Chem. Pharm. Bull. 26(9)2844—2849(1978)

UDC 615.272.4.011.5.015.4.076.9 : 547.918.09

Effect of Ginseng Saponins on Cholesterol Metabolism. III.[1] Effect of Ginsenoside-Rb_1 on Cholesterol Synthesis in Rats fed on High-fat Diet

MASAHIRO IKEHARA, YASUO SHIBATA, TOKUHIKO HIGASHI
SYUICHI SANADA, and JUNZO SHOJI

School of Pharmaceutical Sciences, Showa University[2]

(Received March 29, 1978)

Rats fed on high-fat diet showed a higher level of cholesterol in liver and a lower incorporation of ^{14}C-acetate into liver cholesterol. The activity of HMG-CoA reductase, which is a key enzyme in cholesterol biosynthesis, was repressed in those rats. Ginsenoside-Rb_1 purified from Ginseng (the root of *Panax ginseng* C.A. MEYER) has been found to reverse such effect of high-fat diet when administered on earlier days of feeding.

Keywords——ginseng; saponin; ginsenoside; cholesterol; high-fat diet; HMG-CoA reductase

In the previous paper Sakakibara *et al.*[1] reported the effect of five purified saponins of Ginseng on cholesterol metabolism. Among those saponins, ginsenoside-Rb_1 most enhanced the incorporation of ^{14}C-acetate into serum and liver cholesterol. The evidence suggests a stimulation of cholesterol synthesis in ginsenoside-treated rats.

In the biosynthetic pathway of cholesterol, conversion of 3-hydroxy-3-methyl glutaryl coenzyme A (HMG–CoA) into mevalonate is a rate-limiting step, which is catalyzed by HMG–CoA reductase[mevalonate: NADP oxidoreductase (acylating CoA); EC 1.1.1.34]. Ikehara[3] found an increased activity of HMG–CoA reductase in liver when rats were given 5 mg of ginsenoside-Rb_1 per 100 g body weight, supporting the evidence described above.

The biosynthesis of cholesterol is known to be inhibited by the end product, cholesterol. In the present study rats were fed on high-fat diet, and the effect of ginsenoside-Rb_1 on a repressed synthesis of cholesterol in those rats has been investigated.

Experimental

Animals——Male Wistar rats weighing 120—140 g were used. They were fed on high-fat diet for 1.5, 3.0, 4.5 or 6.0 weeks. The diet contained: raw proteins 25.0%, raw carbohydrates 53.15%, raw fats 15.0%, minerals 5.0%, vitamins 1.7% and choline chloride 0.15%.

Saponin——Ginsenoside-Rb_1 was isolated and purified from Ginseng,[4] and proved to be pure by chemical and physicochemical analysis. Five mg of ginsenoside-Rb_1 per 100 g body weight was injected into rats intraperitoneally. The time of injection was scheduled between 8: 30 and 9: 30 *a.m.* and rats were killed after 4 hr, unless otherwise indicated.

Assay of Cholesterol——Total and free cholesterol in serum was determined by a modification[5] of Zak's method.[6] Liver cholesterol was determined by a modification of the method reported by Ichida,[7] as described previously.[1]

Measurement of ^{14}C incorporated into Liver Cholesterol——Sodium acetate-1-^{14}C (10 μCi per 100 g body weight) was injected 90 min prior to sacrifice, *i.e.*, 2.5 hr after the injection of saponin in the case of saponin-

1) Part I: K. Sakakibara, Y. Shibata, T. Higashi, S. Sanada, and J. Shoji, *Chem. Pharm. Bull.* (Tokyo), **23**, 1009 (1975). Part II: K. Gommori, F. Miyamoto, Y. Shibata, T. Higashi, S. Sanada, and J. Shoji, *Chem. Pharm. Bull.* (Tokyo), **24**, 2985 (1976).
2) Location: *1-5-8, Hatanodai, Shinagawa-ku, Tokyo, 142, Japan.*
3) M. Ikehara, *Thesis for Master Degree* (*Showa University*).
4) S. Sanada, N. Kondo, J. Shoji, O. Tanaka, and S. Shibata, *Chem. Pharm. Bull.* (Tokyo), **22**, 421 (1974).
5) "Hyojun Rinsyo Kensaho," ed. by M. Motegi and H. Suzuki, Igaku-shoin, Tokyo, 1967, p. 214.
6) A. Zlatkis, B. Zak, A.J. Boyle, and D. Mich, *J. Lab. Clin. Med.*, **41**, 486 (1953).
7) T. Ichida, *Hokkaido J. Med. Sci.*, **38**, 67 (1963).

NII-Electronic Library Service

treated rats. Liver cholesterol was isolated as reported in the previous paper,[1] and assayed for the radioactivity using a scintillation spectrometer (Beckman LS-100C).

Microsomal Protein Solution——The preparation of liver microsomes for HMG-CoA reductase activity was carried out by the method of Nicolau *et al.*[8] Liver was homogenized in 8 volumes of a solution containing 0.3 M sucrose, 0.075 M nicotinamide, 0.002 M EDTA and 0.02 M mercaptoethanol. Postmitochondrial supernatant separated by centrifugation at $11000 \times g$ for 15 min, was further spun at $100000 \times g$ for 60 min. Microsomal pellet was washed once by centrifugation at $100000 \times g$ for 60 min, and suspended in a small amount of the solution described above. The protein concentration was measured according to the method of Lowry *et al.*[9]

HMG-CoA Reductase Activity——The method reported by Iijima *et al.*[10] was used with a slight modification. The reaction mixture contained in 50 μl: 10 μmol of potassium phosphate buffer, pH 7.4, 3 μmol of EDTA, 3 μmol of glucose-6-phosphate, 0.3 μmol of NADP, 2 μmol of dithiothreitol and 0.35 unit of glucose-6-phosphate dehydrogenase. To 50 μl of this mixture, 20 μl of microsomal protein solution (enzyme solution) was added and was preincubated at 37° for 5 min. Then 30 μl of a substrate solution containing 15.8 nmol of HMG-CoA (DL-3-^{14}C-HMG-CoA and unlabelled HMG-CoA, 1:1) was added. After incubation at 37° for 15 min, the reaction was stopped by adding 20 μl of a mixture of 1 M mevalonolactone and concentrated HCl (1:1), followed by centrifugation at $1600 \times g$ for 15 min. An aliquot (10 μl) of the supernatant was chromatographed on a silica-gel plate, using benzine and acetone (1:1). Mevalonolactone visualized with iodine vapor was collected and assayed for radioactivity by a scintillation spectrometer (Aloka LSC-502). A reaction mixture without microsomal protein solution was employed as control. The enzyme activity was expressed as cpm/mg protein/15 min.

Chemicals——^{14}C-Acetate (specific radioactivity: 58.8 mCi per mmol) was purchased from The Radiochemical Centre, Amersham, England and ^{14}C-HMG-CoA (specific radioactivity: 49.5 mCi per mmol) was from New England Nuclear, Boston, Mass. U.S.A. Glucose-6-phosphate, NADP and glucose-6-phosphate dehydrogenase were obtained from Boehringer Mannheim.

Results

Cholesterol Metabolism in Rats fed on High-fat Diet

Figure 1 shows the gain in body weight of rats during the period of feeding on high-fat diet (~6 weeks). No remarkable difference was observed from that of control rats fed on regular diet containing 5% of fat.

The amount of cholesterol in serum and in liver of the rats fed on high-fat diet are shown in Fig. 2 and 3. Both total and free cholesterol level in serum don't seem to be affected so much by feeding on high-fat diet (Fig. 3). However, the amount of liver cholesterol, both in total and in free, was found to be larger in rats on high-fat diet than the control.

Incorporation of ^{14}C-acetate into liver cholesterol was apparently reduced in the rats fed on high-fat diet, as shown in Fig. 4. The findings indicate a feed-back inhibition for cholesterol biosynthesis by exogenous cholesterol.

Supporting the repression in cholesterol synthesis, the activity of HMG–CoA reductase in liver was observed to be extremely lowered in rats on high-fat diet (Fig. 5). For approximation of the experimental conditions physiological saline solution was injected intraperitoneally into rats 2 hr before assaying the enzyme activity. Furthermore, the time of killing rats was fixed at or about 1:00 *p.m.* In control rats HMG–CoA reductase activity showed no appreciable fluctuation throughout the period of experiment.

Effect of Ginsenoside-Rb$_1$ on Cholesterol Metabolism in Rats fed on High-fat Diet

Ginsenoside-Rb$_1$ was administered to rats fed on high-fat diet for various period of time and its effect to improve the repressed metabolism of cholesterol was investigated. Table I shows a reduction in the level of liver cholesterol by the treatment of ginsenoside-Rb$_1$. The same effect was previously observed by Sakakibara[1] with normal rats. However, after

8) G. Nicolau, S. Shefer, G. Salen, and E.H. Mosbach, *J. Lipid Res.*, **15**, 94 (1974).
9) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
10) Y. Iijima and Y. Maruyama, *Proceedings of Japanese Conference on the Biochemistry of Lipid*, **15**, 164 (1973).

NII-Electronic Library Service

6-week feeding on high-fat diet the saponin could not produce an elimination of liver cholesterol.

Fig. 6 illustrates the incorporation of ^{14}C-acetate into liver cholesterol in the experimental rats with or without treatment of ginsenoside-Rb_1. After 1.5-week feeding on high-fat

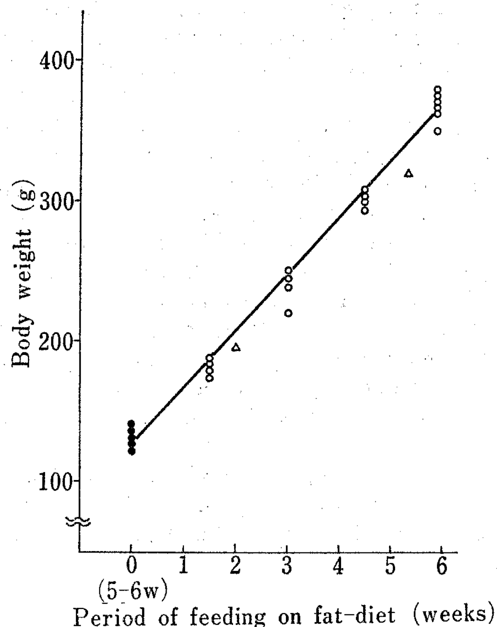

Fig. 1. Body Weight of Rats fed on Fat-diet

Rats were 5—6 weeks of age at the start of the experiments.
○: fat-diet,
●: before starting experiment,
△: average of control rats.

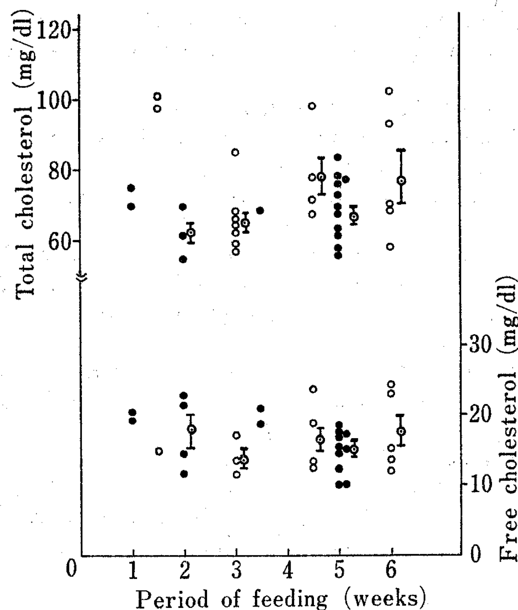

Fig. 2. Serum Cholesterol Level of Rats fed on Fat-diet

○; fat-diet, ●: control-diet, ⊙ (with bars): mean ±S.E.

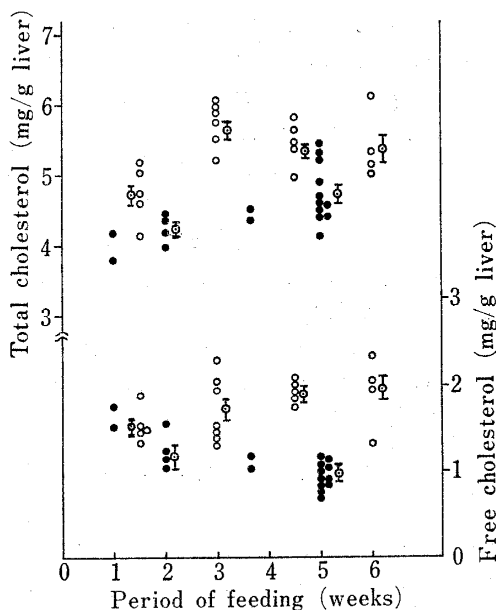

Fig. 3. Liver Cholesterol Level of Rats fed on Fat-diet

○: fat-diet, ●: control-diet.

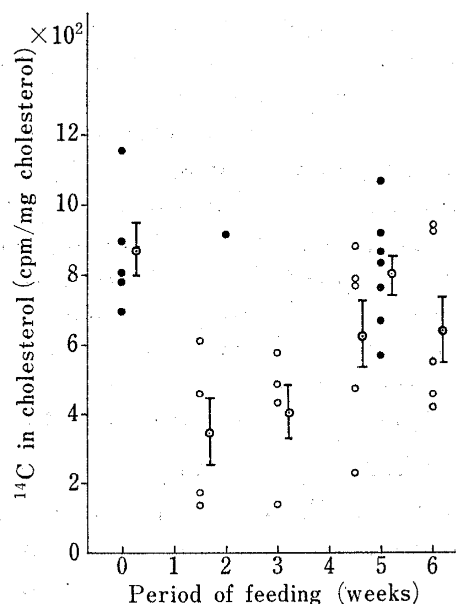

Fig. 4. Incorporation of [1-^{14}C]-acetate into Liver Cholesterol in Rats fed on Fat-diet

Rats were fed on fat-diet (○) or on control-diet (●) for indicated period, and were injected intraperitoneally with [1—^{14}C]-acetate at 90 minutes prior to sacrifice.

NII-Electronic Library Service

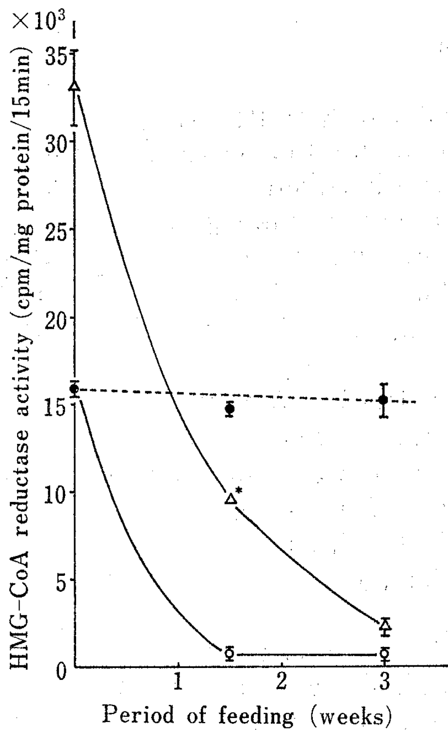

Fig. 5. Effect of High-fat Diet and of Ginsenoside-Rb_1 on HMG-CoA Reductase Activity in Liver

Rats were fed on fat-diet (○, △) or on control-diet (●) for indicated period, and injected intraperitoneally with either ginsenoside-Rb_1 (5 mg/100 g body weight) (△) or physiological saline solution (○, ●). 2 hours prior to sacrifice. The values are the mean ±standard error of 2—3 experiments, except for the one from a single experiment indicated by asterisk.

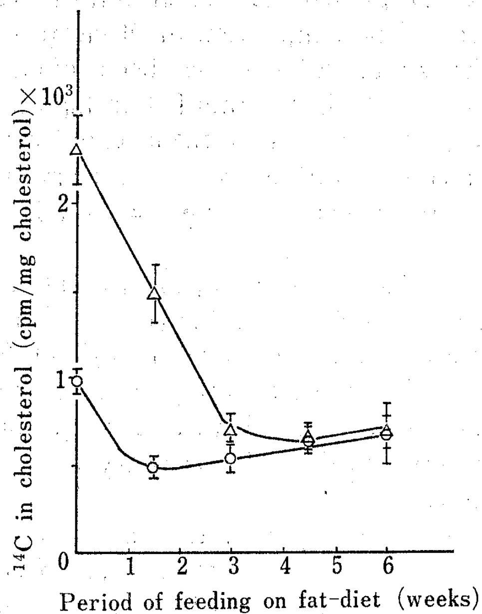

Fig 6. Effect of Ginsenoside-Rb_1 on Incorporation of [1-^{14}C]-acetate into Liver Cholesterol in Rats fed on Fat-diet

△: with Rb_1-treatment. Rats were fed on fat-diet for indicated period and killed at 4 hours after the injection of Rb_1. They were injected intraperitoneally with [1—^{14}C]-acetate at 90 minutes prior to sacrifice. ○: without Rb_1-treatment. Each value is the mean of 2—6 experiments.

Table I. Effect of Ginsenoside Rb_1 on Liver Cholesterol Level in Rats fed on High-fat Diet

Period of feeding (weeks)	Liver cholesterol level					
	Total			Free		
	Rb_1(−) (mg/g liver)	Rb_1(+)	(+)/(−) (%)	Rb_1(−) (mg/g liver)	Rb_1(+)	(+)/(−) (%)
1.5	4.75±0.16	3.99±0.25	84.0	1.52±0.08	1.31±0.12	86.8
3.0	5.71±0.12	5.05±0.27	87.8	1.69±0.14	1.24±0.12	73.4
4.5	5.43±0.12	4.88±0.10	89.9	1.92±0.09	1.68±0.05	87.5
6.0	5.39±0.21	5.38±0.23	100.0	1.96±0.16	2.07±0.05	106.1

Values are the mean ±standard error, calculated from those of 3—5 experiments.

diet the enhancement of cholesterol biosynthesis by the ginsenoside was still detectable even if by a reduced extent compared with that at the start of the experiment. However, feeding for more than 3 weeks abolished the effect of the saponin.

The activity of HMG–CoA reductase in rats fed on high-fat diet was assayed after the treatment with ginsenoside-Rb_1. The rats were killed at 2 hr after injection of the saponin, when the most increased activity of the enzyme was observed in the experiment with normal rats.[3] Stimulation in HMG–CoA reductase by the treatment with the ginsenoside was seen after 1.5-week of the feeding whereas almost none was detected after 3 weeks (Fig. 5). The results coinside with those obtained in the incorporation of ^{14}C-acetate into liver cholesterol (Fig. 6)

NII-Electronic Library Service

Discussion

It is well known that the activity of HMG–CoA reductase regulates the biosynthesis of cholesterol. The stimulation in ^{14}C-acetate incorporation into liver cholesterol by the treatment with ginsenoside-Rb_1 has been found to be associated with the increase in HMG–CoA reductase activity in the rats fed on high-fat diet, as observed in normal rats.[1,3] A high-fat diet produced an increase in the level of liver cholesterol and a repression in cholesterol synthesis with a concomitant decrease in the activity of HMG–CoA reductase in liver. Ginsenoside-Rb_1 could reverse these changes when administered in the earlier stage of exposure to high-fat diet.

Since a feed-back inhibition by cholesterol for HMG–CoA reductase has been known, the ginsenoside may act to increase the activity of this enzyme secondary to eliminating the cholesterol in liver. As the period of high-fat feeding became longer beyond 3 weeks, cholesterol synthesis seemed to recover gradually to the normal level (Fig. 4 and 6), and the effect of ginsenoside-Rb_1 turned out undistinguishable (Fig. 6). Therefore, the feed-back regulation on cholesterol synthesis is necessary to be intact for the appearance of the effect of the ginsenoside.

Yamamoto[11] reported that a crude extract from Ginseng enhanced the excretion of injected 4- ^{14}C-cholesterol into bile and feces. Ikehara[3] investigated the 24-hr excretion of injected ^{14}C-cholesterol into bile, using rats treated daily with 2 mg of ginsenoside-Rb_1 for 5 days. However, he detected no significant difference from that in normal rats. In connection to this, Ikehara has obtained the evidence that the stimulative effect of the ginsenoside on cholesterol synthesis is rather temporary and non-accumulative.[3] Therefore, to confirm a stimulation by the ginsenoside on the degradative process of liver cholesterol to bile acids, a short-term experiment should be desirable. The enzyme assay for cholesterol 7α-hydroxylase, a key enzyme in cholesterol degradation, is one of the candidates under investigation.

According to Myant and Mitropoulos,[12] HMG–CoA reductase and cholesterol 7α-hydroxylase have been reported to show a correlative alteration in many cases. Administration of ginsenoside-Rb_1 possibly increases the activity of the latter enzyme as did the former one. The synthesis of bile acids was reported to increase by feeding dogs[13] or rats[14] on cholesterol-rich diet. In those cases HMG–CoA reductase activity decreased,[15,16] whereas the activity of cholesterol 7α-hydroxylase was found to increase,[17–20] or not to change.[21,22] Therefore, both enzyme activities are not necessarily parallel.

Recently, Tormanen *et al.*[23] have suggested the existence of another regulatory factor than cholesterol, which inactivates HMG–CoA reductase. Furthermore, Srikantaiah *et al.*[24] have pointed out the importance of NADPH/$NADP^+$ ratio *in vivo* in the control mechanism

11) M. Yamamoto, *Metabolism and Disease*, **10**, 184 (1973).
12) N.B. Myant and K.A. Mitropoulos, *J. Lipid Res.*, **18**, 135 (1977).
13) E.H. Mosbach and F.E. Kendall, *Circulation*, **16**, 490 (1957).
14) J.D. Wilson, *J. Lipid Res.*, **5**, 409 (1964).
15) T.C. Linn, *J. Biol. Chem.*, **242**, 990 (1967).
16) D.J. Shapiro and V.W. Rodwell, *Biochemistry*, **11**, 1042 (1972).
17) G.S. Boyd, N.A. Scholau, and J.R. Mitton, *Adv. Exp. Med. Biol.*, **4**, 443 (1969).
18) K.A. Mitropoulos, S. Balasubramaniam, and N.B. Myant, *Biochim. Biophy. Acta*, **326**, 428 (1973).
19) J.V. Cantfort, *C.R. Acad. Sci., Paris*, **275**, 1015 (1972).
20) R.F. Raicht, B.I. Cohen, S. Shefer, and E.H. Mosbach, *Biochim. Biophys. Acta*, **388**, 374 (1975).
21) I. Björkhen and H. Danielson, *Eur. J. Biochem.*, **53**, 63 (1975).
22) E.H. Mosbach, "Regulation of Bile Acid Synthesis," in Bile acids in human diseases, II. Bile Acid Meeting, Freiburg i. Br., June 30—July 1, P. Back, and W. Gerok (ed.), F.K. Schattauer Verlag, Stuttgart., 89—96, (1972).
23) C.D. Tormanen, M.V. Srikantaiah, J.E. Hardgrave, and T.J. Scallen, *J. Biol. Chem.*, **252**, 1561 (1977).
24) M.V. Srikantaiah, C.D. Tormanen, W.L. Redd, J.E. Hardgrave, and T.J. Scallen, *J. Biol. Chem.*, **252**, 6145 (1977).

NII-Electronic Library Service

of cholesterol biosynthesis, considering from the K_m value of a purified HMG–CoA reductase to the cofactor, NADPH. It is of interest to investigate whether ginsenoside-Rb_1 affects those factors to alter cholesterol metabolism.

Diurnal rhythm has been observed with the two key enzymes described above. They are high in activity in the dark, and low in the light.[25–27] Since a similar diurnal changes was reported in the level of corticosterone in circulation,[28,29] Balasubramaniam *et al.*[30] examined the relationship between the activities of the two enzymes and the amount of corticosterone in blood plasma, using adrenectomized rats. They observed a peak of the former a few hours later than that of the latter, and concluded that the diurnal rhythm in the activities of HMG–CoA reductase and cholesterol 7α-hydroxylase was induced by corticosterone. Taking such diurnal rhythm into consideration rats were sacrificed at or about 1:00 p.m. in the present study. Undoubtedly it is a matter worthy to be studied whether the effect of the ginsenoside on cholesterol metabolism is mediated by the secretion of adrenocortical hormones.

25) M. Higgins, T. Kawachi, and H. Rudney, *Biochem. Biophys. Res. Commun.*, **45**, 138 (1971).
26) J. Gielen, J.V. Cantfort, B. Robaye, and J. Renson, *C.R. Hebd. Séances Acad. Sci. Ser. D.*, **269**, 731 (1969).
27) K.A. Mitropoulos and S. Balasubramaniam, *Biochem. J.*, **128**, 1 (1972).
28) R. Guillemin, W.E. Dear, and R.A. Leibelt, *Proc. Soc. Exp. Med.*, **101**, 394 (1959).
29) P.N. Cheifetz, *J. Endocrinol.*, **49**, XI—XII (1971).
30) S. Balasubramaniam, K.A. Mitropoulos, and N.B. Myant, "Hormonal Control of the Activities of Cholesterol 7α-Hydroxylase and Hydroxymethylglutaryl-CoA Reductase in Rats," in Adv. in Bile Acid Res., III. Bile Acid Meeting, Freiburg i.Br., June 13—15, S. Matern, J. Hackenschmidt, P. Back, and W. Gerok(ed.), F.K. Schattrauer Verlag, Stüttgart, pp. 61—67 (1975).

NII-Electronic Library Service