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## Review

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### THE ROLE OF ASCORBIC ACID IN THE REGULATION OF CHOLESTEROL METABOLISM AND IN THE PATHOGENESIS OF ATHEROSCLEROSIS

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## Summary

Strong clinical and experimental evidence suggests that chronic latent vitamin C deficiency leads to hypercholesterolaemia and the accumulation of cholesterol in certain tissues. Ascorbic acid supplementation of the diet of hypercholesterolaemic humans and animals generally results in a significant reduction in plasma cholesterol concentration.

While most studies relating ascorbic acid to atherosclerosis have used the rabbit as a model, those concerned with elucidating the role of ascorbic acid in the regulation of cholesterol metabolism have generally used the guinea pig. Comparatively little use has been made of the non-human primates. A significant advance in recent years has been the development of a model of chronic latent scurvy in the guinea pig.

Chronic dietary inadequacy of vitamin C may influence the pathogenesis of atherosclerosis as it affects not only plasma cholesterol and triglyceride concentrations but also the integrity of the vascular wall.

Ascorbic acid is involved in the regulation of cholesterol metabolism in several ways. Dietary inadequacy of vitamin C is associated indirectly with a lowering of cholesterol absorption, this effect resulting from a reduction in the availability of bile acids, monoglycerides and fatty acids. The excretion of cholesterol as neutral steroids, however, appears not to be affected by ascorbic acid. Although much of the evidence for the involvement of ascorbic acid in cholesterol synthesis is equivocal, it seems likely that cholesterol synthesis is decreased in vitamin C deficiency. A series of studies using guinea pigs with chronic latent vitamin C deficiency has provided clear evidence that bile acid synthesis is re-

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duced in this condition. Indirect evidence strongly suggests that this results from a decrease in the activity of the microsomal enzyme cholesterol 7 $\alpha$ -hydroxylase. However, some evidence suggests that the mitochondrial reactions of bile acid synthesis require ascorbic acid. The role of ascorbic acid in the regulation of steroidogenesis appears to involve selective inhibitory and stimulatory effects on the desmolase, hydroxylase and dehydrogenase reactions which lead to the formation of pregnenolone and its subsequent conversion to steroid hormones.

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Key words: *Acute scurvy — Animal models — Ascorbic acid — Atherosclerosis — Cholesterol metabolism — Chronic latent scurvy — Plasma cholesterol — Vitamin C*

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## I. Introduction

A fundamental metabolic defect in man is the absence of the enzyme L-gulonolactone oxidase in the biosynthetic pathway of ascorbic acid [1]. This defect is also found in non-human primates, guinea pigs and a variety of other animals which thus all have a dietary requirement for vitamin C [2]. A critical dietary deficiency of ascorbic acid in these species results in acute scurvy which is the only clinical pathological condition for which cure and prevention is known specifically to require vitamin C. The incidence of acute or clinical scurvy in human populations, apart from its limited occurrence in infants [3], is considered rare [4,5]. However, a greater potential health risk exists in latent or subclinical scurvy which arises from a chronic dietary inadequacy of vitamin C.

The widespread incidence of latent scurvy in man, first demonstrated about forty years ago [6,7], remains evident in several population groups. Chronic dietary vitamin C deficiency is particularly prevalent in the elderly [8–12], especially the elderly poor [13] and also in the institutionalised young [14]. Chronic latent hypovitaminosis C also occurs in those populations subjected to marked seasonal variations in their dietary vitamin C intake [11,15–17].

The significance of dietary inadequacy of vitamin C in the etiology of hypercholesterolaemia and atherosclerosis first became apparent from clinical studies in Russia. Myasnikova [18] showed in 1947 that serum cholesterol concentrations could be lowered in hypercholesterolaemic patients by the administration of ascorbic acid. In the three decades since this study was carried out a large volume of literature on vitamin C and cholesterol metabolism has accumulated. However, many workers have used animals suffering from severe scurvy which completely masks the more subtle effects of a chronic dietary insufficiency of vitamin C. Only in recent years has the alternative approach of using animals with latent scurvy been adopted.

Since 1968, several authors have examined the problem of the involvement of ascorbic acid in cholesterol metabolism and atherosclerosis. One reviews the evidence for a role of vitamin C in bile acid synthesis [19], while several others give particular emphasis to the potential involvement of vitamin C in the pathogenesis of atherosclerosis [20–22].

In the present review the effects of dietary vitamin C deficiency and supple-

mentation on plasma and tissue cholesterol concentrations are discussed. The advantages and disadvantages of various animal models are then defined. The final sections are concerned with how ascorbic acid is involved in the pathogenesis of atherosclerosis and in the regulation of cholesterol absorption and excretion, its synthesis and its catabolism to bile acids or steroid hormones.

## II. Effect of ascorbic acid on plasma and tissue cholesterol concentrations

The involvement of ascorbic acid in cholesterol metabolism is apparent from the changes that occur in plasma and tissue cholesterol concentrations during dietary vitamin C deficiency and by the hypocholesterolaemic action of ascorbic acid in hypercholesterolaemic animals and human subjects. Furthermore, changes in tissue ascorbic acid concentration often occur in cholesterol-fed animals.

Total body cholesterol is increased in acutely scorbutic guinea pigs [23–25]. Although hypercholesterolaemia has been reported under such conditions [24–26], some studies have shown no change in plasma cholesterol concentration [23,27,28]. In guinea pigs, in whom the onset of a severe deficiency was delayed, the plasma cholesterol concentration first increased and then declined to prescorbutic values with the onset of severe scurvy [29]. As shown by studies on severely scorbutic guinea pigs [28,30–32] and monkeys [33], these changes in plasma cholesterol concentration reflect changes in both the proportion of the various lipoprotein fractions and their respective content of cholesterol. However, there is wide variation in the nature of the changes observed; this probably resulted from the use of semiquantitative methods in the separation and analysis of the lipoprotein fractions.

As guinea pigs show a diminishing food intake and a marked deterioration in physical condition after 17 days on a scorbutogenic diet, it is not surprising that during the later stages of acute scurvy, trends established earlier in the condition reverse. For example, adrenal cholesterol concentration increases in the early stages of scurvy in guinea pigs [34] but subsequently decreases [27,34]. The change in the cholesterol content of the adrenal is less marked than the change in concentration because the adrenal becomes enlarged in the later stages [27]. Thus, the differing reports that the cholesterol concentration of the adrenals shows no change [35] or decreases [23,24] in late scurvy could be explained by differences in the time of sampling in relation to the onset of scurvy and the changes in the size of the adrenal. In guinea pigs with advanced acute scurvy the concentration of cholesterol in intestine and testes increases and in liver and kidneys remains unchanged [23,24].

The many secondary effects of acute vitamin C deficiency on cholesterol metabolism are avoided by using animals with chronic latent hypovitaminosis C. In male guinea pigs with this condition, increased cholesterol concentrations in plasma and liver are consistently observed [36–41].

The studies on how severe dietary vitamin C deficiency affects plasma cholesterol concentration in primates, like those with guinea pigs, have not been definitive. Thus, although monkeys with severe scurvy showed reduced plasma cholesterol concentrations, repletion with ascorbic acid did not reverse this condition [33,42]. In baboons serum cholesterol was unaffected by either high

oral doses of vitamin C or by the withdrawal of all dietary vitamin C [43]. In man, severe vitamin C deficiency was associated with a decrease in serum cholesterol concentration which was reversed on repletion with vitamin C [5,44,45]. However, these decreases were probably not specifically produced by vitamin C deficiency. Thus, in a study of clinical scurvy in Bantus, Bronte-Stewart et al. [5] indicated that the diet of the subjects was altered to counter general malnutrition before initial observations on serum cholesterol concentrations could be made. Similarly, the study of experimental scurvy in humans by Hodges et al. [45] is of limited value because the diets contained no cellulose and breakdown of the steroid nucleus of cholesterol in the gut is known to occur on such diets [46].

Many clinical trials have been undertaken where subjects have received vitamin C at doses of at least an order of magnitude greater than those necessary to prevent clinical scurvy. It has generally been found that vitamin C lowers plasma cholesterol concentration in hypercholesterolaemic human subjects [16,18,47-51] but has no effect on those with normal plasma cholesterol concentrations [51-53]. There are however other reports showing that vitamin C supplementation to hypercholesterolaemic subjects either has no effect on plasma cholesterol concentration [54,55] or increases it [56]. These differences in findings may be related to differences between the trials in the ascorbic acid status of the subjects before the commencement of ascorbic acid supplementation. Unfortunately most of these clinical studies were carried out on only a small number of subjects and did not include measurements of blood ascorbic acid concentration.

The finding that vitamin C has no effect on normocholesterolaemic subjects is consistent with the results of a study by Elwood et al. [57] where no association was found between plasma ascorbic acid and serum cholesterol concentrations. A more recent study on a much smaller number of people showed a positive correlation between the levels of serum cholesterol and both plasma and leucocyte ascorbate [58]. However, the statistical treatment of the data in this study is of doubtful validity.

Seasonal variation in vitamin C intake is an important factor in the etiology of latent scurvy in man. A chronic dietary insufficiency of vitamin C has been demonstrated during the winter and spring months [11,15-17]. Although one study has shown no seasonal effect on plasma cholesterol concentration in man [59], others [16,17,60-63] have revealed distinct hypercholesterolaemia during the period of the year when the vitamin C intake becomes sub-optimal. In one of these studies blood ascorbic acid concentration was also measured and found to be inversely correlated with plasma cholesterol concentration [17]. Similarly, in rats the seasonal variation in plasma cholesterol concentration is related to the variation in adrenal ascorbic acid content [64].

Cholesterol feeding increases the vitamin C requirement of the guinea pig [65,66] and, when prolonged, reduces the ascorbic acid concentration of tissues [67]: there is a significant negative correlation between the concentration of cholesterol and ascorbic acid in liver, adrenal and small intestine but not in plasma [65]. Cholesterol feeding in rabbits and rats, which are species that synthesise ascorbic acid [68,69], results in increased accumulation of vitamin C in the tissues and increased excretion in the urine. This indicates an enhancement

of ascorbic acid biosynthesis [67,70]. Conversely, dietary cholesterol-induced hypercholesterolaemia in rabbits [71,72], rats [72] and pigs [73] is reduced by supplementation with vitamin C. The cholesterolaemic response of rabbits to dietary cholesterol varies much between animals [70,74]. The lower cholesterolaemic response in some rabbits is associated with a greater ability to augment ascorbic acid biosynthesis in response to the increased dietary load of cholesterol [70]. Seasonal variation in the hypocholesterolaemic action of supplementary dietary ascorbic acid in cholesterol-fed rabbits [75] may have been due to a variable content of ascorbic acid in the non-supplemented diet.

### III. Evaluation of animal models

The rabbit has been used more extensively than any other species as a model for studying the effect of vitamin C on the development of atherosclerosis [70-72,75-83], probably because it was the first in which dietary-induced hypercholesterolaemia [84] and atherosclerosis [85] were reported. However, it is held by some workers that some aspects of experimental atherosclerosis in rabbits resemble the human condition only superficially [86]. Furthermore, the rabbit, unlike the human, synthesises ascorbic acid and, on cholesterol feeding, increases the rate of synthesis of the vitamin to compensate for the increased requirement for vitamin C — this response shows marked individual variation [70]. Thus, although studies with the rabbit have generally supported the evidence for a role of vitamin C in cholesterol metabolism and atherosclerosis, its usefulness as a model for future work is limited.

The ideal model for this work would be one which is unable to synthesise ascorbic acid. Although this essentially restricts the selection to various non-human primates and the guinea pig, there are other species unable to synthesise ascorbic acid — such as the Indian fruit-eating bat and various birds, including the red-vented bulbul [2]. These other species may be useful in specific circumstances.

It is currently widely held that atherosclerosis in non-human primates may be particularly relevant to the human disease; it is thus surprising that the relationship between ascorbic acid and atherosclerosis has not been examined in these species. The required level of deficiency as well as the speed and severity of onset of the disease are obvious factors to investigate. There is also scope for further use of the cholesterol-fed guinea pig as a model. Most studies [87-91], contrary to that of Cook and McCullagh [92], have reported that cholesterol-fed guinea pigs develop atherosclerosis. The development of lesions occurs slowly and at plasma cholesterol concentrations closely paralleling those of hypercholesterolaemic human subjects [87,88]. The high individual variation between guinea pigs with dietary-induced cholesterol atherosclerosis, considered a limitation of this model [93], might be overcome if strains of guinea pigs with a more predictable cholesterolaemic response could be selected. This has been achieved with the rabbit [74] and squirrel monkey [94]. Guinea pigs maintained under conditions where they naturally develop latent scurvy during the winter and spring months [95], could be used as models for examining the impact of seasonal variations in vitamin C intake on the pathogenesis of atherosclerosis.

Among the non-human primates, only the baboon and rhesus monkey have been used for studying the role of ascorbic acid in specific aspects of cholesterol metabolism [33,43,96]. The particular value of the baboon is that its lipoproteins are similar to those in man [97], in contrast to the absence of HDL in the guinea pig — except when fed cholesterol [98]. Nevertheless, the guinea pig has over-riding advantages in cheapness and the rapidity with which its ascorbic acid can be depleted [34] (half-life ~ 4 days [99,100]). Although ascorbic acid differs in its catabolism between guinea pig and man [100,101], its amount and tissue-distribution are similar in these two species [6].

Chronic deficiency of ascorbic acid in the guinea pig provides a better model than acute deficiency where the animal develops anorexia, weight-loss, negative nitrogen balance, bleeding and anaemia [102]. Ginter has devised a particularly suitable model of chronic latent hypovitaminosis C [103]: animals are fed a scorbutogenic diet for 14 days and are then maintained on 0.5 mg ascorbic acid per day. This regime results in tissue concentrations comparable with acute scurvy. However, only in males does this chronic deficiency lead to increased cholesterol concentrations [104].

Although the vitamin C requirement of guinea pigs is given as 16 mg/kg body weight/day [105], Williams and Deason have shown a 20-fold variation in the requirement of individual guinea pigs [106]. This is possibly related to individual differences in the rate at which ascorbic acid is catabolised. It may also indicate varying abilities to synthesise ascorbic acid. There is evidence that a small number of female guinea pigs can synthesise ascorbic acid [107], but this is not accepted universally [108,109]. This individual variation could be used to advantage if strains of guinea pigs could be selected for differences in vitamin C requirement.

#### IV. The role of ascorbic acid in the pathogenesis of atherosclerosis

The early studies of Myasnikova (as reported by Myasnikov [76]), and also those by Myasnikov [77,78], showed that ascorbic acid inhibits the development of experimental atherosclerosis and cholesterolaemia in rabbits. These findings were confirmed in clinical trials in Russia in later years [47,48]. Vital statistics for England and Wales show a dicyclic seasonal variation in deaths from myocardial infarction with peaks during the winter and spring [110] and it has been suggested that these peaks coincide with periods of minimum consumption of vitamin C [111]. More recently a correlation analysis between the standardised mortality ratios for various causes of death and the intake of a number of nutrients in different regions of England and Wales in different years has shown strong negative correlations between vitamin C intake and mortality from ischaemic heart disease ( $-0.49$ ) and cerebrovascular disease ( $-0.68$ ) [112]. Such statistical correlations however do not necessarily prove a cause-and-effect relationship [113].

The inference that high intakes of vitamin C reduce the incidence of atherosclerosis is supported by the results of many experimental studies. In guinea pigs fed an atherogenic diet the development of atheroma was directly related to vitamin C intake — the most advanced lesions occurred in the group with the lowest intake [65]. In rabbits, vitamin C-supplementation ameliorated the se-

verity of dietary cholesterol-induced atherosclerosis [71,72,75–80] although some studies demonstrated no such effect [81,82]. In rats receiving a diet containing no added cholesterol, dietary supplements of vitamin C markedly decreased the cholesterol and phospholipid concentration of the aorta [114].

Ascorbic acid may influence the pathogenesis of atherosclerosis, not only by an effect on plasma cholesterol concentration but also on plasma triglyceride concentration and on the integrity of the vascular wall. Sokoloff et al. demonstrated a 50–70% reduction in plasma triglyceride concentration in patients receiving ascorbic acid supplements and this was associated with a marked stimulation of plasma lipoprotein lipase activity [51]. In guinea pigs fed an atherogenic diet, high doses of vitamin C reduced the triglyceride concentration in the plasma, liver and aorta but the effect on lipoprotein lipase activity in various tissues was not uniform [115]. In human arteries those segments with higher concentrations of ascorbic acid had a lower incidence and severity of atheroma [116], indicating that ascorbic acid might regulate arterial wall structure and function. Spittle has suggested that the mobilisation of cholesterol from the arterial wall by an increased intake of ascorbic acid in the diet could account for a reported increase in the plasma cholesterol concentration [56]. However, the magnitude of the reported increase cannot be explained by such a mobilisation [117]. It has also been suggested that ascorbic acid decreases the development of experimental atherosclerosis in rabbits by decreasing the permeability of the aortic wall to cholesterol [75].

The mechanism by which ascorbic acid could probably exert its greatest influence on the structure of the arterial wall is through its effect on collagen formation where it plays an essential role in the hydroxylation of proline. Acutely scorbutic guinea pigs have been shown to develop lesions in the aortic intima, such as the separation of endothelial cells and a depletion of sub-endothelial collagen [118]. Such lesions, which could be produced in guinea pigs without hypercholesterolaemia [119] and which could be readily reversed in their early stages by ascorbic acid therapy [120], were considered a direct result of a disturbance in the formation of the intimal ground substance [119].

The evidence for the positive benefits of ascorbic acid in reducing atherosclerosis would now appear to be as strong as the evidence available in the early 1950's for the positive benefits of dietary polyunsaturated fats in reducing coronary heart disease because of their effect on plasma cholesterol [121,122]. On the basis of these findings, a series of trials was carried out [123–126] but unfortunately did not produce convincing evidence that polyunsaturated fats would benefit the whole community [127]. Be that as it may, the time would now appear to be right for the initiation of large trials to test the efficacy of ascorbic acid in reducing not only atherosclerosis but also mortality, which is a more meaningful endpoint when assessing the health of the whole community. Often therapeutic agents thought to be beneficial may in fact just alter disease patterns, while having no effect on morbidity or mortality

## V. The role of ascorbic acid in regulating various aspects of cholesterol metabolism

### (1) Cholesterol absorption and excretion

The accumulation of cholesterol in the vitamin C-deficient animal does not

appear to result from increased cholesterol absorption. Ginter et al. [128] found that the recovery, after 72 h, in the gut contents and faeces of [4-<sup>14</sup>C] cholesterol administered intra-gastrically was higher in chronically vitamin C-deficient guinea pigs than in controls. Furthermore, the recovery of radioactive cholesterol in the plasma, liver and lungs was lower in the scorbutic animals. Ascorbic acid deficiency could inhibit cholesterol absorption through its inhibition of the synthesis of bile acids (discussed below) and pancreatic lipase [129]. Decreased pancreatic lipase activity would reduce the extent of lipolysis of triglycerides to monoglycerides and fatty acids. Bile acids, monoglycerides and fatty acids are essential prerequisites for the micellar solubilisation of cholesterol [130]. Thus, their reduced availability during vitamin C deficiency would inhibit cholesterol absorption.

As the quantification of neutral steroid output remains one of the major problems in cholesterol balance studies in guinea pigs [131] and in mammals in general [132], it is not surprising that the effect of ascorbic acid on this aspect of cholesterol metabolism has not been studied in detail. In rabbits with advanced experimental atherosclerosis, supplementation of the diet with ascorbic acid enhances excretion of cholesterol in the bile [83]. However, in guinea pigs injected intraperitoneally with [4-<sup>14</sup>C] cholesterol the output of radioactivity in the neutral steroid fraction in both bile and faeces did not differ between guinea pigs with chronic latent vitamin C deficiency and controls [37]. Thus, present evidence would not suggest that ascorbic acid plays a major role in cholesterol excretion *per se*.

Some interesting effects of ascorbic acid-2-sulphate on cholesterol excretion have been reported recently. When rats were injected intracardially with <sup>35</sup>S-labelled ascorbic acid-2-sulphate, the excretion of labelled cholesterol sulphate in the faeces was 50-times that when rats were injected with <sup>35</sup>S-labelled inorganic sulphate [133]. Steroid sulphates usually constitute about 3% of the neutral steroid output in human faeces [134,135]. Ascorbic acid-2-sulphate does not possess anti-scorbutic activity and does not accumulate in the liver when administered either orally or parenterally [136]. Obviously further work needs to be done to evaluate ascorbic acid-2-sulphate as a cholesterol-lowering agent.

## (2) Cholesterol synthesis

The role of ascorbic acid in the regulation of cholesterol synthesis remains uncertain because the results of most studies on the effect of vitamin C deficiency on cholesterogenesis are conflicting. In acutely scorbutic guinea pigs the rate of cholesterol synthesis in various tissues has been reported to increase [137,138] or to show no change [26]. In guinea pigs with chronic latent scurvy cholesterol synthesis has been shown to be either reduced [27] or not to change [40]. More recently, a study of baboons with latent scurvy demonstrated a significant lowering of the rate of cholesterogenesis [96].

It has been suggested that the increase in cholesterogenesis observed in acutely scorbutic guinea pigs could result from an increased availability of acetate for cholesterol synthesis [23,139,140]. Diminished oxidation of acetate occurs in liver preparations of acutely scorbutic guinea pigs. This decrease in the activity of the tricarboxylic acid cycle [139] is thought to result from hypoinsulinism [23,24]. Insulin has a role in the regulation of the activity of the tricar-



boxylic acid cycle [141] and its content in the pancreas of guinea pigs with acute vitamin C deficiency is decreased [142]. In addition, the administration of insulin to these animals increases acetylation (a measure of acetyl coenzyme A availability) to normal levels and lowers the body cholesterol content to normal [23]. The finding that the level of coenzyme A (including its reduced form) does not change in severe vitamin C deficiency was taken as further evidence that there was a genuine increase in cholesterol synthesis in the scorbutic animal. However, it is difficult to accept these proposals in the light of evidence that the availability of acetate is not rate-limiting in cholesterol synthesis [143] and that hypoinsulinism may directly inhibit  $\beta$ -hydroxy- $\beta$ -methylglutaryl coenzyme A reductase (HMG CoA reductase) [144], the rate-limiting enzyme in cholesterol synthesis [143,145].

Although indirect, there is evidence that ascorbic acid regulates the activity of an enzyme in the biosynthetic pathway subsequent to HMG CoA reductase, possibly squalene synthetase [70]. Popják et al. [146] showed that ascorbic acid could act as a cofactor in the conversion of labelled mevalonate to sterol by rat liver microsomes. The recent study of Weight et al. [96] with scorbutic baboons showed that the incorporation of labelled mevalonate into cholesterol was reduced to a greater extent than was the incorporation of labelled acetate.

As discussed in a later section, bile acid synthesis is markedly reduced during vitamin C deficiency. This may in itself accelerate cholesterol synthesis indirectly by impairing the intestinal absorption of endogenous and dietary cholesterol, thereby interrupting the normal feedback inhibition on hepatic cholesterologenesis [147].

Much of the evidence for the involvement of ascorbic acid in cholesterol synthesis is equivocal. The measurement of the activities of enzymes, such as HMG CoA reductase and squalene synthetase, in scorbutic and re-supplemented animals is essential.

### *(3) Bile acid synthesis and excretion*

Several studies with guinea pigs have clearly demonstrated that bile acid synthesis and excretion are significantly reduced in vitamin C deficiency. Guchhait et al. [148] reported that 24 h after the intraperitoneal administration of [4- $^{14}$ C]cholesterol, less radioactivity was recovered from the bile acid fraction of the liver, bile, gut and faeces of severely scorbutic guinea pigs than from paired controls. Similarly, Ginter et al. [37] found that the bile acids recovered from the liver and gallbladder-bile 3 days after intraperitoneal injection of [4- $^{14}$ C]cholesterol were labelled to a lesser extent in guinea pigs with chronic latent scurvy than in the controls. When [26- $^{14}$ C]cholesterol was similarly administered, the recovery of  $^{14}$ CO<sub>2</sub> after 10 days was less in the chronically scorbutic guinea pigs than in the control animals. Furthermore, the recovery of  $^{14}$ CO<sub>2</sub> from the deficient animals significantly increased after the resaturation of the animals with ascorbic acid [39]. Using the [26- $^{14}$ C]cholesterol technique, which slightly underestimates bile acid synthesis [149], Ginter and his colleagues [36,38] subsequently showed that chronically scorbutic guinea pigs produced (on a 500 g body-weight basis) significantly less bile acid (8.3 mg/day) compared with control animals (11.8 mg/day). There was no difference in the rate of cholesterol turnover between the two groups. The rate of bile acid

synthesis was positively correlated with the ascorbic acid concentration of the liver, which in turn was negatively correlated with the cholesterol concentration of both plasma and liver.

The mechanism by which ascorbic acid regulates bile acid synthesis is not fully understood. The conversion of cholesterol to bile acids involves a series of reactions that occur at three subcellular sites [150,151]. While ascorbic acid possibly regulates the oxidative steps in side-chain cleavage in the mitochondria, it seems more likely to exert its major effect on the steroid nuclear hydroxylation reactions in the microsomes. Guchhait and co-workers showed that the formation of bile acids from [4-<sup>14</sup>C]cholesterol by guinea pig mitochondria was enhanced by the addition of ascorbic acid *in vitro* [148]. The effect was more pronounced with mitochondria from acutely scorbutic animals than with those from the pair-fed controls. However, Kritchevsky et al. [152] were not able to demonstrate any stimulatory effect of ascorbic acid on the oxidation of [26-<sup>14</sup>C]cholesterol by mitochondria from normal guinea pigs or rats; probably because the mitochondrial preparations already contained the optimum concentration of ascorbic acid. Neither of these studies take into account that the 7 $\alpha$ -hydroxylation of cholesterol, which is the initial step in the primary route for the conversion of cholesterol to bile acids, occurs primarily in the microsomes. Shefer et al. have shown in studies in the rat that microsomes, on a protein-weight basis, have more than 11 times the 7 $\alpha$ -hydroxylating activity found in mitochondria [153]. Thus, the role of mitochondria should be tested not with cholesterol but with steroids that have been shown to serve as mitochondrial substrates, such as 5 $\beta$ -cholestane-3 $\alpha$ ,7 $\alpha$ -diol [151]. An alternative pathway of bile acid synthesis has been suggested by Mitropoulos and Myant in which cholesterol is first converted to 26-hydroxy-cholesterol [154,155]. It is possible that the effect of ascorbic acid on bile acid synthesis reported by Guchhait and co-workers [148] was on this pathway and not on that in which 7 $\alpha$ -hydroxycholesterol is first formed.

As the basic role of ascorbic acid in metabolism is that of a hydroxylating agent [156,157], it would seem likely that this function is extended to the regulation of bile acid synthesis. Ginter and his colleagues [37-39,65] believe that ascorbic acid controls bile acid synthesis by regulation of the activity of the rate-limiting enzyme in the pathway, cholesterol 7 $\alpha$ -hydroxylase [158, 159], but this is so far supported only by two lines of indirect evidence. Firstly, the concentration of cytochrome P-450, which is involved in the 7 $\alpha$ -hydroxylation of cholesterol [160], is substantially reduced in scorbutic guinea pigs [161,162] and this effect is reversed by repletion with ascorbic acid. Ginter and Nemec [163] have shown that the time course of the enhancement of the oxidation of [26-<sup>14</sup>C]cholesterol to <sup>14</sup>CO<sub>2</sub> produced by injecting ascorbic acid into chronically scorbutic guinea pigs closely parallels the increase in the concentration of cytochrome P-450 observed under these conditions by Leber et al. [161]. Secondly, Ginter has recently demonstrated that there is no difference in the conversion of [26-<sup>14</sup>C]7 $\alpha$ -hydroxycholesterol to <sup>14</sup>CO<sub>2</sub> by normal guinea pigs when compared with those with chronic ascorbic acid deficiency [164]. Although this indicates that ascorbic acid regulates bile acid synthesis at the stage of 7 $\alpha$ -hydroxylation of cholesterol and not in subsequent reactions, it will only be verified by the measurement of cholesterol 7 $\alpha$ -hydroxylase

in scorbutic animals. Such studies with guinea pigs would need first to define the optimal conditions of assay of the  $7\alpha$ -hydroxylation reaction, as the activity is usually very low in this species compared with that in the rat [152,165].

#### (4) Steroid hormone synthesis

Although steroidogenesis constitutes a pathway of negligible importance to the overall cholesterol economy of animals [166], the involvement of ascorbic acid in its regulation has been widely studied. However, many of the results are equivocal and the precise role of ascorbic acid thus remains uncertain. Most of these studies have been made on the adrenal which, on stimulation by adrenocorticotrophic hormone (ACTH), releases ascorbic acid and then increases steroid hormone secretion [167,168].

Ascorbic acid appears to regulate two separate enzyme systems in steroidogenesis, either by a stimulatory effect on some reactions or an inhibitory effect on others. The first of these are the mitochondrial reactions that convert cholesterol to pregnenolone; these comprise two successive hydroxylations to form  $20\alpha,20\epsilon$ -dihydroxycholesterol which, with the action of the 20,22-desmolase, forms pregnenolone. Studies with a soluble enzyme preparation from bovine and porcine adrenal mitochondria showed that, while ascorbic acid was stimulatory to the  $20\alpha$ -hydroxylation reaction, it was inhibitory to 20,22-desmolase activity [169]. An inhibitory effect of ascorbic acid on cholesterol side-chain cleavage in rat ovary [170] and testis [171] has also been reported, although high concentrations of ascorbic acid were necessary to produce such inhibitory effects.

The second series of reactions involving ascorbic acid are the mitochondrial  $11\beta$ - and  $18\beta$ -hydroxylations, the microsomal  $17\alpha$ - and 21-hydroxylations and the dehydrogenations that occur after pregnenolone formation. The  $11\beta$ -hydroxylation of deoxycortisol to cortisol decreases in the adrenals of scorbutic guinea pigs but can be restored by the in-vitro addition of ascorbic acid [172]. However, when added to mitochondria from adrenals of normal guinea pigs, ascorbic acid has no effect on  $11\beta$ -hydroxylation [173]. In bovine adrenal preparations cortisol synthesis is inhibited by ascorbic acid [174-176] but not by dehydroascorbic acid [174]. The 21-hydroxylating activity of bovine adrenocortical microsomes is also inhibited by ascorbic acid [177-179]. However as these preparations probably already contained optimum concentrations of ascorbic acid, the inhibitory effect of the added ascorbic acid may not have reflected a physiological effect of ascorbic acid on 21-hydroxylase activity. Thus, the activity of the various hydroxylases involved in steroidogenesis, as with that of cholesterol  $7\alpha$ -hydroxylase in bile acid synthesis, should be measured in scorbutic animals. There is better agreement about the role of ascorbic acid in the microsomal dehydrogenation reactions, where studies with both guinea pig [180] and rat [181] adrenal preparations suggest a stimulatory effect on dehydrogenase activity.

## VI. Conclusion

There is now substantial evidence that chronic latent scurvy is prevalent in human populations. Studies with guinea pigs, in whom latent scurvy has been

produced experimentally, have convincingly shown that the most critical effect of a chronic dietary inadequacy of vitamin C on cholesterol metabolism is impairment of bile acid synthesis; this leads to the accumulation of cholesterol in the plasma and tissues. Equally good experimental evidence suggests that the integrity of the vascular wall is directly altered by an inadequate intake of vitamin C. These effects imply that chronic dietary insufficiency of vitamin C must be considered a risk factor in the pathogenesis of atherosclerosis. Dietary supplementation with ascorbic acid would, therefore, seem justified in those population groups where chronic latent scurvy is known to be prevalent. However, this supplementation probably needs only to be made to the extent where it brings the sub-optimal intake to a level which corresponds to the recommended daily allowance of vitamin C. Supplementation with "mega" doses, particularly where there is already an adequate intake of vitamin C, may not be expected to provide any additional benefit in reducing the risk of atherosclerosis. This conclusion follows from the observations that in healthy individuals the concentrations of cholesterol and ascorbic acid in the blood are not correlated and that the administration of "mega" doses of vitamin C to normocholesterolaemic subjects does not alter plasma cholesterol concentrations.

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