis were those previously described (3).

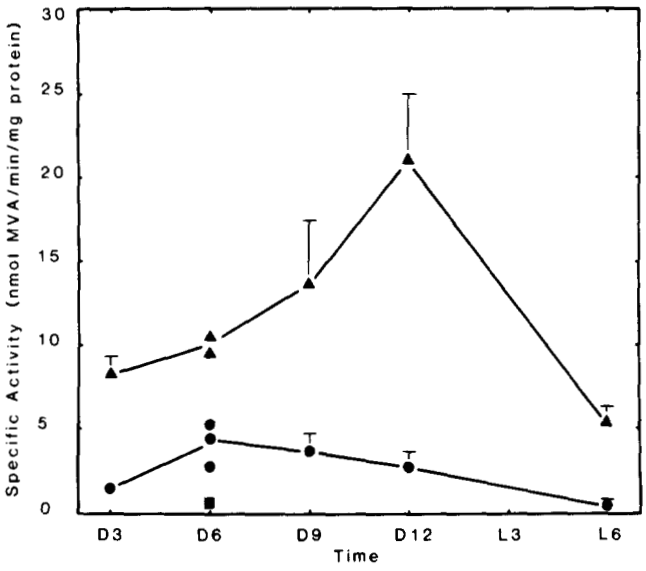

Fig. 1. Administration of cholestyramine or cholestyramine plus Mevinolin to rats and the effects on the diurnal periodicity of hepatic HMG-CoA reductase activity. Rats were fed three different diets: *A*, powdered food containing 5% cholestyramine for 4 days prior to being killed (●); *B*, powdered food containing 5% cholestyramine for 2 days and then switched to food containing 5% cholestyramine plus Mevinolin (112 mg/100 g food) for 2 days (▲); *C*, powdered food only (■). The concentration of Mevinolin was adjusted to deliver 200 mg of Mevinolin/kg body weight per day. Rats were killed at the times noted. Liver microsomes were prepared and the specific activity of HMG-CoA reductase was measured (3). Individual data points are shown for time points at which fewer than four animals were tested. All other time points reflect the mean from at least four animals and the brackets indicate the standard deviation. Only one time point corresponding to the peak in the diurnal cycle is shown for the control animals who received only powdered food. The activities of the other time points were too low to be accurately depicted in the graph. Basal (L-6) specific activity was approximately 0.15 nmol MVA/min per mg microsomal protein. This activity was 133-fold lower than the highest activity in the cholestyramine-Mevinolin-treated animals.

Gels were stained for protein using the silver-staining procedure of Oakley, Kirsch, and Morris (14). All other sources of material and equipment have been previously reported (3).

RESULTS

Diurnal periodicity of hepatic HMG-CoA reductase activity

Feeding cholestyramine to rats increased the specific activity of the hepatic HMG-CoA reductase at all time points tested when compared to control animals (**Fig. 1**, circles). Complete data from control animals are not shown in Fig. 1; however the specific activity at the peak (D-6) in the diurnal cycle of the hepatic enzyme in the normal-fed rat was approximately 0.5 nmol MVA/min per mg microsomal protein. The basal activity at the nadir (L-6) was approximately 0.15 nmol MVA/min per mg microsomal protein (data not shown). The peak

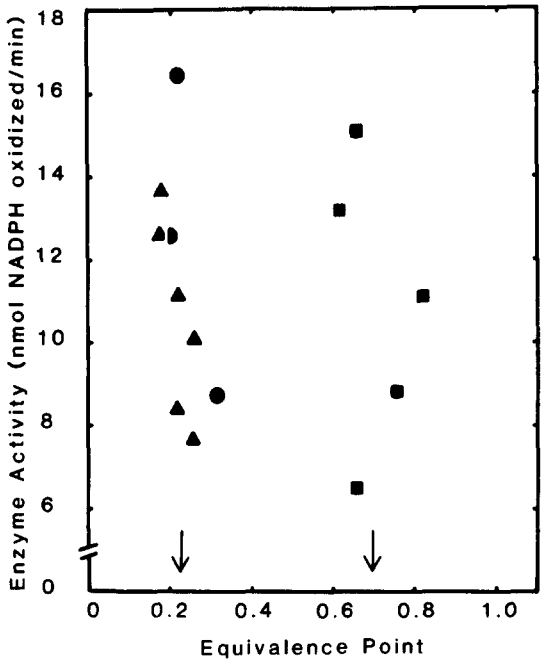

Fig. 2. Equivalence points from immunotitration experiments carried out on hepatic HMG-CoA reductase from rats fed diets containing either cholestyramine, cholestyramine plus Mevinolin, or powdered food alone. HMG-CoA reductase was solubilized from hepatic microsomes (10) and immunotitrations were performed using varying amounts of enzyme activity as noted in the graph. Immunotitrations were performed by adding increasing amounts of antibody to a constant amount of enzyme, incubating 30 min at 37°C, and measuring the remaining reductase activity spectrophotometrically (3). Enzyme activity was expressed as the nmol NADPH oxidized/min. Symbols correspond to: *A*, animals fed a diet containing 5% cholestyramine for 4 days and killed at D-6 (■); *B*, animals fed a diet containing 5% cholestyramine for 2 days, then switched to a diet containing 5% cholestyramine plus Mevinolin (112 mg/100 g food) for 2 days, and killed at D-9 (▲); *C*, animals fed powdered food only and killed at L-6 (●). The arrows depict the mean for the above data points.

in the diurnal cycle occurred at D-6 in both the normal and cholestyramine-fed animals. Maximal activities in the latter group were approximately 4.0 nmol MVA/min per mg microsomal protein.

Augmenting the cholestyramine-containing diet with Mevinolin significantly increased the specific activity of HMG-CoA reductase at all time points tested (Fig. 1, triangles). The peak in the diurnal cycle of the hepatic enzyme was shifted to D-12, and the specific activity at this time point was approximately 20 nmol MVA/min per mg microsomal protein. This was 5-fold and 20-fold greater than the specific activities at the peaks (D-6) in the cholestyramine-fed and normal-fed animals, respectively.

The effect of administering different amounts of Mevinolin with the diet containing 5% cholestyramine indicated that a dose of 200 mg Mevinolin/kg body weight per day gave maximal activities of hepatic HMG-CoA reductase. Animals receiving a dosage of either 0, 25, 200, or 250 mg Mevinolin/kg body weight per day, and killed at D-6 had specific activities of 4.5, 7.9, 10.2, and 9.5 nmol MVA/min per mg microsomal protein, respectively. Preliminary results indicated that rats receiving the diet supplemented with 200 mg Mevinolin/kg body weight for 4 days exhibited a lower level of reductase activity compared to rats fed for only 2 days. These high doses given for 4 days may be toxic since daily food consumption decreased on the fourth day.[2] We have not determined what percentage of the 20 mg of Mevinolin eaten per day by a 100 g rat was in fact absorbed.

Immunotitration studies

Immunotitration experiments were conducted to determine if the changes in hepatic enzyme activity induced by cholestyramine or cholestyramine plus Mevinolin were due to changes in enzyme mass or due to activation of the enzyme. Results from the immunotitration experiments on solubilized enzyme preparations from rats fed cholestyramine (D-6), cholestyramine plus Mevinolin (D-9), and normal diets (L-6) are shown in **Fig. 2.** Enzyme activities were measured using the spectrophotometric assay because of the relatively high amounts of enzyme activity used in each immunotitration experiment. Under the conditions employed and using at least 7 units (nmol NADPH oxidized/min) of enzyme activity per assay, the equivalence point was independent of the initial enzyme activity (Fig. 2). The enzyme solubilized from cholestyramine-fed animals gave an average equivalence point of 0.7 ± 0.08 (SD). In comparison, the enzyme obtained from rats fed either the normal diet or a diet supplemented with cholestyramine plus Mevinolin had similar equivalence points with an average value of 0.23 ± 0.04 (SD). Based on the 3-fold difference in equivalence points, we concluded that the enzyme from cholestyramine-fed animals had 3-fold greater activity than the enzyme solubilized from microsomes isolated from either normal-fed or cholestyramine-Mevinolin-treated animals. Hence the increase in hepatic enzyme activity induced by cholestyramine feeding resulted in part from a 3-fold activation of the enzyme. This is similar to previously reported results (3, 6). However, the cholestyramine-induced enzyme activation was blocked when Mevinolin was added to the cholestyramine-containing diet. Therefore, the 133-fold increase in hepatic enzyme activity (Fig. 1) that occurred after cholestyramine-Mevinolin treatment resulted solely from increased enzyme mass.

[2] Tanaka, R. D., P. A. Edwards, S. Lan, E. M. Knöppel, and A. M. Fogelman. Unpublished data.

Variation in equivalence point with low levels of enzyme activity

Enzyme solubilized from the same preparation of microsomes isolated from a cholestyramine-fed animal (D-6) was diluted to the enzyme activities specified in **Fig. 3,** and immunotitration experiments were conducted. Due to the low amounts of enzyme, the reductase activity was measured using the radioassay and enzyme activity was expressed as nmol MVA/min. Since there was no change in the specific activity of the enzyme after dilution (i.e., no enzyme inactivation) the equivalence point would be expected to remain constant as noted in Fig. 2. However, as shown in Fig. 3, the equivalence point was proportional to the initial level of enzyme activity. These data could be erroneously interpreted to suggest that enzyme inactivation or activation occurred since the equivalence point fluctuated with the amount of enzyme activity used in each immunotitration. This would be inconsistent with the absence of any change in the specific activity of the enzyme. Similar data were obtained using either another antibody preparation made in a different rabbit or microsomal-bound enzyme. We have also observed this relationship between initial enzyme activity levels and the equivalence point in studies with the low levels of enzyme found in human monocyte-macrophages (15). Therefore we propose that the results and conclusions drawn from immunotitration studies on HMG-CoA reductase will only be valid when it can be shown that the equivalence point is independent of the initial level of enzyme activity.

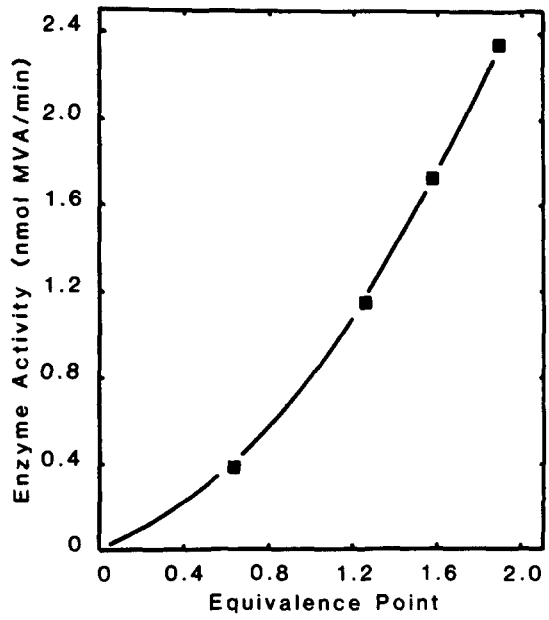

Fig. 3. Equivalence points from immunotitration experiments using different amounts of hepatic HMG-CoA reductase from rats fed a diet containing 5% cholestyramine for 4 days and killed at D-6. Conditions were similar to those described in Fig. 2 except that reductase activity was measured using a ^{14}C radioassay (3). All immunotitrations were performed on one enzyme preparation and the sample was diluted to attain the enzyme activities noted in the graph. There was no decrease in specific activity following dilution. Similar results have been obtained with other enzyme preparations.

Purification of hepatic HMG-CoA reductase from cholestyramine-Mevinolin-treated animals

The immunotitration data (Fig. 2) indicated that the enzyme from cholestyramine-fed animals had 3-fold greater activity than enzyme taken from animals fed either a normal diet or a diet containing cholestyramine and Mevinolin. To further corroborate these data, hepatic HMG-CoA reductase was purified from cholestyramine-Mevinolin-treated animals. The specific activity of the purified enzyme was 4,000–7,700 nmol NADPH oxidized/min per mg protein. This is 3–4-fold lower than the corresponding values of 17,000–23,000 that we have always obtained when enzyme was purified by an identical method from rats fed cholestyramine for 4 days and killed at D-6 (3).

The enzyme from cholestyramine-Mevinolin-treated animals was essentially homogeneous as demonstrated by SDS polyacrylamide gel electrophoresis (**Fig. 4**). The purified enzyme produced one major protein staining band corresponding to a molecular weight of approximately 53,000 when the gel was deliberately over stained to reveal minor components. (Fig. 4, Lane A). A minor protein contaminant (M_r = 98,000) was present but constituted less than 1% of the total protein. The major polypeptide (M_r = 53,000) was identical to that obtained after electrophoresis of pure reductase obtained from cholestyramine-treated animals and with a specific activity of 20,778 nmol NADPH/min per mg protein (data not shown). Also when the SDS polyacrylamide gels were not overstained, the enzyme obtained from both cholestyramine-Mevinolin-treated and cholestyramine-treated animals appeared to be composed of two equal-staining polypeptides with molecular weights of 52,000 and 54,000 (data not shown). Hence these data corroborate our immunotitration results, and we conclude that the 3-fold activation normally occurring after cholestyramine feeding is blocked by Mevinolin.

Since the increased hepatic enzyme activity due to cholestyramine-Mevinolin treatment resulted solely from increased enzyme mass, we predicted that the total yield of purified enzyme per g liver would also increase. In the enzyme purification noted above, a total of 2.6 mg of essentially homogeneous reductase (Fig. 4) was obtained from 21 rats (total liver weight approximately 200 g). This represented an overall recovery of 38%. Compared to our purification of the enzyme from cho-

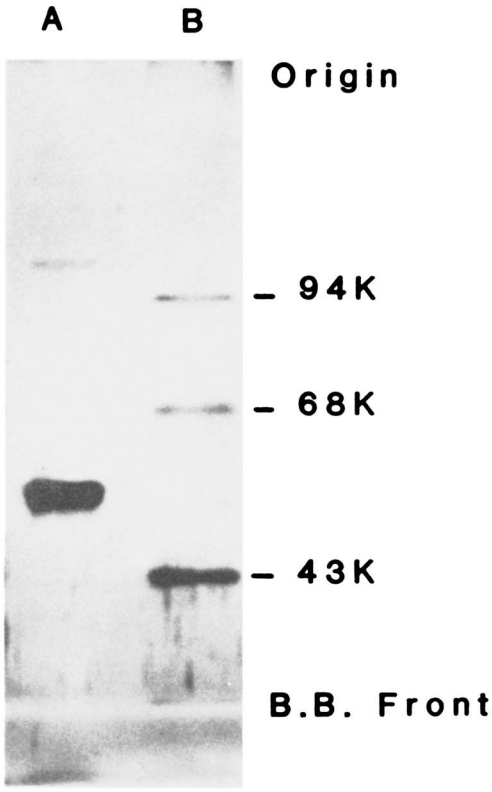

Fig. 4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified HMG-CoA reductase from rat liver. The enzyme was purified (3) from rat livers taken from animals fed a diet containing 5% cholestyramine for 2 days, then switched to a diet containing 5% cholestyramine plus Mevinolin (100 mg/100 g food) for 2 days, and killed at D-9. Conditions for electrophoresis were those previously described (3). Lane A contained 180 ng of the pure reductase and lane B contained 140 ng of the Bio-Rad protein standards. Gels were stained for protein using the silver staining procedure (14). The relative positions of the protein standards are indicated on the right of the figure.

lestyramine-treated animals killed at D-6 (3), there was approximately 5-fold more enzyme mass/g liver in the cholestyramine-Mevinolin-treated animals. This is an estimation and may not accurately reflect the actual magnitude. However, these data demonstrate that more enzyme mass can be purified from cholestyramine-Mevinolin-treated rats. The total yield of 2.6 mg of pure enzyme from 21 animals would be predicted to be approximately 1.7-fold higher if the enzyme was purified at the peak in the diurnal cycle in cholestyramine-Mevinolin-treated animals (i.e., D-12, see Fig. 1).

DISCUSSION

Both cholestyramine, a bile acid sequestrant, and cholestyramine plus Mevinolin induced higher levels of hepatic HMG-CoA reductase activity during the diurnal cycle of the enzyme. However, administration of Mevinolin, a potent competitive inhibitor of HMG-CoA reductase (9), caused a much larger increase in the specific activity of the enzyme. The peak activity (D-12) in the cholestyramine-Mevinolin-treated rats was 5-fold greater than the highest level of activity observed in rats fed cholestyramine alone. Mevinolin may increase the reductase activity by a mechanism similar to that of Compactin. The latter drug was reported to elevate the level of the enzyme in hepatocytes and fibroblasts by production of large amounts of latent enzyme which became active during enzyme isolation and subsequent removal of the inhibitor (3, 8, 16).

Based on the immunotitration experiments and using levels of enzyme activity at which the equivalence point was constant, the large increase in hepatic reductase activity due to administration of cholestyramine plus Mevinolin resulted solely from increased enzyme mass since the equivalence point was identical to that from control basal animals (Fig. 2). This was very different from the effect observed after feeding cholestyramine alone. In the latter instance and in agreement with earlier studies (3, 6), the observed increase in hepatic reductase activity resulted in part from a 3-fold activation of the enzyme (Fig. 2). If these data from the equivalence points were valid, we predicted that the enzyme purified from rats fed the cholestyramine-Mevinolin diet should have specific activities approximately one-third of the values (17,000–23,000 nmol NADPH oxidized/min per mg protein) obtained from rats fed cholestyramine alone (3). Experimentally, the specific activity of the purified enzyme (4,000–7,700 nmol NADPH oxidized/min per mg protein) was shown to be approximately 3- to 5-fold lower than that obtained from cholestyramine-fed animals (3). Hence cholestyramine-Mevinolin treatment induced large increases in the amount of HMG-CoA reductase present in the liver and, unlike treatment with cholestyramine alone, the increased activity did not result from enzyme activation.

How Mevinolin blocked the cholestyramine-induced activation of the hepatic enzyme is currently unknown and is being investigated. However, our observation that cholestyramine-Mevinolin treatment induced large increases in the mass of enzyme present in rat liver may be of great importance in future studies since this method affords a novel system for isolation of milligram quantities of HMG-CoA reductase. This method may also be suitable for increasing production of messenger RNA coding for hepatic HMG-CoA reductase, and purification of this RNA would facilitate experiments involving the regulation of the reductase gene. Studies are being conducted to elucidate this point.

The variation in the equivalence point which occurred when conducting immunotitration studies with low amounts of enzyme activity demonstrated the potential pitfalls in interpreting immunotitration data (Fig.

3). The precise cause of this variation is unknown. There has been controversy in the study of rat hepatic HMG-CoA reductase activity regarding whether the changes in enzyme activity during the diurnal cycle or following diet- or drug-induced perturbations were due to activation/inactivation of the enzyme or due to changes in enzyme mass (3, 6, 16–18). Delineation has been based largely upon the outcome of immunotitration experiments. However, these studies may not have noted the possibility for fluctuation in the equivalence point based on the amount of enzyme used in the experiment. Our findings clearly demonstrate how immunotitration experiments may yield erroneous data if care is not taken to determine the effect of using low levels of enzyme activity. Therefore, the variation in the equivalence point may be common to all the previously reported work using immunotitration experiments on HMG-CoA reductase, and future studies should be cognizant of this point in order to avoid ambiguous conclusions.

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