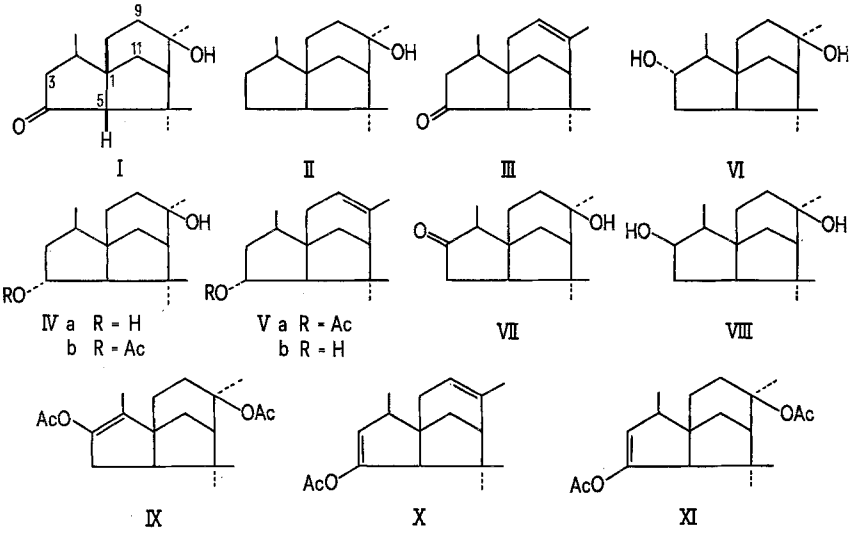
carbonyl group must be located at C_3 or C_4. Recently, microbial hydroxylation of cedrol has been reported[3]. Three metabolites, 3α-hydroxycedrol VI, 3-ketocedrol VII and 3β-hydroxycedrol VIII, were isolated and identified. The proof of oxidation at C_3 was demonstrated by the preparation of IX from VII[4]. I and VII were obviously different compounds by comparison of their physical data. Therefore the carbonyl group of I must be located at C_4. This assignment was further supported when I gave X and XI by treatment with *p*-toluenesulphonic acid in isopropenyl acetate at 96°. The minor product X exhibits IR-absorption bands at 1755 and



1695 cm^{-1} (—C=C—OAc) and NMR-spectrum signals at τ 7.85 (3H, s, CH_3COO—) and 4.66 (2H, br s, CH_3—C=C—H, and AcO—C=C—H). The major product XI shows two acetate absorption bands in IR-spectrum at 1755 and 1725 cm^{-1} and NMR-spectrum signals at τ 8.03 and 7.85 (each of 3H, s, CH_3COO- and CH_3COO—C=C—) and 4.59 (1H, br s, CH_3COO—C=C—H). Finally, the assignment of the hydroxyl group at C_4 in IVa is in good agreement with the observation that on sodium borohydride reduction of I, the reducing agent approaches from the sterically less hindered β-side, thus giving exclusively IVa.

[3] K. C. WANG, L. Y. Ho and Y. S. CHENG, J. Chinese biochem. Soc. *1*, 53 (1972).
[4] Unpublished data (private communication from Dr. K. C. WANG).

Ascorbic Acid and Cholesterol: Effect of Graded Oral Intakes on Cholesterol Conversion to Bile Acids in Guinea-Pigs

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Summary. A significant correlation between liver ascorbic acid (AA) and total bile acids or liver bile acids has been established in guinea-pigs by direct determination of the bile acids, confirming an earlier hypothesis. The oxidation of cholesterol to bile acids is dependent on the AA status, but it cannot be further stimulated by AA when the animals are already on an adequate intake of the vitamin. This suggests that AA has a hypocholesterolaemic effect over a limited range of AA status.

In guinea-pigs with chronic ascorbic acid (AA)[1] hyposaturation, cholesterol accumulation in the liver was significantly enhanced and even more pronounced when feeding an atherogenic diet with added cholesterol resulting in significantly raised cholesterol levels in several tissues[2-4]. Catabolism of cholesterol was found to be decreased in scorbutic guinea-pigs, and addition of AA enhanced the conversion of (4-^{14}C) cholesterol to bile acids by liver mitochondrial preparations from AA-deficient guinea-pigs[5]. The rate of conversion of cholesterol to bile acids is significantly correlated with the hepatic AA concentration in guinea-pigs, as concluded from experiments on the catabolism of (26-^{14}C) cholesterol using $^{14}CO_2$ exhalation as an indirect measure of the formation of bile acids[6-9]. It was postulated that AA is essential in the hydroxylation of cholesterol and that the action of AA on its catabolism is mediated via cytochrome P-450[8]. The rate-limiting steps in the degradation of cholesterol are side-chain oxidation and 7α-hydroxyla-

[1] Abbreviations: AA, ascorbic acid; Na-AA, sodium ascorbate.
[2] E. GINTER, P. BOBEK, Z. KOPEC, M. OVECKA and L. CEREY, Z. Versuchstierk. *9*, 228 (1967).
[3] E. GINTER, P. BOBEK, J. BABALA and E. BARBIERIKOVA, Cor Vasa *11*, 65 (1969).
[4] E. GINTER, J. BABALA and J. CERVEN, J. atheroscler. Res. *10*, 341 (1969).
[5] R. GUCHHEIT, B. C. GULIA and N. C. GANGULI, Biochem. J. *86*, 193 (1963).
[6] E. GINTER, J. CERVEN, R. NEMEC and L. MIKUS, Am. J. clin. Nutr. *24*, 1238 (1971).
[7] E. GINTER, R. NEMEC and P. BOBEK, Br. J. Nutr. *28*, 205 (1972).
[8] E. GINTER, Science *179*, 702 (1973).
[9] E. GINTER, R. NEMEC, J. CERVEN and L. MIKUS, Lipids *8*, 135 (1973).

Table I. Effect of feeding graded oral doses of sodium ascorbate to male guinea-pigs on various parameters

	Na-ascorbate (twice daily, mg)			
	0.75	5	25	300
Ascorbic acid				
Plasma (mg/100 ml)	0.08 ± 0.02 (9)	0.19 ± 0.02 (14) [c]	0.36 ± 0.07 (10) [c]	0.65 ± 0.19 (10) [c]
Liver (mg)	0.31 ± 0.05 (9)	0.79 ± 0.04 (14) [c]	1.08 ± 0.16 (9) [c]	1.64 ± 0.46 (10) [b]
Plasma				
Cholesterol (mg/100 ml)	49.8 ± 10.4 (9)	39.5 ± 8.0 (9) [a]	37.4 ± 5.3 (10)	42.8 ± 10.5 (10)
Triglycerides (mg/100 ml)	24.7 ± 7.9 (9)	28.7 ± 4.7 (8)	24.7 ± 6.6 (10)	25.9 ± 9.7 (9)
Free fatty acids (μeq/l)	—	465.5 ± 116.7 (9)	444.4 ± 73.6 (10)	501.8 ± 85.9 (9)
Bile acids (mg)				
Liver	83.3 ± 17.7 (9)	107.8 ± 17.6 (14) [b]	97.9 ± 23.4 (10)	101.5 ± 18.1 (10)
Gall bladder bile	9.1 ± 4.0 (9)	6.7 ± 0.9 (14) [a]	6.9 ± 3.1 (8)	9.3 ± 5.1 (10)
Small intestine	18.5 ± 4.6 (9)	33.9 ± 6.5 (10) [c]	35.1 ± 6.5 (10)	31.5 ± 5.5 (10)
Total	110.0 ± 19.0 (9)	142.2 ± 25.9 (10) [b]	141.1 ± 30.2 (8)	142.2 ± 22.4 (10)

The animals received the supplement twice daily over a period of 15 days and were maintained on an ascorbic acid-free diet. Figures represent mean values ± SD, number of animals is given in brackets. [a] $p < 0.025$; [b] $p < 0.005$; [c] $p < 0.0005$; always to preceding group.

tion[10]. Whereas the 7α-hydroxylation by rat liver microsomes is unaffected by addition of AA, hydroxylation by guinea-pig liver microsomes is stimulated, but not significantly[11]. On the other hand, a marked reduction in hepatic microsomal 7α-hydroxylic activity in scorbutic guinea-pigs as compared to AA-supplemented animals was shown, suggesting that AA affects the rate of synthesis or breakdown of the cholesterol 7α-hydroxylating system[12] involving cytochrome P-450[13]. The influence of AA on the enzymes involved in the side-chain cleavage of cholesterol suggests a potential role of the vitamin as a physiological control mechanism, since high levels inhibit side-chain cleavage in immature rat ovary and bovine and porcine adrenal glands, while at lower levels the reaction proceeds[14,15].

The effect of graded oral intakes of AA on total bile acids (liver, gallbladder bile, small intestine) was thought to be a direct means of establishing the predicted involvement of AA in cholesterol catabolism. In addition, the retention by various tissues of radiaoctivity derived from (4-^{14}C) cholesterol was studied in guinea-pigs in comparison with animals on a marginal intake of AA.

Materials and methods. Male guinea-pigs were maintained prior to the experiment on an AA-free diet (Nutrition Biochemicals Co.) supplemented with 1 g AA/kg of diet, then subdivided into 4 groups and given the AA-free diet for 15 days and orally supplemented daily as follows: group 1: 2×0.75 mg Na-AA; starting weight 253.1 ± 5.8 (g ± SEM), $n = 9$; group 2: 2×5 mg Na-AA; 259.7 ± 2.9, $n = 14$; group 3: 2×25 mg Na-AA, 261.9 ± 11.5, $n = 10$; group 4: 2×300 mg, 249.2 ± 16.6, $n = 10$. On the 12th day of supplementation, each animal was orally administered a single dose of (4-^{14}C) cholesterol (29.77 μCi; 263 μg) and sacrificed 72 h later following a 6 h fasting period. Liver AA was determined by a colourimetric and plasma AA by a fluorometric method[16]. Bile acids were determined by an enzymatic method using 3-hydroxy-steroid dehydrogenase (Worthington)[17], cholesterol, triglycerides and free fatty acids by autoanalyzer methods. Radioactivity was counted by means of a Nuclear Chicago Unilux II liquid scintillation spectrometer in solubilized tissue samples (disintegrations per

Table II. Total radioactivity (dpm × 10^5 ± SD) accumulated by tissues 72 h after a single orally administered dose of 29.77 μCi (263.0 μg) (4-^{14}C)cholesterol to guinea-pigs maintained on an ascorbic acid-free diet and supplemented twice daily with graded oral doses of Na-ascorbate over 15 days

Tissues	Radioactivity (dpm × 10^5)			
	Na-ascorbate (twice daily, mg)			
	0.75	5	25	300
Liver	12.7 ± 0.88	7.34 ± 1.08 [b]	5.25 ± 2.91	5.50 ± 3.30
Spleen	4.82 ± 0.86	2.52 ± 1.23 [c]	1.73 ± 0.97	1.51 ± 0.91
Kidneys	5.25 ± 0.88	2.74 ± 0.33 [c]	2.12 ± 1.23	2.40 ± 1.42
Lungs	16.1 ± 3.3	8.5 ± 3.7 [c]	6.2 ± 4.0	7.9 ± 5.8
Adrenals	4.44 ± 1.09	1.87 ± 0.34 [c]	1.79 ± 1.18	1.99 ± 1.44
Small intestine	28.9 ± 3.0	17.8 ± 9.4 [b]	16.2 ± 8.0	16.3 ± 7.8
Gall bladder	5.57 ± 2.82	2.91 ± 1.96 [a]	3.24 ± 2.46	3.57 ± 2.11
Heart [d]	—	9.31 ± 4.64	7.69 ± 4.52	7.70 ± 4.47
Testes [d]	4.60 ± 1.41	2.66 ± 1.47 [c]	1.97 ± 1.18	1.88 ± 1.11
Brain [e]	—	5.4 ± 2.2	4.6 ± 2.7	4.4 ± 2.6

The label was administered at day 12 (9 or more animals per group). [a] $p < 0.01$; [b] $p < 0.0025$; [c] $p < 0.0005$; always to preceding group; [d] dpm × 10^4; [e] dpm × 10^3.

Table III. Correlation coefficients r (Pearson-Bravais) of total liver ascorbic acid to total bile acids in liver, total bile acids, and plasma ascorbic acid concentrations, and of plasma ascorbic acid levels to plasma levels of cholesterol (n in brackets)

	Na-Ascorbate (twice daily, mg)			
	0.75; 5.0		0.75; 5.0; 25.0; 300	
Liver ascorbic acid to mg liver bile acids	0.522[b]	(23)	0.312[a]	(42)
Liver ascorbic acid to mg total bile acids	0.572[c]	(23)	0.408[c]	(42)
Liver ascorbic acid to plasma ascorbic acid	0.800[d]	(23)	0.913[d]	(42)
Plasma ascorbic acid to plasma cholesterol	— 0.112	(23)	0.037	(42)

[a] $2\alpha \leqslant 0.05$; [b] $2\alpha \leqslant 0.025$; [c] $2\alpha \leqslant 0.01$; [d] $2\alpha \leqslant 0.001$.

min, dpm). For statistical evaluation Student's t-test was used, correlation coefficients were determined according to Pearson-Bravais.

Results. Plasma AA concentration and liver AA depend directly on the intake, suggesting that saturation of plasma and liver does not occur with intakes of up to 600 mg Na-AA/day. Whereas triglycerides and free fatty acids were not influenced by the varying intake of Na-AA, the cholesterol levels were significantly higher in guinea-pigs receiving only twice daily 0.75 mg Na-AA than in those supplemented with 5 mg Na-AA twice daily ($p \leqslant 0.025$). A higher Na-AA supplementation (twice 25 and 300 mg) did not further significantly reduce the cholesterol plasma concentration. Total bile acids were significantly decreased in guinea-pigs on the marginal intake of AA ($p \leqslant 0.005$) and intakes higher than twice 5 mg Na-AA had no further stimulating influence on bile acid formation. Marginal supplementation with Na-AA (twice 0.75 mg) caused a significant reduction in bile acids in liver ($p \leqslant 0.0025$) and small intestine ($p \leqslant 0.0005$), whereas bile acids in gall bladder bile were increased (most probably an effect of volume) in comparison with values in animals supplemented twice 5 mg or 25 mg Na-AA (Table I). The disposition of labelled material accumulated by 10 tissues 72 h after single oral administration of (4-^{14}C) cholesterol showed that animals supplemented with only twice 0.75 mg Na-AA/day have a significantly higher retention of ^{14}C-radioactivity by the tissues than those supplemented twice daily with 5 mg Na-AA; in animals receiving more than twice 5 mgAA/day the retention of label by the tissues was slightly, but not significantly, further decreased (Table II).

Correlation coefficients r were calculated on the basis of pooled data from all 4 groups of animals, as well as from the 2 low-dose groups only. The correlation of total liver to plasma AA concentration was highly significant. The correlation of total liver AA to liver or total bile acids was stronger among the 2 low-dose groups than among all 4 groups together. The plasma AA and cholesterol correlation was not significantly affected by supplementation of AA (Table III).

Discussion. This finding confirms the hypothesis that the rate of conversion of cholesterol to bile acids is correlated with the concentration of AA in the liver as drawn from indirect evidence using $^{14}CO_2$ exhalation after (26-^{14}C) cholesterol as a measure of bile acid production[7,9,18]. In addition, our results demonstrate that the formation of bile acids is inhibited only in the low-dosed animals, and that supplementing high amounts of AA does not further stimulate bile acid formation when the animals are already on an adequate[19] intake of AA (twice 5 mg daily). This suggests that the oxidation of cholesterol to bile acids is dependent on the AA status only over a limited range of concentration, implying a correlation between liver AA and bile acid formation in marginal or hypovitaminotic conditions. This view is in accordance with studies on the 7α-hydroxylation of cholesterol by hepatic microsomal preparations from normally supplied guinea-pigs which failed to show a statistically significant increase in the rate of hydroxylation by addition of AA[11]. The correlation of total liver AA to the bile acid pool was far stronger when considering only the values obtained from guinea-pigs supplemented twice daily 0.75 and 5 mg Na-AA ($r = 0.572$) than when using the pooled data from all 4 groups ($r = 0.408$). Similarly, a stronger correlation was found in the low-dosed groups between liver AA and liver bile acids, which contribute the largest amount of the total pool ($r = 0.522$ and 0.312). With the decrease in bile acid formation from cholesterol under marginal supplementation with AA the plasma cholesterol concentration was increased, but doses of Na-AA larger than twice 5 mg daily had no additional cholesterol-lowering effect, suggesting AA to be of advantage as a hypocholesterolaemic substance in guinea-pigs only up to a daily intake of twice 5 mg under our experimental conditions (i.e. low fat-containing diet). From our experiments, one could deduce a minimal plasma AA concentration (at least 0.20 mg/100 ml plasma following a 6 h fasting period) and a minimal liver AA content (at least 0.8 mg) at which the oxidation of cholesterol to bile acids cannot be further stimulated by additional AA intake. If this finding is applicable to humans, it might explain the rather contradictory conclusions drawn from studies on the effect of AA on plasma cholesterol levels, and would seem to indicate a substantial difference in the AA status of the probands[18,20-24].

The increased retention capacity of tissues for ^{14}C-radioactivity from (4-^{14}C) cholesterol in guinea-pigs with a marginal supply of AA in comparison with animals receiving larger supplements, suggests a higher accumulation of cholesterol or of its metabolic products, or a faster turnover of the newly introduced cholesterol in animals on sufficient AA supplementation, as suggested by GINTER and ZLOCH[25].

[10] S. BERGSTRÖM and S. LINDSTEDT, Biochim. biophys. Acta *19*, 556 (1956).
[11] D. KRITCHEVSKY, S. A. TEPPER and J. A. STORY, Lipids *8*, 482 (1973).
[12] I. BJÖRKHEM and A. KALLNER, J. Lipid Res., in press (1976).
[13] I. BJÖRKHEM and H. DANIELSSON, Molec. Cell Biochem. *4*, 79 (1974).
[14] S. SULIMOVICI and G. S. BOYD, Steroids *12*, 127 (1968); Eur. J. Biochem. *3*, 332 (1968).
[15] K. SHIMIZU, Biochim. biophys. Acta *210*, 333 (1970).
[16] G. B. BRUBACHER and J. P. VUILLEUMIER, in *Clinical Biochemistry, Principles and Methods* (Eds. H. CH. CURTIUS and M. ROTH; De Gruyter, Berlin 1974), vol. 2, p. 989.
[17] D. MAYER, H. HAINDL, F. W. KOSS and W. LAMPRECHT, Z. Analyt. Chem. *243*, 242 (1968).
[18] E. GINTER, Ann. N.Y. Acad. Sci. *258*, 410 (1975).
[19] F. J. STARE, Nutr. Rev. *19*, 278 (1961).
[20] E. GINTER, I. KAJABA and O. NIZNER, Nutr. Metab. *12*, 76 (1970).
[21] A. B. HANCK, Z. Ernährungswiss. *12*, 152 (1973).
[22] C. R. SPITTLE, Lancet *2*, 1280 (1971).
[23] L. PEDERSEN, Scand. J. Gastroenterol. *10*, 311 (1975).
[24] V. E. PETERSON, P. A. CRAPO, J. WEININGER, H. GINSBERG and J. OLEFSKY, Am. J. clin. Nutr. *28*, 584 (1975).
[25] E. GINTER and Z. ZLOCH, Int. J. Vit. Nutr. Res. *42*, 72 (1972).