Lowered cholesterol catabolism in guinea pigs with chronic ascorbic acid deficiency

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There is much disagreement regarding the effects of acute ascorbic acid deficiency on cholesterol metabolism in the guinea pig (1-6). The contradictions in results may be explained to a certain extent by the dynamic character of avitaminosis C development, during which the character of metabolic disturbances is changed according to development of scurvy. When following the dynamics of the changes in adrenal cholesterol levels during development of avitaminosis C in guinea pigs, we have found (7) that distinct cholesterol accumulation can be observed at the beginning. During further stages, the cholesterol level reached its normal value and only in the last stage of scurvy, which was accompanied by a decrease in body weight, did the cholesterol concentration in adrenals decrease to below control values. Results attained in animals with acute scurvy may be, however, affected by secondary non-specific effects, such as food refusal followed by a sudden drop in body weight, hemorrhaging, total metabolic disorganization, etc. Some of these changes (e.g., hemorrhage) cannot be simulated by pair-feeding. Moreover, acute experimental scurvy does not reproduce the latent ascorbic acid deficiency observed in humans.

The aim of our experiments was to approach the contemporary state in human nutrition and to use a metabolically better defined state than acute scurvy. All these reasons have led us to elaborate a model of chronic hypovitaminosis C in guinea pigs (8). When using this model, we have found that in male guinea pigs with chronic ascorbic acid deficiency a significant accumulation of cholesterol occurs in the liver (9. 10). When guinea pigs were fed an atherogenic diet supplemented with 0.3% cholesterol, the effect of ascorbic acid deficiency was reflected in an increased accumulation of cholesterol in a number of other tissues, including the aorta wall (11). A significant negative correlation was confirmed as existing between the tissue cholesterol concentration and saturation of tissues with ascorbic acid, i.e., with decreasing saturation of tissues with the vitamin, the cholesterol accumulation in the relevant tissue was increasing (12). The increased cholesterol accumulation in the tissues of hypovitaminotic guinea pigs can be explained neither by increased synthesis of endogenous cholesterol from acetate-1-14C (7, 13) nor by increased absorption of exogenous cholesterol (14).

Speed of these processes is either unchanged or even decreased in guinea pigs with hypovitaminosis C. The aim of the experiments reported here was to determine whether an increased cholesterol accumulation in the tissues of guinea pigs with an ascorbic acid deficiency is caused by retarded cholesterol catabolism.

Materials and methods

Growing male guinea pigs were used in all experiments; they were fed a scorbutigenic diet ad libitum (10). All experiments were arranged as follows: the guinea pigs were divided into a control and a hypovitaminotic group. The control guinea pigs were administered 10 mg ascorbic acid/animal per 24 hr perorally. Hypovitaminosis C was evoked in the other group by 14-day administration of a scorbutigenic diet without any addition of ascorbic acid, and after that, by oral administration of a maintenance dose of 0.5 mg ascorbic acid/animal per 24 hr. Ascorbic acid in a 20% sucrose solution was administered by peroral probe three times weekly thereafter. Such a nutritional regimen was followed by a sudden decrease in tissue vitamin C levels of hypovitaminotic animals compared with the ascorbic acid concentrations.

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observed in the tissues of acute scorbutic guinea pigs (9). The maintenance dose of ascorbic acid, however, prevented manifestations of distinct avitaminosis C. Hypovitaminotic guinea pigs consumed the same diet as the control group; their behavior was normal and their weight curves were similar to those of control animals that were given doses of ascorbic acid 20 times higher than the maintenance dose. All experiments were long term, i.e., the guinea pigs were in the state of chronic hypovitaminosis C for 5 to 7 months.

The first experiment, lasting 22 weeks, was carried out with 10 control and 8 hypovitaminotic guinea pigs initially weighing about 400 g. Twenty days before the end of the experiment, the guinea pigs were given intraperitoneally solutions of cholesterol-4-¹⁴C (Amersham, Great Britain; specific activity 24.6 mCi/m mole) in a dose about 4.4 μCi/animal. The exact dose administered was determined by differential weighing of the injection needle with syringe before and after application of labeled cholesterol. Labeled cholesterol was dissolved in methanol with Tween 80; the methanol was evaporated and the residue was dissolved in sterile, physiological saline solution. The same application of labeled cholesterol was used also in the remaining experiments. After administration of cholesterol-4-¹⁴C, the guinea pigs were placed in metabolic cages and were allowed free access to food. At 2-day intervals their stools were quantitatively collected. These were dried at 105 °C to constant weight; the samples obtained from individual animals were thoroughly mixed together. For analysis, average samples were used which represented approximately 10% of the total weight of dry stool. The samples were extracted for 30 min with boiling ethanol, and then for 12 hr with ethanol in a Soxhlet apparatus. Only negligible traces of activity were found in extracted stool. Five milliliters of the extract was mixed with 5 ml 10% NaOH and hydrolyzed for 8 hr at 160 °C. Neutral sterols were extracted from the hydrolysate into five 10-ml portions of petroether; bile acids after acidification to pH 1 with concentrated sulfuric acid were extracted into five 10-ml portions of ethylether (15). Absolute activity of ¹⁴C in samples was determined by scintillation spectrometry in a Nuclear Chicago scintillation counter model Mark I, using external standard for quenching correction. Twenty days after the cholesterol-4-¹⁴C application, the guinea pigs were decapitated and total activity of ¹⁴C in their blood serum and thoracic aorta was determined by scintillation spectrometry, after being dissolved in a Nuclear Chicago solubilizer.

In the second experiment, 11 control and 12 hypovitaminotic guinea pigs initially weighing about 450 g were used. The experiment lasted 31 weeks. Seventy-two hours before its end, the guinea pigs were given intraperitoneally a solution of cholesterol-4-¹⁴C (the same preparation as in the first experiment) in a dose of 0.5 μCi/100 g body wt. After 72 hr, the guinea pigs were decapitated. In their blood serum and liver after extraction (16), concentration of total cholesterol was determined by the Liebermann-Burchard reaction (17). The concentration of vitamin C in liver and spleen was determined after extraction in 6% trichloroacetic acid (18). The appropriate amount of liver and pooled samples of gallbladder bile were hydrolyzed for 8 hr at 160 °C in a mixture of 5 ml 10% aqueous NaOH and 2.5 ml 96% ethanol. Extraction of neutral sterols and bile acids from the hydrolysate and determination of the ¹⁴C activity was performed, as already mentioned in the first experiment. More than five sterol extractions are not necessary because in the next five petrolether extracts only negligible traces of activity were found. Thin-layer chromatography revealed that the fraction of bile acids did not contain any detectable amounts of cholesterol.

The third experiment was carried out with 10 control and 5 hypovitaminotic guinea pigs initially weighing about 300 g. This experiment lasted 28 weeks. At the end of the experiment, guinea pigs were given intraperitoneally a solution of cholesterol-26-¹⁴C (Amersham, Great Britain; specific activity 24 mCi/m mole) in a dose of 0.6 to 0.7 μCi/100 g body wt. The guinea pigs were individually placed in the metabolic cages (diameter 29 cm), in which they had free access to food. CO₂-free air was admitted to the cages, aspirated at a rate of 400 to 600 ml/min and bubbled into two washing bottles with fritted-glass stoppers connected in a series and filled with 2.5 N water solution KOH. Expired ¹³CO₂ was collected in 48 hr-intervals for 10 days. The known quantity of KOH solution with absorbed ¹³CO₂ was acidified and ¹³CO₂ was absorbed into ethanolamine in an apparatus according to Saba and Diluzio (19). The radioactivity of ¹³CO₂ absorbed in ethanolamine was assayed as in the first experiments by scintillation spectrometry.

Results of all experiments were statistically evaluated by Student's t test and correlations were determined by the method of the least squares on the Olivetti Programma 101 computer.

Results

Results of the first experiment, in which output of ¹⁴C in stool was followed after intraperitoneal administration of cholesterol-4-¹⁴C in the fraction of neutral sterols and bile acids, are shown in Fig. 1. Hypovitaminosis C had no effect on excretion of ¹⁴C in the fraction of neutral sterols. The average output of ¹⁴C in the fraction of bile acids slightly decreased in hypovitaminotic guinea pigs. The ratio of activity of ¹⁴C-bile acids to ¹⁴C-sterols was lower in the hypovitaminotic group in almost all intervals. In regard to the fact that in this experiment only pooled samples of stool were analyzed, it was not
blood serum and thoracic aorta of hypovitaminotic guinea pigs than in the control group (blood serum: control 854 ± 73 dpm, hypovitaminosis C, 1,151 ± 61 dpm/100 μl, \( P < 0.01 \); thoracic aorta: control 355 ± 61 dpm, hypovitaminosis C, 577 ± 52 dpm/100 mg, \( P < 0.01 \)). Statistical analysis of the combined sets of control and hypovitaminotic guinea pigs has shown that there is a middle closed correlation between the level of \(^{14}\)C in the blood serum and thoracic aorta (\( P < 0.01 \)) (Fig. 2).

Table 1 lists the concentrations of ascorbic acid and total cholesterol in control and hypovitaminotic guinea pigs from the second experiment. The results prove that ascorbic acid concentrations in the liver and spleen of hypovitaminotic guinea pigs significantly decreased. Hypovitaminosis C caused a significant increase in the level of total cholesterol in liver, which is in agreement with results of our previous works (7, 9, 10).

Table 2 gives the distribution of \(^{14}\)C in the fraction of neutral sterols and bile acids in liver and gallbladder bile 72 hr after intraperitoneal administration of cholesterol-\(^{4}\)C. These results prove that distribution of \(^{14}\)C between the fraction of neutral sterols and bile acids was significantly changed. In the liver of ascorbic acid-deficient guinea pigs a not sufficiently significant tendency to higher values of activity in the fraction of sterols and significantly lower activity in the

### Table 1

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<th>Vitamin C and total cholesterol concentration in control and vitamin C hyposaturated guinea pigs</th>
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<td><strong>Parameter</strong></td>
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<td>Ascorbic acid</td>
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<td>mg/100 g, wet tissue</td>
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<td>Total cholesterol</td>
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<td>mg/100 ml</td>
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<td><strong>Values are means ± SEM. NS = not significant.</strong></td>
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possible to determine the degree of statistical significance of the differences observed.

Twenty days after intraperitoneal administration of cholesterol-\(^{4}\)C, significantly higher \(^{14}\)C-total activity was found in the
fraction of bile acids was observed. Due to these changes, the $^{14}$C-bile acids: $^{14}$C-sterols decreased in liver and gallbladder bile. (It reached almost one-half of the control values in liver.) These results prove that in liver of guinea pigs with chronic vitamin C deficiency, transformation of cholesterol to bile acids is significantly retarded.

Results of the third experiment, in which a different method for following cholesterol catabolism was used, prove the above conclusion. Figure 3 represents cumulative oxidation of intraperitoneally administered cholesterol-26-$^{14}$C to $^{14}$CO$_2$ in control and hypovitaminotic guinea pigs. In agreement with the results of the first two experiments, the rate of this process is significantly decreased in ascorbic acid-deficient guinea pigs. Differences in the amount of expired $^{14}$CO$_2$ between the control and hypovitaminotic group are highly significant in all intervals ($P < 0.01$-$0.001$).

**Discussion**

Cholesterol level in blood and tissues under a defined nutrition regimen is the result of a combination of many processes. Theoretically the increased cholesterol levels, regularly observed in the tissues of hypovitaminotic guinea pigs could result from any or a combination of the following alternatives in cholesterol metabolism: 1) a redistribution of cholesterol between various tissues and blood; 2) an increase in absorption of exogenous cholesterol; 3) an increase in endogenous cholesterol synthesis; 4) a decrease in fecal excretion of cholesterol; and 5) a decrease in cholesterol transformation to bile acids.

Theoretically, we could consider also other alternatives, e.g., transformation of cholesterol to steroid hormones. However, quantitatively, these processes are negligible for the total cholesterol balance (20).
The first alternative is highly improbable because in hypovitaminotic guinea pigs an accumulation of cholesterol was observed in many organs, especially in those which contribute to a substantial degree to the body pool of cholesterol (e.g., liver, small intestine, adrenals) (11, 12). The second alternative was examined by an experiment in which distribution of intragastrically applied cholesterol-4-14C was followed (14). The results obtained have shown that an increased accumulation of cholesterol in hypovitaminotic guinea pigs cannot be explained by an increased absorption of exogenous cholesterol. On the contrary, this process is slowed during chronic hypovitaminosis C. Increased levels of tissue cholesterol in hypovitaminotic guinea pigs cannot be explained either by the third alternative, as endogenous synthesis of cholesterol from acetate-1-14C is not changed during chronic hypovitaminosis C, or even a slight decrease (7, 13).

As the increased accumulation of cholesterol in vitamin C-deficient guinea pigs can be explained neither by an increased absorption of exogenous cholesterol nor by an increased biosynthesis of endogenous cholesterol, it is probable that chronic vitamin C-deficiency affects the metabolism of cholesterol within the sphere of its catabolism. The first in the series of experiments, results of which are presented in this paper, has shown that the output of 14C-neutral sterols in stools after intraperitoneal administration of cholesterol-4-14C is not affected by hypovitaminosis C. Thus, the only explanation is in the last alternative, i.e., retarded transformation of cholesterol to bile acids.

Catabolism of cholesterol to bile acids can be followed by using two isotopic methods. When cholesterol labeled with 14C in position 4 of the cyclic structure is administered, the activity that occurs in the bile acid fraction is the measure of the speed of this process. Mammals do not have an enzymatic system that is able to split the cholesterol nucleus (21). When cholesterol labeled in position 26 on the side chain is given, the isopropyl fragment from the side chain is split off during cholesterol catabolism and the 13C is released in the form of carbon dioxide (22). The speed of cholesterol catabolism is measured according to the amount of 14C in expired CO2. In order to prove our hypothesis that chronic hypovitaminosis C causes retardation of cholesterol catabolism to bile acids, both above-mentioned techniques were employed. The results we attained proved this hypothesis to be correct: in guinea pigs with long-term vitamin C-deficiency, transformation of cholesterol-4-14C to bile acids is slowed down and so is the oxidation of cholesterol-26-14C to 14CO2.

The mechanism of ascorbic acid participation in the transformation of cholesterol to bile acids is unknown. It seems that the basic biological role of ascorbic acid consists in its participation in hydroxylation reactions, e.g., in the synthesis of hydroxyproline from proline (23), in catabolism of aromatic amino acids (24), in hydroxylation of dopamine (25), tryptophan (26), acetanilid (27), tyramine (28), and other substances. Mechanism of the majority of the above-mentioned ascorbic acid-dependent hydroxylation reactions consists, according to Staudinger (29), in the fact that ascorbic acid forms with molecular oxygen, in an enzymatically catalyzed reaction, hydroxyl radicals that can be used for hydroxylation of various substrates. For the enzymes, catalyzing reactions of this sort in which one oxygen atom incorporates into hydroxylated substrate and the second atom is simultaneously reduced to water, the term "mixed-function oxidases" is used. A similar function to that of vitamin C can be played in these reactions by other electron donors such as nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), nicotinamide adenine dinucleotide, reduced form (NADH), dihydroxyfumaric, and tetrahydrofolic acids.

Hydroxylation reactions also play an important role in the transformation of cholesterol to bile acids (30). Björkhem et al. (31) have described the major pathway in the conversion of cholesterol to chenodeoxycholic acid in guinea pigs: cholesterol → cholest-5-ene-3β,7α-diol → 7α-hydroxycholesterol-4-en-3-one → 7α-hydroxy-5β-cholane-3-one → 5β-cholane-3α,7α-diol → 5β-chenodeoxycholic acid. During the first reaction, cholesterol
nucleus is hydroxylated in position 7; during the second to last, hydroxylation of side chain in position 26 proceeds. As the function of ascorbic acid in hydroxylation reactions is generally accepted, we suggest as a working hypothesis that ascorbic acid has a similar function in the transformation of cholesterol into bile acids. The scheme of this hypothetic role of ascorbic acid is in Fig. 4. The direct participation of ascorbic acid in the above-mentioned processes is supported also by data on the stimulatory effect of ascorbic acid on the synthesis of bile acids in vitro in liver mitochondria from scorbutic guinea pigs (32), as well as by our finding of substantially increased oxidation of cholesterol-26-14C to 14CO2 shortly after resaturation of ascorbic acid-deficient guinea pigs with a large intraperitoneally administered dose of ascorbic acid (unpublished observations). In this connection also, the data on the stimulatory effect of ascorbic acid on oxidation of 7-dehydrocholesterol in the mouse liver microsomal system (33) and on transformation of deoxycholic acid to cholic acid in nonbiological systems (34) are very interesting. There are other authors who emphasize the important role of Fe2+ ions during reactions of this nature (28, 33-36).

The question of the potential role of latent hypovitaminosis C in the pathogenesis of atherosclerosis is interesting. According to Willis (37), within a short period of time scorbutic guinea pigs show atheromatous changes in the aorta without being fed a cholesterol atherogenic diet. Gore et al. (38, 39) examined a great number of scorbutic guinea pigs, but did not find any similar changes. We have not found any significant morphological changes in the aorta and coronary arteries of scorbutic guinea pigs that were given an atherogenic cholesterol diet during the entire period of development of acute avitaminosis C (11). There are more data on protective effects of massive doses of ascorbic acid on experimental cholesterol atheromatosis (40-42). Unfortunately, these experiments were carried out with the animals (rabbits and rats) synthesizing ascorbic acid. Metabolic response of these animal species to the large amounts of exogenous vitamin C differs from that of the organisms that are not able to synthesize ascorbic acid (43), and the results attained must be very carefully extrapolated to the human organism. In guinea pigs fed atherogenic cholesterol diets, large amounts of the vitamin did not prevent the appearance of morphological changes in the vascular system, but pathomorphological changes in vessels of guinea pigs with chronic ascorbic acid deficiency were more distinct (12). In our opinion, the fact that chronic ascorbic acid deficiency with decreasing cholesterol catabolism causes an increasing accumulation of cholesterol in tissues creates a metabolic situation that increases the risk that pathomorphological changes in vessels will occur. There is a prospect that knowledge of the function of ascorbic acid in catabolism of cholesterol, obtained on the model of latent chronic hypovitaminosis C in guinea pigs, will make it possible to extrapolate also to a human organism, as when we succeeded in decreasing the level of serum cholesterol in hypercholesteremic persons with seasonal vitamin C deficiency through resaturation with massive doses of ascorbic acid (44).

Summary

Chronic ascorbic acid deficiency was provoked in male guinea pigs by 14-day administration of a scorbutigenic diet followed by peroral administration of a maintenance dose of vitamin C (0.5 mg/animal per day). A control group was fed the same scorbutigenic diet supplemented perorally by 10 mg vitamin C/animal per day. The experiments lasted 5 to 7 months. The course of weight
curves was not affected significantly by hypovitaminosis C.

Hypovitaminosis C caused a substantial decrease in vitamin C levels in tissues of guinea pigs and accumulation of total cholesterol in the liver. Hypovitaminosis C did not affect fecal excretion of \( ^{14} \text{C} \) in the fraction of neutral sterols in guinea pigs injected intraperitoneally with cholesterol-4-\( ^{14} \text{C} \), and it slightly decreased fecal excretion of \( ^{14} \text{C} \) bile acids (during 20 days following administration of labeled cholesterol). Twenty days after application of cholesterol-4-\( ^{14} \text{C} \) more \( ^{14} \text{C} \) was found in the blood serum and thoracic aorta of hypovitaminotic guinea pigs than in the control group. Three days after intraperitoneal injection of cholesterol-4-\( ^{14} \text{C} \), less \( ^{14} \text{C} \) in the fraction of bile acids was found in liver and gallbladder bile of hypovitaminotic guinea pigs than in the control group. Chronic hypovitaminosis C decreased significantly the oxidation of intraperitoneally injected cholesterol-26-\( ^{14} \text{C} \) to \( ^{14} \text{CO}_2 \) during 10 days following administration of labeled cholesterol.

The results prove that in guinea pigs with chronic ascorbic acid deficiency, the catabolism of cholesterol to bile acids is significantly decreased. A working hypothesis is postulated that ascorbic acid is required for cholesterol hydroxylation during its transformation to bile acids.

References


