ASCORBIC ACID IN CHOLESTEROL AND BILE ACID METABOLISM

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This overview deals with the function of ascorbic acid in the metabolism of cholesterol and bile acids purely from the biochemical aspect. The pathophysiological implications of this problem, particulary the potential role of vitamin C deficiency in the pathogenesis of atherosclerosis, have been dealt with in another review.[1]

Cholesterol Metabolism in Acute Scurvy in Animals and Men

Avitaminosis C is a state difficult to define metabolically, being complicated by a lowered food intake, an abrupt loss of body weight, a negative nitrogen balance, hemorrhages, etc. It is not surprising, therefore, that a relatively large number of reports on disorders of cholesterol metabolism in experimental scurvy should be mutually contradictory. More detailed analyses of them may be found elsewhere.[2, 3] There is an apparent lack of agreement on the action of acute avitaminosis C on cholesterol levels in the blood and tissues of experimental animals. As a rule, an accumulation of cholesterol is found in the carcass of scorbutic guinea pigs [4, 5] because of an enhanced synthesis of cholesterol from acetate [6] and a slowed rate of cholesterol catabolism.[7] Since these disorders have been described predominantly in severely scorbutic animals, they are probably only of a secondary character. Thus, for instance, an enhanced cholesterologenesis may be plausibly explained by an impaired function of the tricarboxylic acid cycle in the liver of scorbutic guinea pigs,[8] which leads to an augmented utilization of the acetate pool for cholesterol synthesis. In scorbutic persons the cholesterol concentration in blood serum remains unaltered [9] or is slightly lowered.[10, 11] Cholesterolemia in these subjects usually increases following vitamin C therapy.[10, 11] An interpretation of this phenomenon is made more difficult, just as in the case of vitamin-C-deficient animals, by the complex metabolic disorganization induced by scurvy.

A great deal of confusion has been introduced into the vitamin-C-cholesterol relationship by studies following the effect of high doses of ascorbic acid on cholesterol metabolism in animals synthesizing vitamin C, such as the rabbit, the rat,[3, 12] and the hen.[13] As a matter of fact, their tissues are saturated with ascorbic acid. In our experience not even an addition of 1% ascorbic acid to the diet of rats will induce an increase of ascorbic acid concentration in the tissues. Consequently, pool and kinetic parameters of cholesterol metabolism in rabbits with alimentary hypercholesterolemia are independent of the quantity of ascorbic acid in their diet.[1] However, in situations with increased demands on vitamin C, ascorbic acid may have a positive effect also in vitamin-C-synthesizing animals, e.g., in weanling or hypothyroid rats.[14, 15]

Cholesterol Metabolism in Latent Chronic Vitamin C Deficiency

In order to simulate the real situation in the nutrition of humans, a model of latent vitamin-C deficiency has been worked out for guinea pigs.[16] These animals are kept on a scorbutogenic diet [17] for a fortnight, which results in a substantial depletion of their body pool of vitamin C, but without any apparent signs of an evident ascorbic acid deficiency. After this period the guinea pigs receive an oral maintenance dose of ascorbic acid (0.5 mg per animal per 24 hours). The controls are fed the same diet, with the addition of substantially higher doses of ascorbic acid (usually 10 mg per animal per 24 hours).

The growth rate, appearance, behavior, and food intake are the same in guinea pigs with a latent vitamin C deficiency as in the controls, but ascorbic acid levels in the tissues of the former are considerably lower. Figure 1 shows

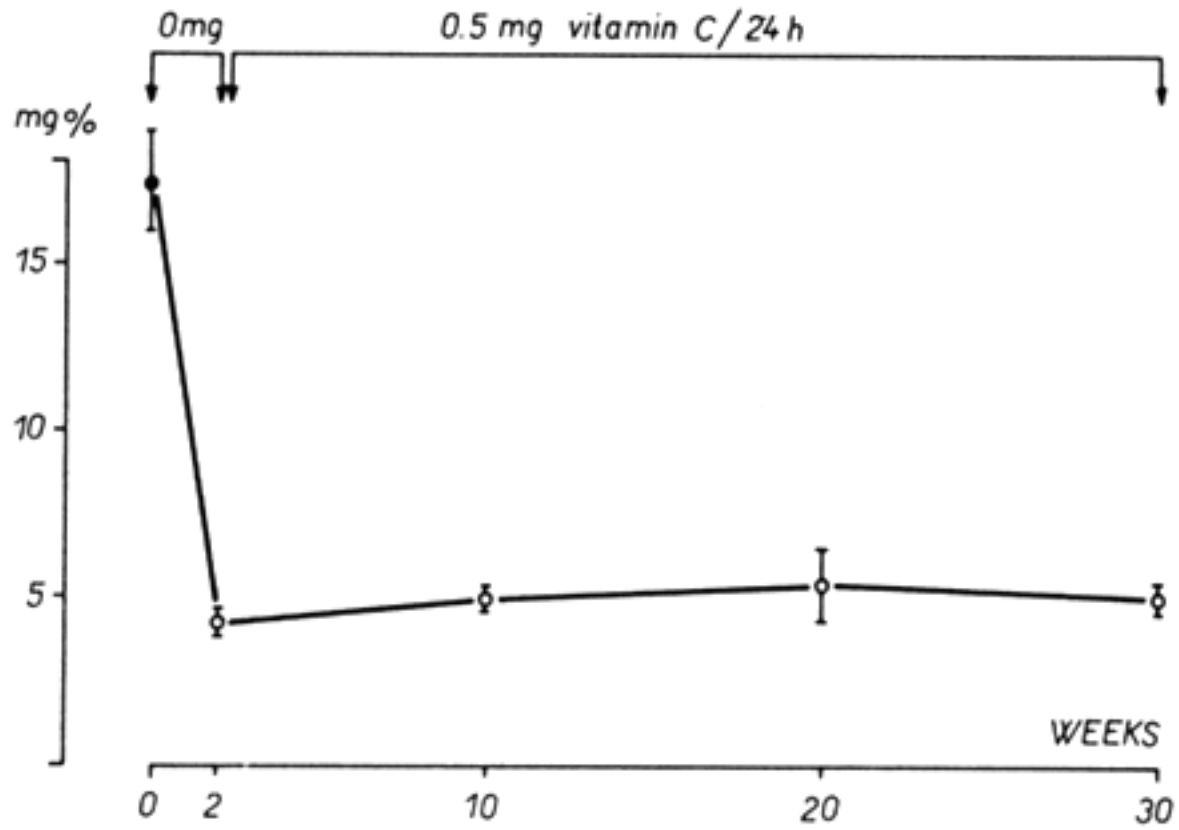


Figure 1. Concentration of ascorbic acid in the spleen of guinea pigs during development of chronic latent vitamin C deficiency. The vertical bars represent the standard errors of the mean.

the course of ascorbic acid concentration in the spleen of guinea pigs during the development of latent vitamin C deficiency. During the first fortnight of feeding on a scorbutogenic diet, the ascorbic acid level drops abruptly. In the ensuing period (maintenance dose of vitamin C) ascorbic acid concentration persists at a steady low level close in values to those observed in guinea pigs with incipient scurvy. The constant supply of ascorbic acid in the maintenance doses prevents the onset of manifest scurvy, and this state is here termed hypovitaminosis C. The interpretation of data obtained from hypovitaminous animals is essentially simpler than in the case of acute scurvy. The only variable to be considered here is the substantial decrease in the body pool of vitamin C, while the various interfering factors associated with scurvy (e.g. drop in body weight and hemorrhages) are left out of account. Moreover, this model is far more realistic as regards present-day status in the nutrition in developed countries.

TABLE 1

INFLUENCE OF CHRONIC LATENT ASCORBIC ACID DEFICIENCY ON TOTAL CHOLESTEROL CONCENTRATION IN BLOOD PLASMA AND LIVER OF MALE GUINEA PIGS *

Blood Plasma			Liver		
Duration of Deficiency (weeks)	Control (mg per 100 ml blood plasma)	Deficiency	Duration of Deficiency (weeks)	Control (mg per 100 g wet tissue)	Deficiency
16–20	118±14	171±18	15	368±29	659±40
22	99±6	132±6	16–20	456±56	627±65
23	95±7	140±8	17–21	395±35	616±70
24	94±7	140±7	20–22	411±33	592±77
26	88±7	139±8	28	325±14	586±106
28	110±6	135±5	31	357±22	661±96

* Means from 9–15 animals ± SEM. All differences between the control and deficient groups are statistically significant ($p < 0.05$–0.001).

In short-term hypovitaminosis C, cholesterol levels in the blood and tissues of guinea pigs are not generally altered to any great extent. However, if the hypovitaminous state persists for about three months or more, hypercholesterolemia ensues, with cholesterol accumulation in the liver (TABLE 1). Cholesterol concentration in the other organs remains unchanged, except that the quantity of Liebermann-Burchardt-positive sterols augments in the skin.[18] If, however, cholesterol (0.3%) is added to the guinea pigs' diet, hypovitaminosis C causes cholesterol to be stored in various organs including the thoracic aorta (TABLE 2).

TABLE 2

INFLUENCE OF LATENT ASCORBIC ACID DEFICIENCY ON TOTAL CHOLESTEROL CONCENTRATION IN TISSUES OF GUINEA PIGS FED CHOLESTEROL DIET *

Duration of Experiment	Tissue	Doses of Ascorbic Acid (mg per animal per day)		
		0.5 (deficiency)	5.0	50.0
12 weeks	Liver	4,017±485	3,652±310	3,404±42
	Adrenal	10,774±1,621	10,646±1,047	8,651±527
	Small intestine	387±19 †	345±35	272±32
	Thoracic aorta	548±48 ‡	545±96	409±29
20 weeks	Liver	6,622±548 †	5,611±416	3,509±350
	Adrenal	7,942±890 ‡	7,782±671	5,186±840
	Small intestine	364±23 ‡	317±17	282±20

* Means from 9–14 animals ±SEM. Values given in mg of cholesterol per 100 g of wet tissue.

† ‡ Significantly higher in comparison with group given 50 mg ascorbic acid daily: † $p < 0.002$–0.001; ‡ $p < 0.05$–0.01.

TABLE 3 summarizes the results of a mathematical analysis of hyperbolic curves of plasma cholesterol in terms of a two-pool model [19] in hypovitaminous guinea pigs following a one-pulse labeling of their body cholesterol with [4-^{14}C]-cholesterol. The half-time of the linear part of the hyperbolic curve in hypovitaminous guinea pigs became significantly prolonged, while the value of the rate constant for the irreversible cholesterol excretion from the organism and the total cholesterol turnover were significantly lowered. Hypovitaminosis C in guinea pigs thus produced similar changes as did alimentary hypercholesterolemia in rabbits.[1] That is it increased cholesterol concentration in blood plasma and liver and slowed down cholesterol release from blood and the whole organism.

MODE OF HYPOVITAMINOSIS C INTERVENTION IN CHOLESTEROL METABOLISM

Cholesterol turnover and cholesterol levels in blood and tissues are the result of a great number of processes mutually bound by feedback mechanisms, such as cholesterol distribution between blood and tissues, absorption of exogenous cholesterol, biosynthesis of endogenous cholesterol, cholesterol secretion into the bile and the gastrointestinal tract, and cholesterol transformation into bile acids. Still other variables could be considered, such as cholesterol transformation into steroid hormones. From the quantitative point of view however, these processes are negligible for a determination of total cholesterol turnover.[20]

We have followed the passage of labeled cholesterol from blood plasma into 14 different tissues of guinea pigs, forming the major part of cholesterol pools in the body. The results obtained from controls and hypovitaminous animals did not differ statistically. Since the total quantity of cholesterol in these tissues is practically equal in the two groups, the accumulation of cholesterol in the liver and the blood of hypovitaminous animals cannot be accounted for by a lower cholesterol deposition in other parts of the body. Following an intragastric application of [4-^{14}C]cholesterol, hypovitaminous guinea pigs had a significantly higher ^{14}C-activity in the gastrointestinal tract and stool and, on the other hand, a substantially lower activity in the blood and tissues.[21] Hence, an enhanced cholesterol accumulation in the blood and liver of hypovitaminous guinea pigs cannot be ascribed to an increased absorption of exogenous cholesterol either. Hypovitaminosis C, rather, tends to inhibit this process. Similar results have also been obtained in humans with an acute vitamin C deficiency.[10]

The rate of *in vivo* [1-^{14}C]acetate incorporation into hepatic cholesterol in experiments with hypovitaminous guinea pigs was found to be unchanged, or only moderately lowered.[3] Similar results have been reported also by other authors in guinea pigs in a state of moderate avitaminosis C.[6] A cholesterol accumulation in a hypovitaminous organism cannot therefore be explained by enhanced cholesterologenesis. In a further experiment, guinea pigs were given an intraperitoneal injection of [4-^{14}C]cholesterol, and excretion of [^{14}C]sterols and [^{14}C]bile acids in their stools was measured for a period of 20 days.[22] Excretion of [^{14}C]sterols in the controls and hypovitaminous animals proved to be practically equal, so that this factor also failed to explain cholesterol accumulation in the blood and liver of hypovitaminous animals. However, this experiment was instrumental in the discovery of the key to the problem: hypovitaminous guinea pigs excreted less [^{14}C]bile acids in the stool. Subsequent tests revealed in hypovitaminous guinea pigs a slower rate of radioactivity

transfer from [4-^{14}C]cholesterol into bile acids in the liver and a lowered oxidation of [26-^{14}C]cholesterol to $^{14}CO_2$.[22] These results indicated that the rate of cholesterol transformation into its principal catabolic product, bile acids, is slowed down in hypovitaminosis C.

Role of Ascorbic Acid in Cholesterol Transformation to Bile Acids

The aim of a further experiment was to find out whether a slowed transformation of cholesterol to bile acids is a specific outcome of vitamin C deficiency. Guinea pigs in the stage of incipient avitaminosis C were given intraperitoneally 100 mg ascorbic acid and, 24 hours later, [26-^{14}C]cholesterol by the same mode of application. On subsequent days they received an oral dose of 50 mg ascorbic acid per 24 hours. Part of the group was fed *ad libitum*, the other was pair-fed with the vitamin-deficient group, in which ascorbic acid deficiency began to be apparent by a lowered food intake. The amount of expired $^{14}CO_2$ was

Table 3

Influence of Latent Ascorbic Acid Deficiency on Kinetic Parameters of Cholesterol Turnover in Guinea Pigs *

Parameter	Control	Deficiency
Half-time of first exponential (days)	8.0±1.3	7.1±2.0
Half-time of linear part of hyperbolic curve (days)	24.0±1.1	30.1±1.7 †
Turnover rate: production rate in pool A (mg per day)	37.8±2.0	31.8±1.5 †
Rate constant for irreversible excretion of cholesterol from pool A (days^{-1})	0.036±0.001	0.031±0.002 †

* Means from 9–12 animals ± SEM.
† Significantly different from the control group ($p < 0.05$–0.01).

followed daily. The results summarized in Table 4 show that a resaturation of guinea pigs with high doses of ascorbic acid significantly stepped up the rate of cholesterol catabolism.[23]

A simultaneous measurement of expired $^{14}CO_2$ and of the specific activity of cholesterol in the liver or plasma enables the rate of cholesterol transformation to bile acids to be quantified.[24, 25] This experiment[26] indicated that a chronic, latent vitamin C deficiency significantly reduced the rate of cholesterol transformation to bile acids (controls: 11.8 ± 0.6; hypovitaminosis C: 8.3 ± 0.4 mg cholesterol per 24 hours per 500 g body weight; $p < 0.001$). Cholesterol is transformed to bile acids in the liver, and the rate of this process very probably depends on ascorbic acid concentration in the hepatic cell, for there exists a relatively close linear correlation between the rate of bile acids synthesis and ascorbic acid concentration in the liver (Figure 2).

A chronic latent hypovitaminosis C apparently is associated with a decline of ascorbic acid concentration in liver cells. As a result, the rate of cholesterol transformation to bile acids is reduced. The lowered synthesis of bile acids is

TABLE 4

INFLUENCE OF ASCORBIC ACID ON [26-^{14}C]CHOLESTEROL OXIDATION TO $^{14}CO_2$ IN VITAMIN-C-DEFICIENT GUINEA PIGS *

Days After Injection of [26-^{14}C]Cholesterol	Vitamin-C-Deficient Group	Groups Treated with Ascorbic Acid	
		Group Fed ad Libitum	Pair-Fed Group
	Percentage of injected dose oxidized		
1	1.6±0.1	2.6±0.4 †	1.8±0.2
2	3.4±0.1	6.2±0.4 †	4.7±0.6 †
3	4.9±0.2	9.0±0.4 ‡	6.9±0.7 †
4	6.0±0.3	11.1±0.5 ‡	8.8±0.8 †
5	6.9±0.3	12.8±0.6 ‡	10.4±0.8 ‡
6	7.7±0.3	14.1±0.6 ‡	11.7±0.9 ‡
7	8.5±0.3	15.0±0.6 ‡	13.0±0.9 ‡
8	9.2±0.3	16.0±0.7 ‡	14.1±1.0 ‡
9	9.9±0.3	16.8±0.6 ‡	15.3±1.1 ‡
10	10.7±0.3	17.8±0.7 ‡	16.8±1.1 ‡

* Means from 5–6 animals ± SEM.

†‡ Significantly higher in comparison with vitamin-C-deficient group. † $p < 0.05$–0.01; ‡ $p < 0.001$.

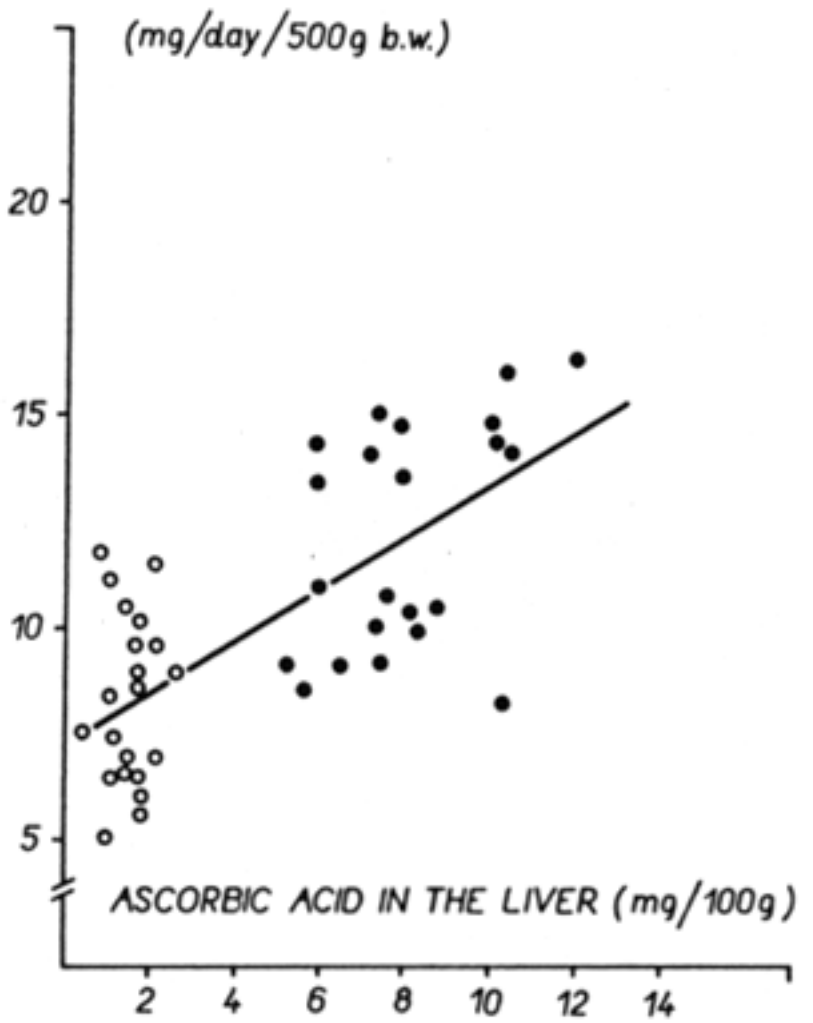
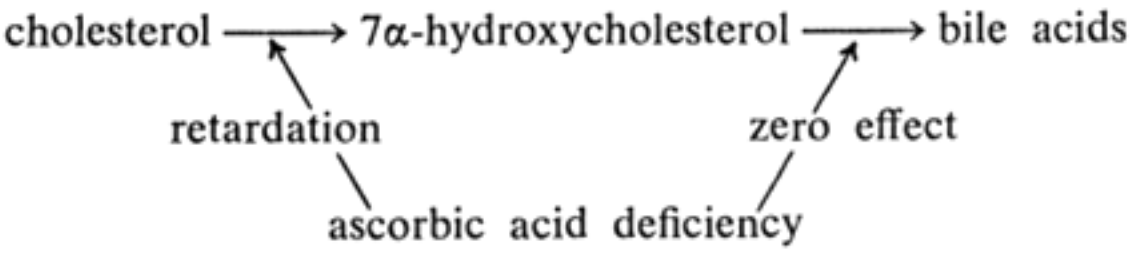


FIGURE 2. Linear correlation between ascorbic acid concentration in the liver and the rate of cholesterol transformation to bile acids in control guinea pigs (●) and guinea pigs with latent vitamin C deficiency (○). (From Ginter *et al.*[18] By permission of *Lipids*.)

indeed associated with a lowered absorption of exogenous cholesterol from the gastrointestinal tract,[21] but this homeostatic mechanism is not sufficiently effective to compensate fully for the slowed cholesterol catabolism. This results in a slowed cholesterol turnover, a slowed cholesterol release from the circulation (TABLE 3), and a cholesterol accumulation in blood plasma and the liver of hypovitaminous organism.

Cholesterol transformation to bile acids is a multistage process taking place successively in the microsomes, supernatant fraction, and mitochondria of the liver cell. It involves hydroxylation, dehydrogenation, saturation of a double bond in the nucleus, 3-ketone reduction, ω- and β-oxidation of cholesterol side chain. The transformation of cholesterol into the principal bile acid of guinea pigs—chenodeoxycholic acid—entails two hydroxylations: at position 7 in the cholesterol nucleus and at position 26 on its side chain. In contrast to ovarian and adrenal tissue,[27, 28] ascorbic acid seems not to have any effect on the oxidation of the side chain (i.e., on 26-hydroxylation) of cholesterol in liver mitochondria.[29]

The first step in cholesterol transformation to C_{24} bile acids is the production of 7α-hydroxycholesterol, and this reaction is rate-limiting for cholesterol catabolism. If our assumption [22] is correct that the role of ascorbic acid in the biosynthesis of bile acids is localized at the level of 7α-hydroxylation of the cholesterol nucleus, then, in contrast to cholesterol catabolism, that of 7α-hydroxycholesterol should not be affected by vitamin C deficiency:



In order to verify this assumption, [26-^{14}C]7α-hydroxycholesterol ([26-^{14}C]-cholest-5-ene-3β, 7α-diol) was synthesized and its oxidation to $^{14}CO_2$ followed *in vivo*. The results, summarized in FIGURE 3, show that contrary to the significantly slowed oxidation of [26-^{14}C]cholesterol to $^{14}CO_2$, oxidation of [26-^{14}C]7α-hydroxycholesterol is not affected significantly by hypovitaminosis C. In *in vitro* experiments Kritchevsky et al.[29] found a higher 7α-hydroxylation of [1,2-^{3}H]-cholesterol in liver microsomes of normal guinea pigs after adding ascorbic acid. Probably because of the small number of animals used, the results are not statistically convincing, but a relatively close correlation appears between the quantity of added ascorbic acid and the rate of 7α-hydroxylation of cholesterol ($r_{xy} = +0.899$). Even though it would be desirable to supplement these results with further studies at the subcellular level or on perfused liver, it appears that ascorbic acid intervenes in the biosynthesis of bile acids at the stage of 7α-hydroxylation of the cholesterol nucleus.

Cholesterol-7α-hydroxylase is localized in the microsomal fraction of the liver cell and requires NADPH and oxygen for maximal activity. These requirements are characteristic for the group of enzymes classified as mixed-function oxidases. It is very probable that cytochrome P-450 has a role in 7α-hydroxylation of cholesterol.[30, 31] Concentration of cytochrome P-450 in liver microsomes of guinea pigs decreases within 24 hours after discontinuing supply of ascorbic acid.[32] There is a parallel increase in the cytochrome P-450 level in the liver microsomes and in [26-^{14}C]cholesterol oxidation to $^{14}CO_2$ after the administration of ascorbic acid to vitamin-C-deficient guinea pigs [33, 34] (FIGURE 4). It

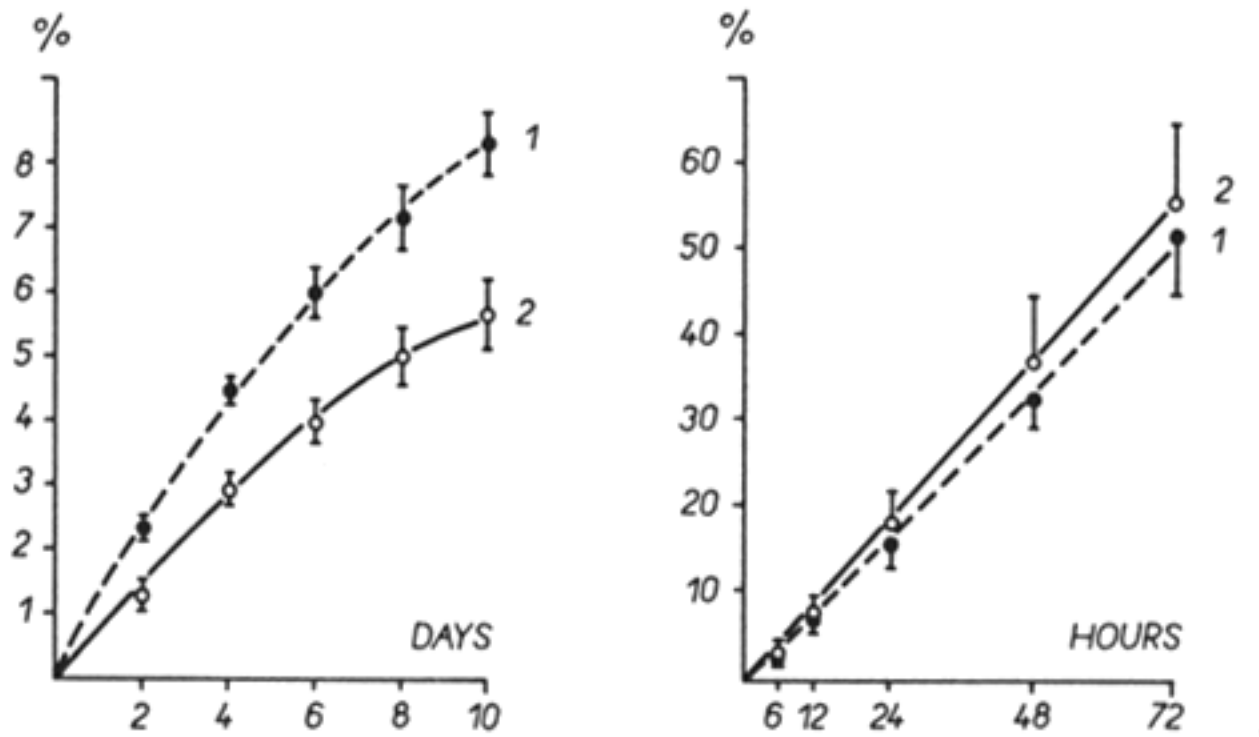


FIGURE 3. Oxidation of [26-^{14}C]cholesterol (*left*) and [26-^{14}C]7α-cholesterol (*right*) to $^{14}CO_2$ as percentage of the dose injected in control (1, ● - - ●) and hypovitaminous (2, ○——○) guinea pigs. Hypovitaminosis C lowered significantly the oxidation of [26-^{14}C]cholesterol. The oxidation of [26-^{14}C]7α-cholesterol is not significantly influenced. The vertical bars represent the standard errors of the mean.

appears plausible that the stimulating action of ascorbic acid on 7α-hydroxylation of cholesterol is mediated through its action on cytochrome P-450 level in the microsomes of liver cell.

There may be a further mechanism through which ascorbic acid increases cholesterol excretion from the organism. Verlangieri and Mumma [35] found *in vivo* sulfation of cholesterol by ascorbic acid 2-sulfate. The resulting product, cholesterol sulfate, was excreted in the stool. However, another group of authors have failed to support these results.[36]

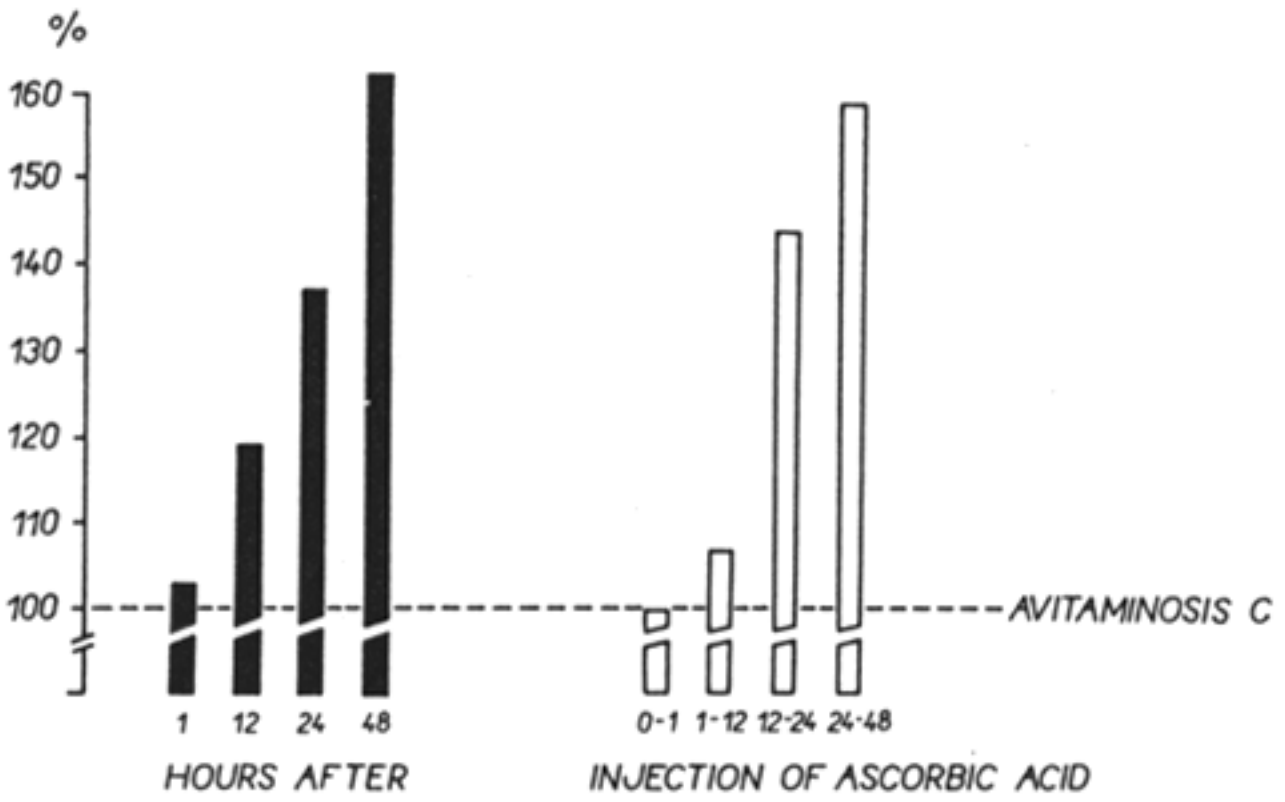


FIGURE 4. Parallel increase in [26-^{14}C]cholesterol oxidation (*right*) and cytochrome P-450 level (*left*) in liver microsomes of vitamin-C-deficient guinea pigs after administration of ascorbic acid. Values found in vitamin-C-deficient animals = 100%. The left part of the figure was constructed on the basis of the data of Leber et al.,[33] who kindly consented to publication.

Remaining Questions

A question that remains at least partially unanswered is whether or not the results of our studies can be extrapolated to human beings. Reports in the literature describing the effect of ascorbic acid on human blood serum cholesterol levels are unusually contradictory in their conclusions.[1, 3] In our experience, administration of 1,000 mg ascorbic acid daily for a period of three months to subjects 50–75 years of age with a starting concentration of plasma cholesterol below 200 mg%, had no effect on plasma cholesterol levels. On the other hand, in a group of subjects of similar age with a starting concentration of plasma cholesterol above 200 mg%, the same dose given for six months brought about a very significant decline in cholesterolemia (Table 5). In an

Table 5

Influence of Ascorbic Acid on Plasma Cholesterol Concentration in Humans

Person Examined, Age (yr) and Sex	Before Treatment	Intake of 1,000 mg Ascorbic Acid Daily	
		After 3 months	After 6 months
		Total Cholesterol (mg per 100 ml blood plasma)	
N.J., 75, F	228	214	178
J.M., 74, F	241	222	183
N.T., 72, F	227	200	207
K.I., 72, F	246	208	215
P.J., 71, M	206	192	159
H.J., 69, M	207	184	193
R.A., 69, F	248	192	175
S.F., 68, F	252	216	205
P.A., 67, F	327	253	303
K.M., 65, F	258	240	228
P.Z., 64, F	276	232	189
K.R., 63, F	285	258	243
S.M., 63, F	274	264	261
J.J., 60, F	248	227	179
B.R., 58, F	244	235	217
Č.P., 58, F	276	213	235
M.V., 57, F	240	243	175
M.J., 57, M	213	202	157
G.M., 56, F	289	267	210
B.A., 55, F	232	210	179
M.A., 52, F	255	273	228
N.M., 52, F	213	218	186
M.M., 52, M	264	227	239
S.R., 51, F	311	338	302
Mean ± SEM	253±6	230±7	210±8
Statistical significance (comparison with starting level)		$p < 0.02$	$p < 0.001$

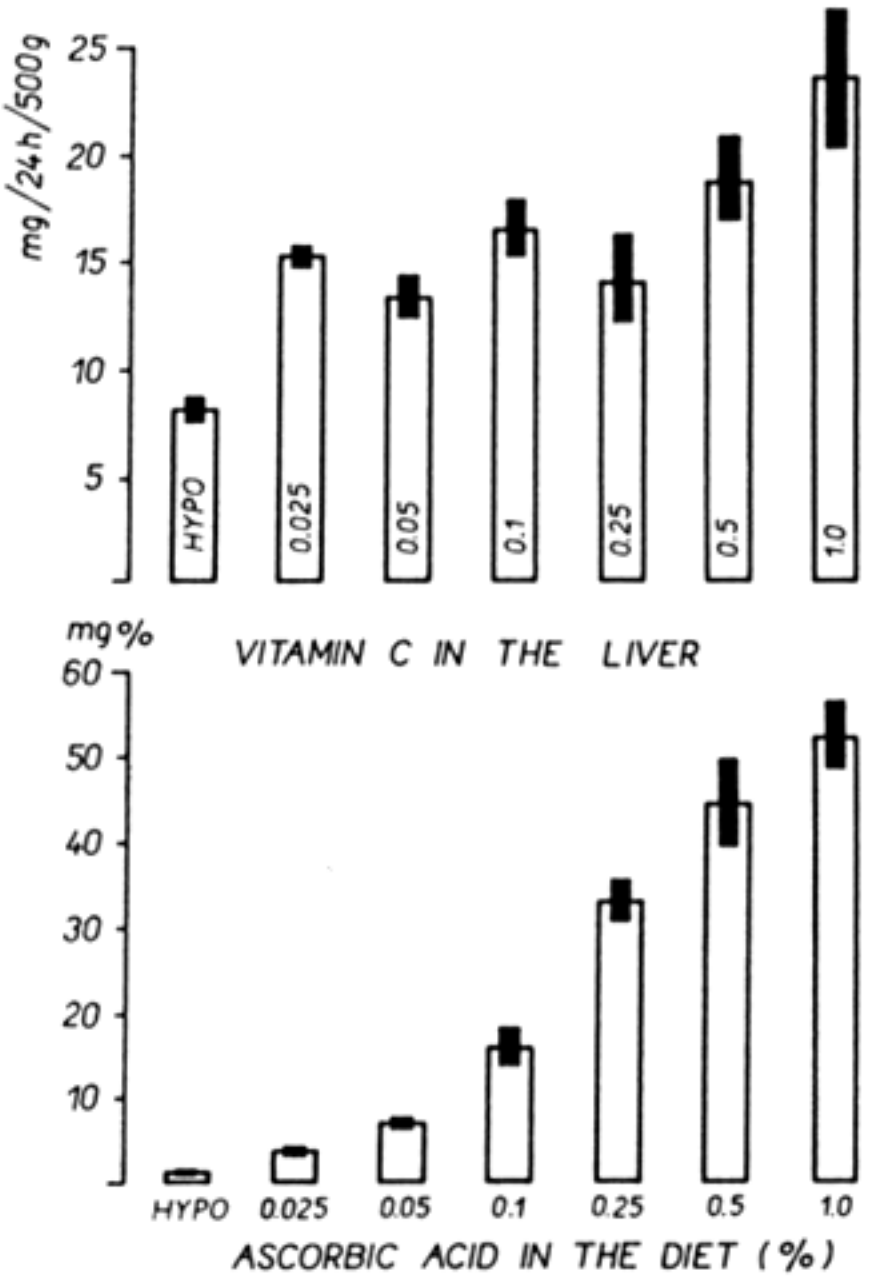


FIGURE 5. Cholesterol catabolism. Influence of graded doses of ascorbic acid (HYPO=hypovitaminosis C, 0.025, 0.05, 0.1, 0.25, 0.5, and 1% ascorbic acid in the diet) on the rate of cholesterol transformation to bile acids and on ascorbic acid concentration in the liver of guinea pigs. In the group given 1% ascorbic acid in the diet, cholesterol transformation to bile acids is significantly higher in comparison with groups given 0.25, 0.05, and 0.025% ascorbic acid in the diet and in hypovitaminous group. Black columns represent the standard errors of the mean.

earlier study on a selected group of subjects with a seasonal vitamin C deficiency and hypercholesterolemia, we succeeded in reducing the cholesterol concentration by using lower doses of ascorbic acid (300 mg daily for a period of 7 weeks).[37] Administration of very high doses of ascorbic acid (4,000 mg daily for 3 weeks) lowered cholesterolemia even in healthy normocholesterolemic subjects.[38] Yet, despite these positive results, further convincing data on the hypocholesterolemic effect of ascorbic acid in humans are still necessary.

Another question remaining is what is an optimal dóse of ascorbic acid? In the great majority of our experiments, a hypovitaminous group of guinea pigs were compared with controls receiving a daily dose of 10 mg ascorbic acid each. The concentration of ascorbic acid in the liver of these animals proved to be far below the saturation point. In a recent experiment we added ascorbic acid directly into the diet and followed the effect of graded doses on the rate of cholesterol transformation to bile acids. We found that the maximal dose used in this experiment (1% ascorbic acid in the diet) increased cholesterol transformation to bile acids threefold above the values found in guinea pigs with a latent vitamin C deficiency (FIGURE 5). The question of the optimal dose of ascorbic acid for the human organism should be urgently dealt with. Doses recommended thus far [39] may be far below optimum.

SUMMARY

Latent chronic ascorbic acid deficiency provokes in guinea pigs a metabolic disorder in the liver, causing an impaired cholesterol transformation to its

principal catabolic product, bile acids. This metabolic disorder induces hypercholesterolemia and accumulation of cholesterol in the liver and slows the release of cholesterol from the circulation. Ascorbic acid probably intervenes into the biosynthesis of bile acids at the stage of 7α-hydroxylation of the cholesterol nucleus. High doses of ascorbic acid significantly stimulate cholesterol transformation to bile acids in guinea pigs and decrease plasma cholesterol concentration in humans.

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