

Lack of effect of experimental ascorbic acid deficiency on bile acid metabolism, sterol balance, and biliary lipid composition in man

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Abstract Extensive studies in animal models indicate that subclinical ascorbic acid deficiency impairs the conversion of cholesterol to bile acid, elevates plasma cholesterol levels, and predisposes to development of cholesterol cholelithiasis. The present study was designed to see if this is also true in man. Five normal volunteers were hospitalized in a metabolic ward and placed on a controlled diet containing 3–4 mg of ascorbic acid each day. Ascorbic acid supplementation was given as follows: control period I (days 1–33), 75 mg/day; deficient period (days 34–96), 0 mg/day; and repletion period (days 97–101), 1000 mg/day. In addition, three of the subjects were studied during a second control period (days 102–139) during which they were given 75 mg/day of ascorbic acid. Ascorbate levels at the end of both control periods were 0.87–1.34 mg/dl in plasma and 19.4–29.5 $\mu\text{g}/10^8$ cells in leukocytes. At the end of the deficient period these levels were 0.09–0.15 mg/dl in plasma and 6.2–10.0 $\mu\text{g}/10^8$ cells in leukocytes, levels approaching those seen in scurvy. There was no effect of ascorbic acid deficiency on plasma cholesterol and triglycerides; plasma cholesterol in high, very low, and low density lipoprotein fractions; biliary lipid composition and saturation index of gallbladder bile; synthesis, fractional turnover, or pool size of either cholic or chenodeoxycholic acids; output of fecal acid or neutral sterols; and fecal sterol balance. Total bile acid pool size calculated by the one-sample technique was reduced 11% in the deficient period compared to control period I ($P < 0.005$), and increased to 98.7% of the baseline levels in control period II. However, total bile acid pool calculated by the Lindstedt method did not change during deficiency. These data demonstrate that short-term subclinical ascorbic acid deficiency near the scorbutic range has no significant effect on bile acid and cholesterol metabolism in man.—**Duane, W. C., and S. W. Hutton.** Lack of effect of experimental ascorbic acid deficiency on bile acid metabolism, sterol balance, and biliary lipid composition in man. *J. Lipid Res.* 1983. **24**: 1186–1195.

Supplementary key words cholesterol • lipoproteins

Extensive scientific evidence indicates that both scurvy (1) and subclinical² ascorbic acid (AA) deficiency alters bile acid metabolism in the guinea pig (2–5), but studies in man have not been done. It seemed especially im-

portant to evaluate this potential determinant of cholesterol homeostasis in human subjects, because even the most affluent Western societies include substantial population groups subclinically deficient in AA (6–9).

In the guinea pig, AA deficiency has been most effectively isolated from other variables by feeding an AA-deficient diet supplemented with just enough AA to prevent hemorrhagic phenomena, anorexia, weight loss, and inanition. Ginter (2) and Holloway and Rivers (5) report that guinea pigs in this state of subclinical AA deficiency have elevated levels of cholesterol in both plasma and liver compared to control animals. These elevated levels of cholesterol seem to result in part from a diminished conversion of cholesterol to bile acid. Four laboratories have found 30–50% reductions in bile acid synthesis in AA-deficient guinea pigs by a variety of techniques including isotope dilution (5), fecal acidic sterol output (4), ^{14}C production after loading with [26- ^{14}C]cholesterol (2), and ^{14}C -labeled bile acid production after administration of [4- ^{14}C]cholesterol (1). Activity of cholesterol 7 α -hydroxylase, the rate limiting enzyme in bile acid synthesis, is reduced 60% or more in guinea pigs with AA deficiency (3, 5). Many of these changes occur as early as 7–14 days after removal of ascorbic acid from the diet (4). Finally, the combination of reduced bile acid synthesis and increased cholesterol accumulation results in the formation of cholesterol gallstones in AA-deficient guinea pigs given a high cholesterol diet (10).

Abbreviations: AA, ascorbic acid; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

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² The term "subclinical" as applied here to guinea pigs indicates that the animals had no signs of scurvy. The terms "latent" and "chronic" have also been used by some authors in reference to the ascorbate-deficient guinea pig without signs of scurvy.

In contrast to the large body of information available in animal models, very few studies of vitamin C deficiency and lipid metabolism have been conducted in human beings. Ginter, Kajaba, and Nizner (11) reported that large doses of ascorbate given to hypercholesterolemic subjects with borderline ascorbate deficiency slightly decreased plasma cholesterol over a period of months. On the other hand, both subhuman primates (12) and humans with scurvy have low levels of plasma cholesterol (13, 14). However, since frank scurvy is complicated by severe metabolic derangements (15), studies in scurvy patients may not apply to subclinical AA deficiency. In that latter situation no assessment of lipid metabolism is available. For these reasons we designed the present study to see if subclinical AA deficiency affects sterol metabolism in man as it does in the guinea pig.

METHODS

Subjects

Five male volunteers were admitted to the Special Diagnostic and Treatment Unit of the Minneapolis Veterans Administration Medical Center. All subjects were without disease by previously published criteria (16) and all had normal biliary ultrasonography. Subjects were fed an ascorbic acid-deficient diet similar to that previously described by Rivers (17). The diet was controlled with respect to ascorbic acid, calories, fat, and cholesterol and provided 3–4 mg of ascorbic acid per day. It was composed of normal foods, excluding foods such as fresh fruits and vegetables, citrus juices, and milk, which contain significant amounts of ascorbic acid. The menu was varied each day of the week and repeated on a weekly basis. Dietary supplements were provided as described previously (18). The supplemented diet provided nutrients in amounts equal to or exceeding the Recommended Dietary Allowances (19) except for folate which was present at 50–61% of the RDA. Levels of folate in both serum and red blood cells were monitored throughout the study and remained within the normal range for all subjects (18). Dietary cholesterol was adjusted for each individual, largely through addition or subtraction of eggs, in order to match previous intake as assessed by dietary recall. The subjects were weighed daily and caloric intake was adjusted as necessary to maintain constant body weight during the course of the study. The subjects' ages, weights, heights, and smoking habits are recorded in **Table 1**.

The diet of all subjects was supplemented with ascorbic acid as follows: control period I (days 1–33), three 25-mg capsules per day; depletion period (days 34–96), no supplement; repletion period (days 97–101), four

TABLE 1. Age, weight, height, and smoking habits of volunteers

Subject	Age yr	Weight kg	Height cm	Cigarettes/Day
A	31	80	174	0
B	55	76	160	40
C	50	68	175	0
D	61	77	172	0
E	54	69	169	20

250-mg capsules per day. Three subjects were also studied during a second control period (days 102–139) during which they were given three 25-mg capsules per day. Informed consent was obtained from all subjects prior to beginning the study. All procedures and protocols were approved by the Subcommittee for Human Studies of the Minneapolis Veterans Administration Medical Center and by the Committee on the Use of Human Subjects in Research of the University of Minnesota.

Blood sample analysis

Levels of ascorbic acid in plasma and white cells were determined in fasting blood samples by standard techniques (20, 21) every 1–2 weeks throughout the study. Plasma high density lipoproteins (HDL) were isolated for cholesterol analysis by the heparin-manganese precipitation technique described by Warnick and Albers (22). Plasma very low density lipoproteins (VLDL) were isolated by ultracentrifugation at 100,000 *g* for 18 hr. Aliquots of these two fractions and of whole plasma were saponified by the method of Abell et al. (23) and extracted with hexane containing 5 α -cholestane as an internal standard. An aliquot of the hexane was dried for cholesterol determination by gas-liquid chromatography as previously described (24). Amount of cholesterol in the low density lipoprotein (LDL) fraction was calculated by subtracting HDL + VLDL cholesterol from total cholesterol. Serum triglycerides were determined by the clinical laboratory of the Minneapolis Veterans Administration Medical Center.

Fecal sterol determinations

Beginning 20 days prior to the end of each of the three study periods, all subjects ingested capsules containing 100 mg of chromic oxide (Fisher Chemical, Fair Lawn, NJ), and 200 mg of β -sitosterol (ICN Pharmaceuticals, Cleveland, OH) three times each day. Ten days after the start of these fecal markers, all stool was collected for the next 10 days. Each volunteer's daily stool output was homogenized with approximately two volumes of water in a Waring blender. Duplicate aliquots of this homogenate were analyzed for chromic oxide as described by Davignon, Simmonds, and Ahrens (25).

Neutral sterols were determined on aliquots of these fecal homogenates by the method of Miettinen, Ahrens, and Grundy (26), except that gas-liquid chromatography was done on a Hewlett-Packard 5830A chromatograph using SP-2250 (Supelco, Bellefonte, PA) as the stationary phase. Acidic sterols were determined by the method of Subbiah (27) using a gas-liquid chromatography column of SP-2250 and acetate derivatives of the bile acid methyl esters, formed as described previously (24). Calculation of cholesterol balance from these analyses required subtraction of dietary cholesterol which was determined on lipid extracts (chloroform-methanol 2:1) of an aliquot of each of the 21 meals fed in rotation to the volunteers.

Biliary lipid and isotope dilution measurements

Four days prior to the end of each of the three study periods, each volunteer was given known amounts ($\sim 2 \mu\text{Ci}$) of $[24\text{-}^{14}\text{C}]$ cholic acid and $[24\text{-}^{14}\text{C}]$ chenodeoxycholic acid (New England Nuclear, Boston,

MA) via a duodenal tube about 9:00 in the evening. For the four subsequent mornings, samples of gallbladder bile were obtained via this tube following intraduodenal administration of about 100 cc of a protein hydrolyzate (Amigen, Baxter Laboratories, Deerfield, IL). Each morning after sampling, the tube was removed and the subject ate normally.

Each of the four gallbladder bile samples was analyzed for specific activity of cholic acid and chenodeoxycholic acid, for bile acid composition, and for cholesterol, phospholipid, and total bile acid as described in previous publications (16, 24). These analyses permitted calculation of pool size, fractional turnover rate, and synthesis rate of cholic and chenodeoxycholic acids by the method of Lindstedt (28) in addition to biliary lipid composition and lithogenic index of gallbladder bile using the equations of Thomas and Hofmann (29) and the solubility limits of Holzbach et al. (30). In addition, an aliquot of the bile sample obtained the day after isotope administration was analyzed for total radioactivity for calculation of the "one-sample" total bile acid

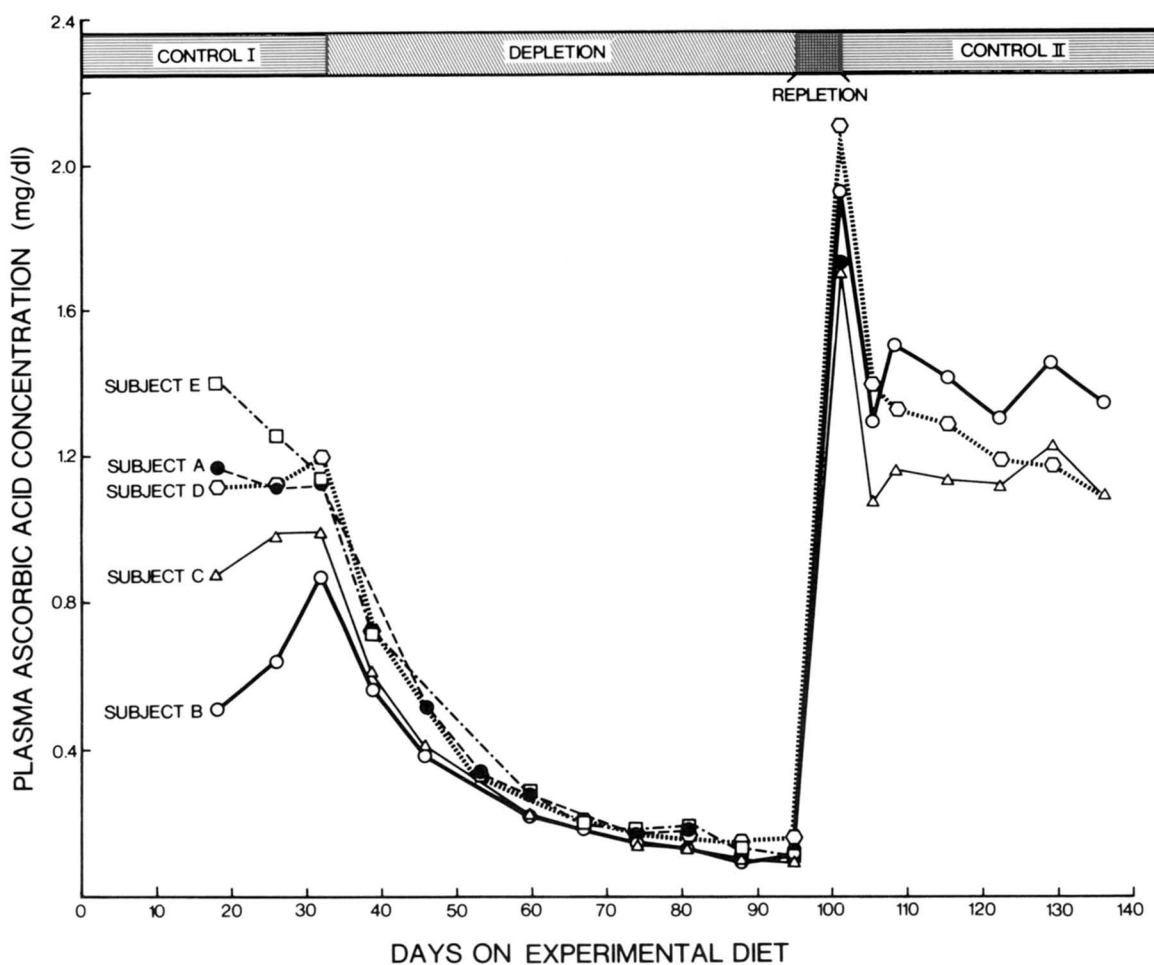


Fig. 1. Plasma ascorbic acid.

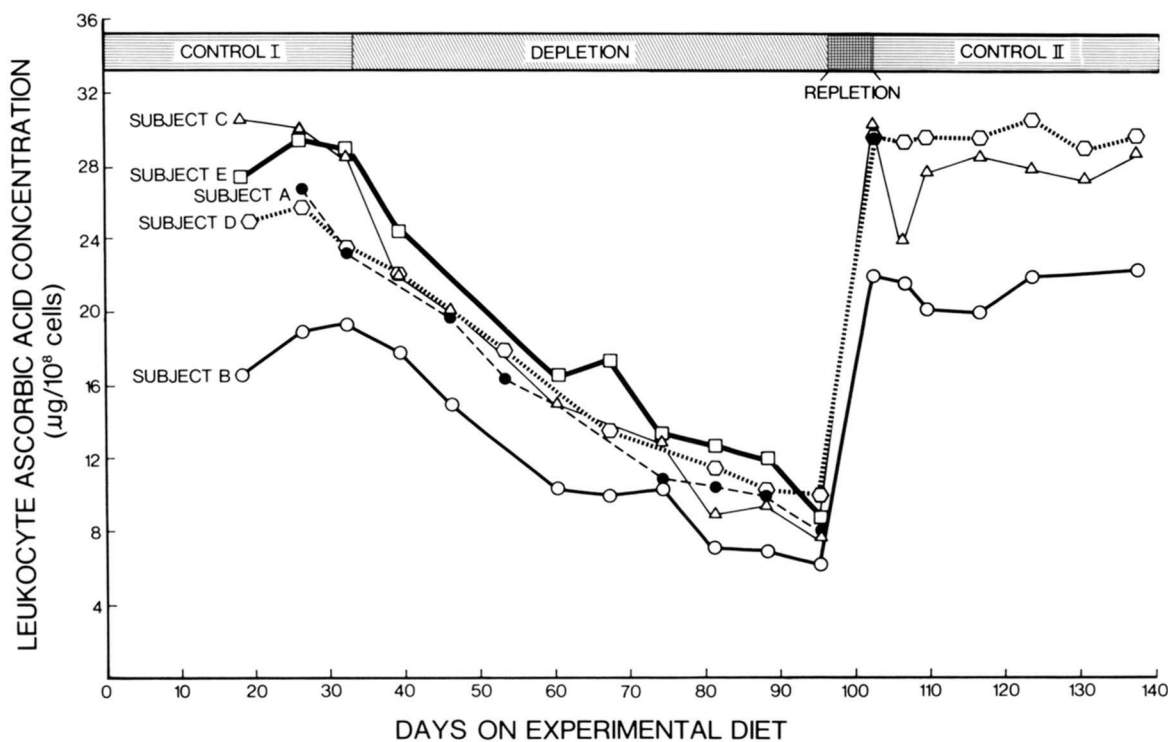


Fig. 2. Leukocyte ascorbic acid.

pool size according to a technique which we have previously described and validated (31).

Statistical analysis was by paired *t*-test.

RESULTS

Plasma and leukocyte levels of AA are depicted graphically in **Fig. 1** and **Fig. 2**. Mean values determined

at the end of each experimental period are given in **Table 2**. At the end of the first control period, plasma levels ranged from 0.77 to 1.20 mg/dl and at the end of the second control period they ranged from 1.08 to 1.34 mg/dl. Leukocyte ascorbic acid levels at the end of these two control periods ranged from 19.4 to 29.0 $\mu\text{g}/10^8$ cells and from 22.2 to 29.5 $\mu\text{g}/10^8$ cells, respectively. At the end of the deficient period plasma ascorbic acid levels ranged from 0.09 to 0.15 mg/dl

TABLE 2. Ascorbate levels, plasma lipids, and biliary lipid composition

	Absolute Mean		Mean (SEM) Percent of C _I Value ^a	
	C _I	Def	Def	C _{II}
Plasma AA (mg/dl)	1.06	0.12 ^b	10.8 (0.8)	118.0 (18.9)
Leukocyte AA ($\mu\text{g}/10^8$ cells)	24.8	8.13 ^b	33.1 (2.6)	113.3 (7.1)
Plasma cholesterol (mg/dl)	228	232	102.5 (4.5)	101.2 (5.5)
LDL cholesterol (mg/dl)	175	178	103.5 (5.9)	99.4 (6.4)
VLDL cholesterol (mg/dl)	13.7	12.2	93.5 (8.0)	91.8 (12.8)
HDL cholesterol (mg/dl)	40.6	42.1	103.6 (3.0)	104.9 (7.7)
Plasma triglycerides (mg/dl)	146	145	101.4 (5.2)	95.4 (10.6)
Bile acid (molar percent)	71.2	71.1	99.8 (2.2)	101.7 (1.0)
Lecithin (molar percent)	20.2	20.1	98.8 (8.8)	93.0 (1.3)
Cholesterol (molar percent)	8.6	8.7	101.3 (6.5)	105.0 (7.5)
Cholesterol saturation index	1.35	1.39	104.4 (8.3)	109.4 (7.0)

^a Each value in deficient and C_{II} periods was converted to a percentage of the corresponding measurement in the C_I period. Mean and SEM of these percentages are shown.

^b *P* < 0.001.

having been at or below 0.20 mg/dl for 30 days in all subjects. Leukocyte ascorbic acid levels at the end of the deficient period ranged from 6.2 to 10.0 $\mu\text{g}/10^8$ cells. According to Sauberlich (32), based in part on the careful work of Hodges et al. (13), levels below which signs of experimental scurvy begin to appear are about 0.2 mg/dl in plasma and about 10 $\mu\text{g}/10^8$ cells in leukocytes, although earlier work from several laboratories suggested that the signs of experimental scurvy did not appear until plasma and leukocyte ascorbic levels were nearly undetectable (33–36). Very recently in a group of patients with spontaneous scurvy, Schorah (37) reported mean levels of ascorbate in plasma of 0.08 mg/dl and in leukocytes of 4.0 $\mu\text{g}/10^8$ cells. The range of levels in these patients was 0–0.15 mg/dl in plasma and 0–0.8 $\mu\text{g}/10^8$ cells in leukocytes. We have had the op-

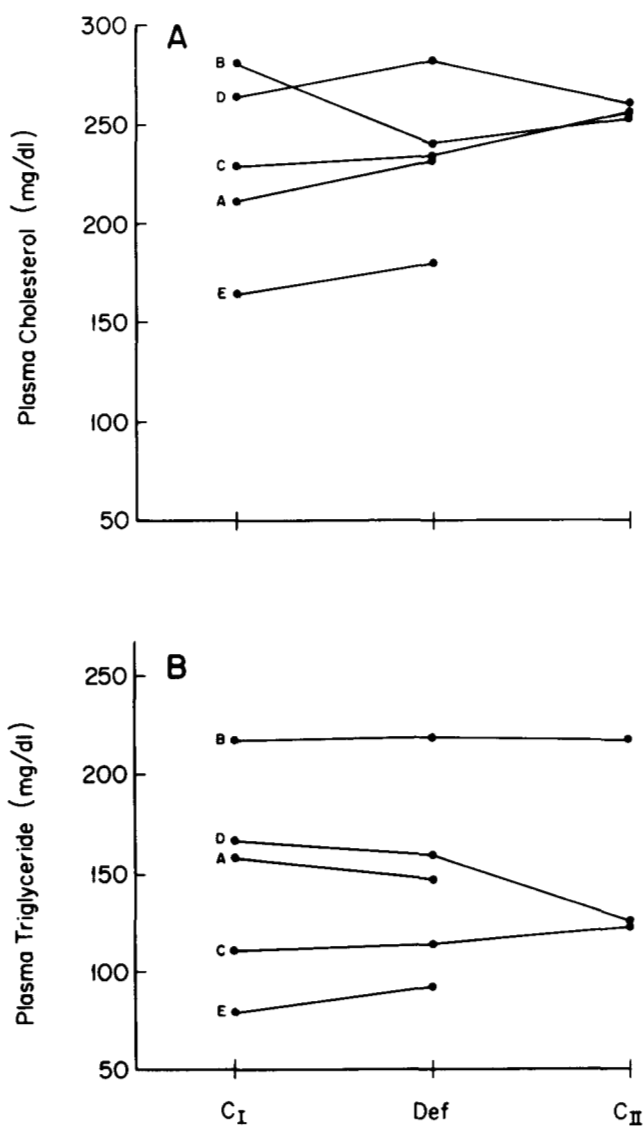


Fig. 3. Plasma lipids: A, total cholesterol; B, triglyceride.

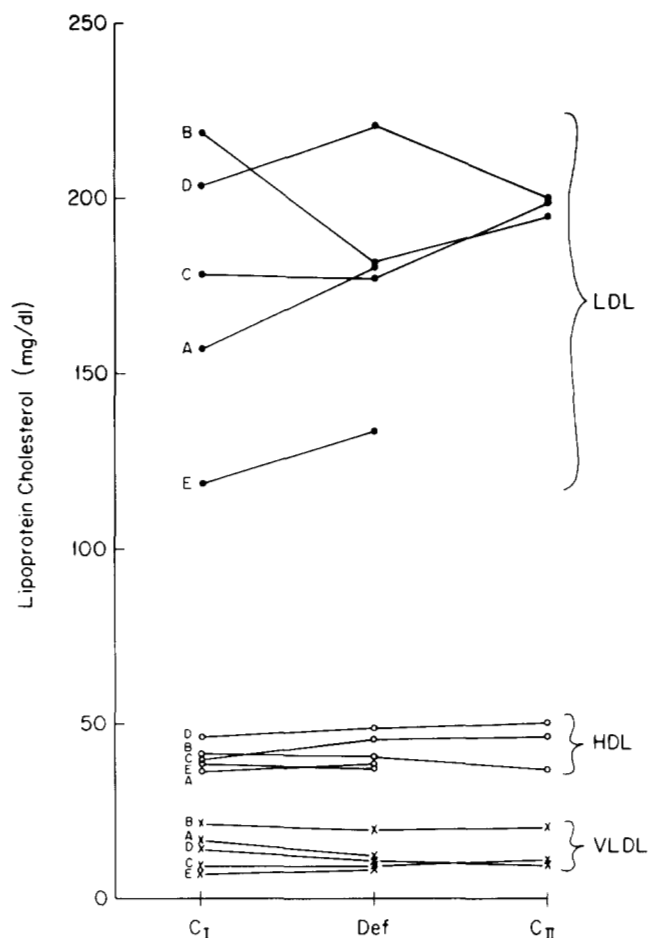


Fig. 4. Plasma lipoprotein cholesterol.

portunity to study one patient with spontaneous scurvy who in our laboratory had levels of ascorbate in plasma of 0.07 mg/dl and in leukocytes of 4.9 $\mu\text{g}/10^8$ cells. Thus the plasma and leukocyte levels of ascorbic acid reached in the present study are close to those seen in patients with clinical signs of scurvy.

At the end of the deficiency period plasma cholesterol and triglyceride levels were unchanged from levels in either the first control or second control period (Fig. 3, Table 2). Similarly, cholesterol levels in LDL, VLDL, and HDL fractions were unaffected by subclinical AA deficiency (Fig. 4, Table 2).

Biliary lipid composition and cholesterol saturation index of gallbladder bile did not change as a result of AA depletion (Fig. 5, Table 2). Isotope dilution measurements indicated no effect of AA deficiency on fractional turnover of either cholic acid or chenodeoxycholic acid (Fig. 6, Table 3). Synthesis rates of cholic, chenodeoxycholic, and total bile acid were identical in the deficient period as compared to the first control period (Fig. 7, Table 3). Mean synthesis of both primary bile acids increased slightly in the second control period

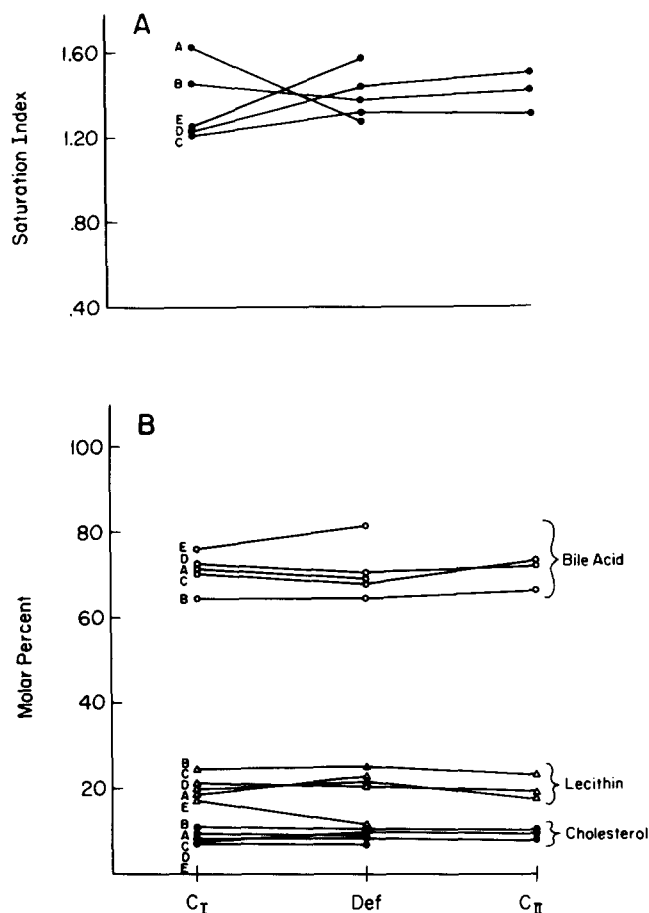


Fig. 5. Bile composition: A, cholesterol saturation index of gallbladder bile; B, individual biliary lipid composition (molar percent).

(Fig. 7, Table 3); however, the overall change in synthesis during depletion relative to both control periods was not statistically significant. Pool sizes of cholic, chenodeoxycholic, and deoxycholic acid determined by the Lindstedt technique were unaffected by AA depletion (Fig. 8, Table 3). Similarly, total bile acid pool by the Lindstedt technique was unchanged by AA deficiency (Fig. 9, Table 3). However, as shown in Fig. 9 and Table 3, total bile acid pool determined by the one-sample method (27) fell an average of 11% from a mean of 4920 μmol in the first control period to a mean of 4400 μmol in the deficient period ($P < 0.005$). In the three subjects who were studied in a second control period, total bile acid pool by the one-sample technique returned to 99% of the value in the first control period (Fig. 9, Table 3).

Mean fecal acidic sterol output fell slightly during AA deficiency, but this drop was not statistically significant (Fig. 10, Table 3). Mean neutral sterol output (Fig. 10, Table 3) and mean cholesterol balance (Fig. 11, Table 3) were unchanged in deficient versus control periods.

DISCUSSION

There seems to be no question that in the guinea pig ascorbic acid deficiency short of the scorbutic level substantially lowers the conversion of cholesterol to bile acid (2-5). However, no investigation of bile acid and cholesterol metabolism in ascorbate-deficient humans has ever been reported, presumably in large part because of difficulty recruiting subjects who will submit both to the required long-term dietary restrictions and to the duodenal and/or fecal sampling procedures required for sterol metabolic measurements. We therefore designed the present study to obtain the most reliable information possible from a relatively small number of intensively studied subjects.

This was accomplished in several ways. First, the entire study was carried out on a metabolic ward with subjects eating only food of controlled composition pre-

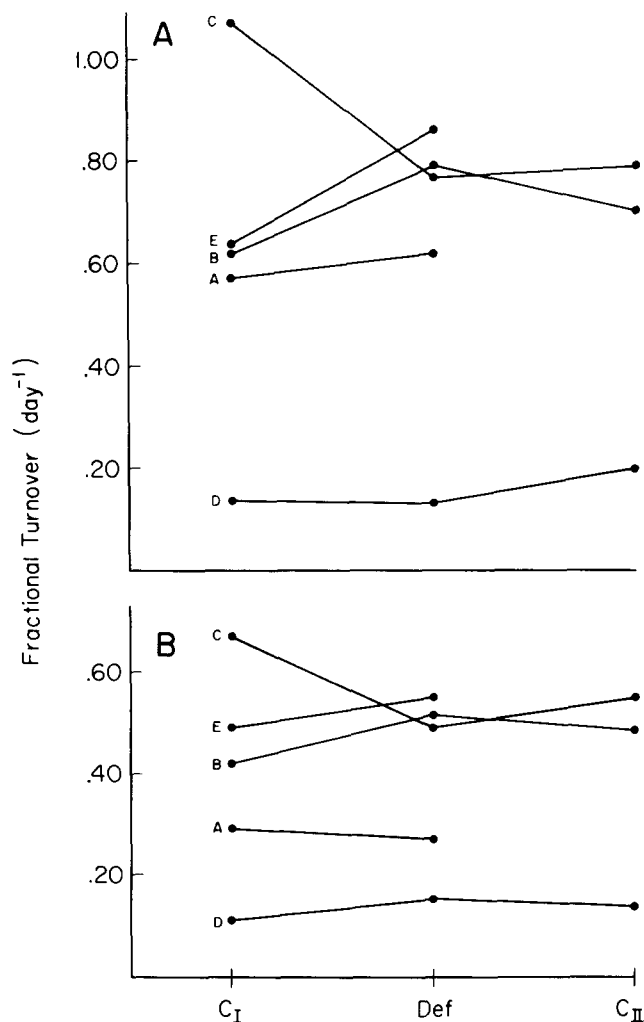


Fig. 6. Bile acid fractional turnover rate: A, cholic acid; B, chenodeoxycholic acid.

TABLE 3. Isotope dilution measurements and fecal sterols

	Absolute Mean		Mean (SEM) Percent of C _I Value ^a	
	C _I	Def	Def	C _{II}
Cholic pool (μmol)	1130	1110	97.0 (13.4)	116.2 (20.8)
Chenodeoxycholic pool (μmol)	1510	1490	99.5 (10.5)	107.3 (9.3)
Deoxycholic pool (μmol)	1500	1480	103.7 (11.9)	87.3 (19.9)
Total pool (μmol)	4140	4170	101.8 (7.9)	101.2 (12.4)
One-sample pool (μmol)	4920	4400 ^b	89.0 (2.3)	98.7 (4.8)
Cholic fractional turnover (day ⁻¹)	0.606	0.632	107.7 (11.4)	110.3 (20.0)
Chenodeoxycholic fractional turnover (day ⁻¹)	0.394	0.391	107.8 (11.6)	108.3 (13.7)
Cholic synthesis (μmol/day)	533	524	102.5 (10.8)	122.9 (20.5)
Chenodeoxycholic synthesis (μmol/day)	437	428	99.9 (7.1)	114.5 (13.1)
Total synthesis (μmol/day)	971	952	101.1 (8.9)	121.1 (15.5)
Acidic sterols (mg/day)	476	448	95.9 (7.8)	107.6 (4.3)
Neutral sterols (mg/day)	872	915	106.2 (8.3)	106.6 (9.1)
Sterol balance (mg/day)	775	790	106.9 (15.0)	114.0 (16.7)

^a Each value in deficient and C_{II} periods was converted to a percentage of the corresponding measurement in the C_I period; mean and SEM of these percentages are shown.

^b *P* < 0.005 versus C_I.

pared under the supervision of a dietitian. Second, each subject served as his own control since each was studied during an initial period of ascorbate supplementation

followed by a period of ascorbate deficiency. In addition, three subjects were studied in a post-deficiency period of ascorbate supplementation to detect any hidden effect of prolonged adherence to the study protocol. Third, both plasma and leukocyte ascorbate were lowered to levels approaching those found in experimental and spontaneous scurvy (13, 32, 37). Finally, bile acid

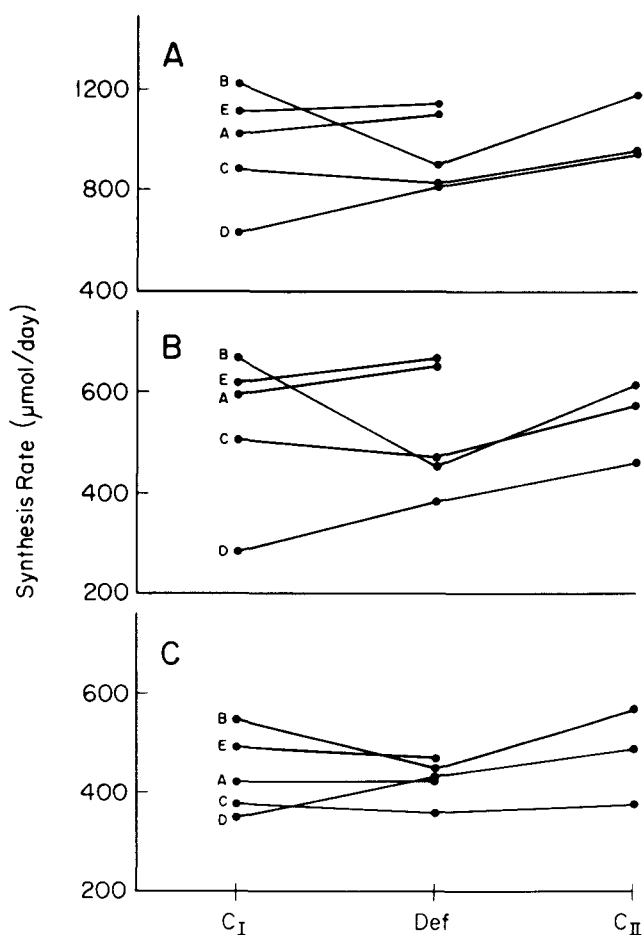


Fig. 7. Bile acid synthesis rate: A, total; B, cholic acid; C, chenodeoxycholic acid.

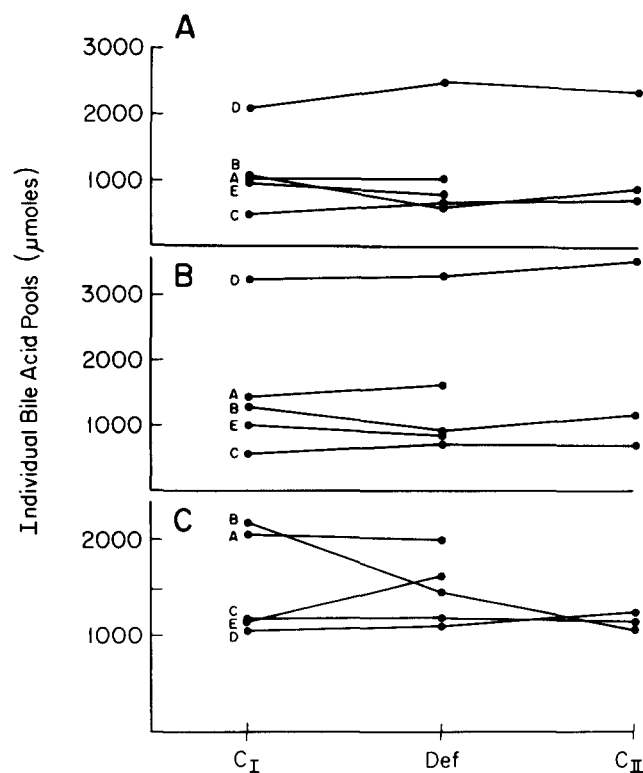


Fig. 8. Individual bile acid pool sizes, calculated from biliary bile acid specific activity decay curves (Lindstedt technique): A, cholic acid; B, chenodeoxycholic acid; C, deoxycholic acid.

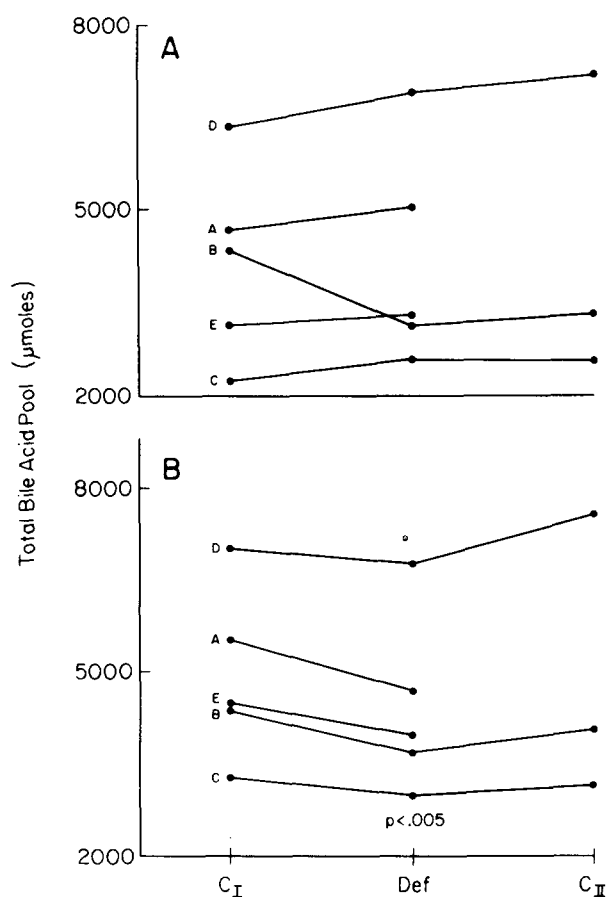


Fig. 9. Total bile acid pool size: A, determined by Lindstedt technique; B, determined by one-sample technique.

synthesis rate, which is the parameter most consistently abnormal in the ascorbate-deficient guinea pig, was measured by two independent methods, isotope dilution and fecal acidic sterol output.

Of all the parameters measured, only total bile acid pool size, determined by the one-sample method (31), proved to be affected by AA deficiency in our group of subjects as a whole. Mean one-sample pool size was about 10% lower in the deficient period than in the first control period ($P < 0.005$) and returned to baseline levels in the post-deficient control period (Fig. 9, Table 3). However, when bile acid pool size was determined by the Lindstedt method (28), there was no difference between values in the deficient period and those in either control period (Fig. 9, Table 3). The reason for this discrepancy is unclear. In either case, this degree and duration of ascorbic acid deficiency in man apparently alters total bile acid pool size only slightly, if at all. It should be noted that in our group of subjects as a whole both fractional turnover rate (Fig. 6) and proportion of deoxycholate (Fig. 8) were somewhat higher than usually reported. Other than random variability, we have no explanation for this unusual feature. How-

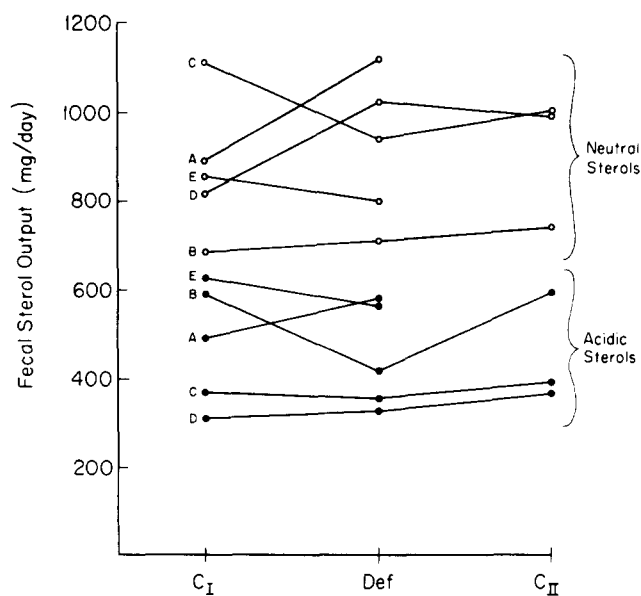


Fig. 10. Fecal sterol excretion.

ever, there is no reason to believe that it obscured effects of ascorbic acid deficiency.

Bile acid synthesis, which has so consistently fallen 30–50% in the guinea pig subclinically deficient in ascorbic acid (2–5), did not change during ascorbate deficiency in our group of human subjects whether measured by isotope dilution (Fig. 7, Table 3) or fecal acidic sterol output (Fig. 10, Table 3). One explanation for this discrepancy between findings in the guinea pig and in man is that the two species differ qualitatively in the way ascorbic acid deficiency affects bile acid synthesis. Alternatively, since the standard technique for producing subclinical vitamin C deficiency in the guinea pig is to administer just enough ascorbate to prevent manifestations of scurvy (2), it is possible that such animals become somewhat more deficient than our subclinically deficient human subjects. Supporting this possibility are the data for subject B, who achieved the lowest levels of leukocyte ascorbate (Fig. 2) and simul-

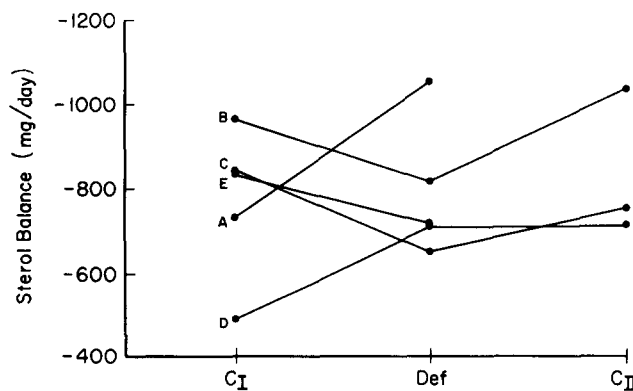


Fig. 11. Calculated sterol balance.

taneously had a 30% reduction in bile acid synthesis, both by isotope dilution (Fig. 7) and acidic sterol output (Fig. 10), which then returned to baseline control levels during the post-depletion period of ascorbate supplementation. On the other hand, leukocyte ascorbate levels in all five of our subjects were close to those found in experimental and spontaneous scurvy (13, 37). It seems likely, therefore, that depletion of appreciably greater magnitude would produce clinical scurvy, rather than subclinical deficiency, and be ethically unjustifiable.

Our subjects as a group had no change in total plasma cholesterol (Fig. 3A, Table 2) or in fractionated lipoprotein cholesterol (Fig. 4, Table 2) during ascorbic acid deficiency. In man and subhuman primates, ascorbate depletion to the point of scurvy lowers plasma cholesterol (12–14). Since none of our subjects had clinical scurvy, it is not surprising that their cholesterol levels did not fall. Subclinical depletion of vitamin C in the guinea pig increases plasma cholesterol (2, 5); however, such animals also have reductions in bile acid synthesis not found in our subjects. Nevertheless, our data do not exclude the possibility that more prolonged subclinical ascorbate deficiency might affect plasma cholesterol in man.

Finally, the data in Fig. 5 and Table 2 demonstrate that ascorbate depletion in our subjects did not cause an increase in either the molar percent cholesterol of gallbladder bile or the cholesterol saturation index. Thus, in humans, subclinical ascorbate deficiency does not seem to increase the lithogenic potential of gallbladder bile as it does in guinea pigs fed a high cholesterol diet (10). We conclude that this degree of human ascorbic acid deficiency does not induce abnormalities in sterol homeostasis that would predispose to atherosclerosis or cholesterol cholelithiasis. ■■

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