# Stimulation of [26-$^{14}$C]cholesterol oxidation by ascorbic acid in scorbutic guinea-pigs

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1. Oxidation of intraperitoneally injected [26-$^{14}$C]cholesterol to $^{14}CO_2$ was significantly increased after resaturation of vitamin C-deficient guinea-pigs with one injection of 100 mg ascorbic acid and subsequent peroral administration of 50 mg ascorbic acid per animal per d.

2. Stimulation of cholesterol oxidation was observed in resaturated guinea-pigs either fed *ad lib.* or pair-fed.

3. A highly significant direct correlation was found between ascorbic acid concentration in liver and rate of cholesterol oxidation.

A hepatic accumulation of cholesterol occurs in male guinea-pigs with chronic ascorbic acid deficiency. If guinea-pigs are given an atherogenic cholesterol-containing diet, chronic hypovitaminosis C causes an increased accumulation of total cholesterol in many tissues, including the thoracic aorta (Ginter, Babala & Červeň, 1969; Ginter, 1970). In experiments with two kinds of labelled cholesterol we have found that the cause of an increased cholesterol accumulation in tissues of hypovitaminotic animals was retarded catabolism of cholesterol to bile acids. Thus, hypovitaminotic animals dosed with [4-$^{14}$C]cholesterol excreted smaller amounts of faecal $^{14}$C-bile acids, and in the bile acid fraction of their liver and gall-bladder bile significantly less $^{14}$C was found than in that of the control group. Oxidation of [26-$^{14}$C]cholesterol to $^{14}CO_2$ was significantly decreased in guinea-pigs with chronic ascorbic acid deficiency (Ginter, Červeň, Nemec & Mikuš, 1971).

The aim of the present work was to examine whether resaturation of vitamin C-deficient guinea-pigs with high doses of ascorbic acid increases significantly cholesterol catabolism.

## EXPERIMENTAL

### *Animals and experimental design*

Eighteen male guinea-pigs initially weighing about 480 g were fed *ad lib.* on a scorbutogenic diet (Ginter, Ondreička, Bobek & Šimko, 1969). After 16 d they were divided into three groups, each of six animals. The first group was kept on the scorbutogenic regimen. Animals in the remaining two groups were given an intraperitoneal injection of 100 mg ascorbic acid per animal on the 16th day, and from then on they were given perorally 50 mg ascorbic acid per animal per d. On the 17th day after the scorbutogenic regimen started, all animals were given intraperitoneally a solution of [26-$^{14}$C]cholesterol (Radiochemical Centre, Amersham, England; specific activity 24 mCi/mmol) in a dose of 0·70–0·75 $\mu$Ci/100 g body-weight. The labelled cholesterol was dissolved in methanol containing Tween 20, the methanol was evaporated and the residue was redissolved in sterile physiological saline.

The guinea-pigs were individually placed in metabolic cages of diameter 29 cm. Air, free of $CO_2$, was admitted to the cages and aspirated at a rate of 400–600 ml/min and bubbled into two washing bottles with fritted-glass stoppers filled with 2·5 M-aqueous KOH. Expired $CO_2$ was collected for successive 24 h periods during the next 10 d. In this period vitamin C-deficient guinea-pigs had free access to food. Some of the resaturated animals were given a scorbutogenic diet *ad lib.*; the other group was pair-fed with the deficient guinea-pigs, in which food consumption was markedly reduced (on the 18th day of avitaminosis C about 20 g of food per animal per d; on the 23rd day about 10 g per animal per d; in the last 2 d of the experiment scorbutic guinea-pigs totally refused food). On the 27th day from the beginning of the scorbutogenic regimen the guinea-pigs were killed by decapitation.

### *Analytical methods*

Ascorbic acid in tissues was determined after extraction in 6% (w/v) trichloroacetic acid (Roe & Kuether, 1943). Total cholesterol was determined manually after extraction (Folch, Lees & Stanley, 1957) by the Liebermann – Burchard reaction (Cook, 1958). Samples of blood serum and liver were dissolved in Nuclear Chicago Solubilizer for determination of total $^{14}C$ activity. A known quantity of the KOH solution containing dissolved $^{14}CO_2$ was acidified and $^{14}CO_2$ was absorbed into ethanolamine in an apparatus described by Saba & Di Luzio (1966). Radioactivity of $^{14}C$ samples was assayed in toluene-based scintillator in a Nuclear Chicago liquid-scintillation spectrometer (model Mark I) using an external standard to correct for quenching. The results were statistically evaluated by Student's *t* test, and correlations were determined by the method of least squares.

## RESULTS

Fig. 1 shows the weight changes of the experimental groups. Body-weights of vitamin C-deficient guinea-pigs began to decrease at about the 18th day after the scorbutogenic regimen started. The weight curve of the resaturated pair-fed guinea-pigs (one animal died as a result of an accident) was almost identical with that for the vitamin C-deficient animals. Weights of resaturated guinea-pigs fed *ad lib.* increased a little during the experiment.

Table 1 gives the ascorbic acid concentrations and total cholesterol concentrations in tissues of guinea-pigs of all groups. Ascorbic acid concentrations in tissues of avitaminotic guinea-pigs were very low; resaturation with high doses of ascorbic acid caused a significant increase in the concentrations; the concentrations in the guinea-pigs fed *ad lib.* were significantly higher than those in the pair-fed resaturated group. This phenomenon might have been caused by an increased decomposition of ascorbic acid in the pair-fed group, which was underfed and during the last 2 d of the experiment completely starving. Total cholesterol concentrations in tissues of guinea-pigs from the individual groups did not differ significantly. Cholesterol concentrations in blood serum of the resaturated pair-fed group were significantly increased.

In Table 2 total $^{14}C$ activity and specific activity of blood serum and liver cholesterol are presented. In agreement with results on total cholesterol concentrations (Table 1),

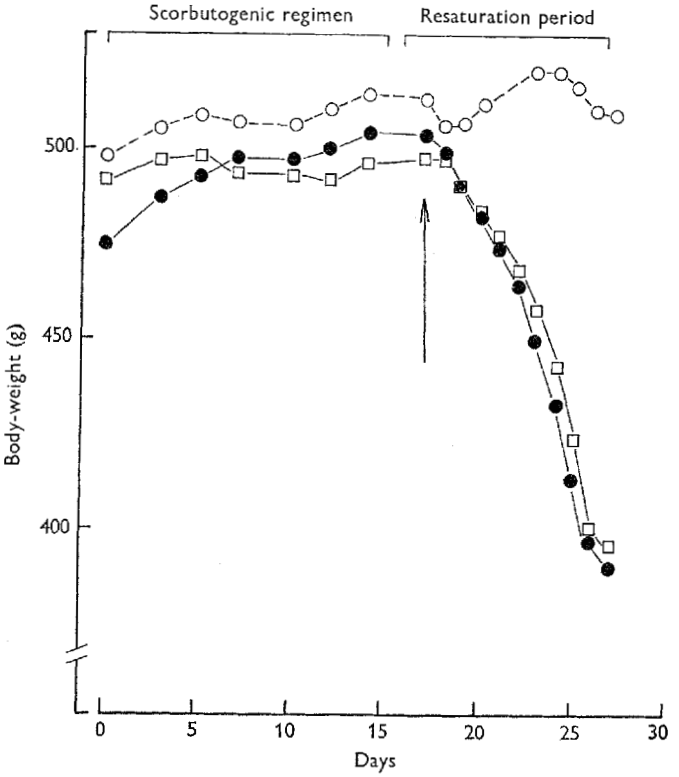


Fig. 1. Weight curves of vitamin C-deficient (●—●, six animals), resaturated pair-fed (□—□, five animals) and resaturated *ad lib.*-fed (○- - -○, six animals) guinea-pigs. The arrow indicates the day of injection of all groups with [26-$^{14}$C]cholesterol.

Table 1. *Mean values with their standard errors for ascorbic acid and cholesterol concentrations in the tissues of vitamin C-deficient and resaturated guinea-pigs*

| Organ | Vitamin C-deficient group (6) A | Vitamin C-resaturated groups: Fed *ad lib.* (6) B | Vitamin C-resaturated groups: Pair-fed (5) C | Statistical significance (value of *P*): A *v.* B | A *v.* C | B *v.* C |
|---|---|---|---|---|---|---|
| | | Ascorbic acid (mg/100 g wet tissue) | | | | |
| Liver | 0·45 ± 0·23 | 11·06 ± 0·49 | 6·72 ± 0·51 | < 0·001 | < 0·001 | < 0·001 |
| Adrenals | 1·37 ± 0·73 | 87·38 ± 7·93 | 45·91 ± 2·25 | < 0·001 | < 0·001 | < 0·002 |
| Small intestine | 1·13 ± 0·26 | 18·95 ± 1·45 | 13·31 ± 1·34 | < 0·001 | < 0·001 | < 0·05 |
| Spleen | 0·99 ± 0·15 | 31·07 ± 0·98 | 23·53 ± 0·76 | < 0·001 | < 0·001 | < 0·001 |
| | | Total cholesterol (mg/100 g or /100 ml) | | | | |
| Liver | 464 ± 61 | 409 ± 35 | 640 ± 133 | NS | NS | NS |
| Adrenals | 4459 ± 598 | 4453 ± 977 | 5279 ± 604 | NS | NS | NS |
| Small intestine | 348 ± 47 | 302 ± 14 | 275 ± 23 | NS | NS | NS |
| Blood serum | 105 ± 13 | 90 ± 9 | 170 ± 14 | NS | < 0·01 | < 0·001 |

Figures in parentheses are the numbers in the groups at the end of the experiment.
NS, not significant ($P > 0·05$).

Table 2. *Mean values with their standard errors for total $^{14}C$ activities and specific activities of cholesterol in the blood serum and liver of vitamin C-deficient and resaturated guinea-pigs*

| Organ | Vitamin C-deficient group (6) A | Vitamin C-resaturated groups: Fed *ad lib.* (6) B | Vitamin C-resaturated groups: Pair-fed (5) C | Statistical significance (value of $P$): A *v.* B | A *v.* C | B *v.* C |
|---|---|---|---|---|---|---|
| | Total activity (disintegrations/min per g wet tissue) | | | | | |
| Blood serum | 8398 ± 1292 | 6380 ± 1145 | 13063 ± 1647 | NS | < 0·05 | < 0·01 |
| Liver | 27175 ± 2076 | 27890 ± 4976 | 46197 ± 5683 | NS | < 0·01 | < 0·05 |
| | Specific activity (disintegrations/min per mg total cholesterol) | | | | | |
| Blood serum | 7784 ± 914 | 7081 ± 871 | 8142 ± 1662 | NS | NS | NS |
| Liver | 6199 ± 633 | 6816 ± 1104 | 7950 ± 1197 | NS | NS | NS |

Figures in parentheses are the numbers in the groups at the end of the experiment.
NS, not significant ($P > 0·05$).

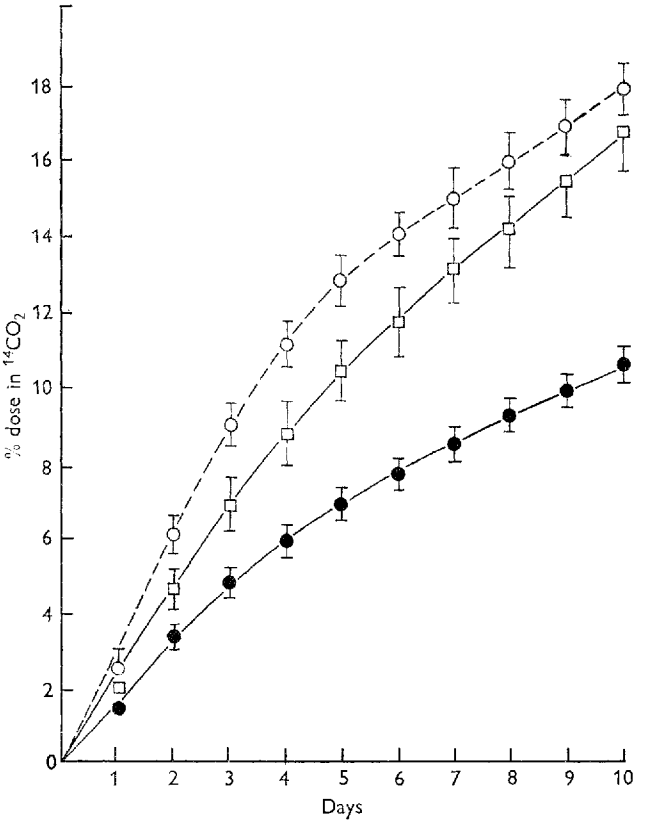


Fig. 2. Oxidation of [26-$^{14}$C]cholesterol to $^{14}CO_2$ as percentage of the dose injected in vitamin C-deficient (●—●, six animals), resaturated pair-fed (□—□, five animals) and resaturated *ad lib.*-fed (○- - -○, six animals) guinea-pigs. The vertical bars represent the standard errors of the mean.

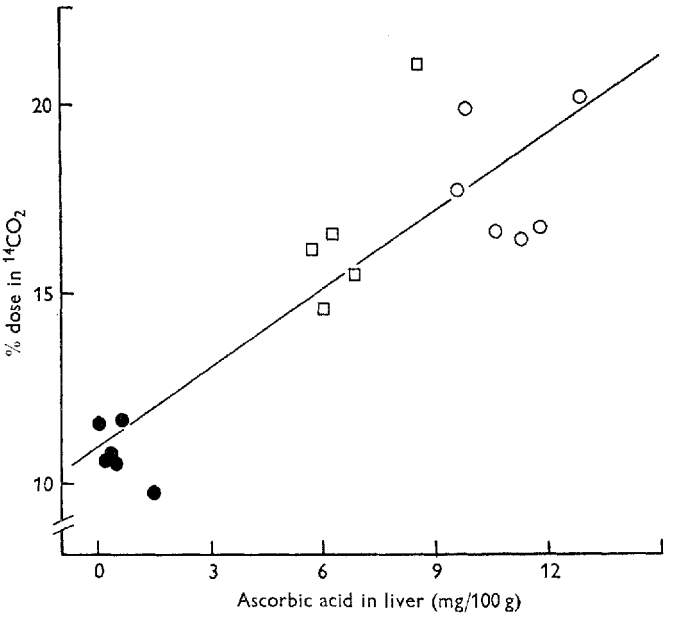


Fig. 3. Linear direct correlation ($P < 0.001$) between ascorbic acid concentration in liver and percentage of [26-$^{14}$C]cholesterol oxidized to $^{14}CO_2$ within 10 d, in vitamin C-deficient (●), resaturated pair-fed (□) and resaturated *ad lib.*-fed (○) guinea-pigs.

the total $^{14}$C activity in blood serum of pair-fed resaturated guinea-pigs was significantly increased. There were no significant differences between resaturated *ad lib.*-fed and vitamin C-deficient animals. We have found that, in guinea-pigs given [26-$^{14}$C]-cholesterol, incorporation of label into digitonin-precipitable sterols is practically identical with the total $^{14}$C activity of blood serum and liver tissue (Bobek, Ginter & Červeň, 1971). This allows calculation of the specific activity of cholesterol from the values for total $^{14}$C activity and cholesterol concentration. The values obtained in such a way were very similar for blood serum and liver and for all three experimental groups.

Fig. 2 shows production of $^{14}CO_2$ cumulatively within 10 d of [26-$^{14}$C]cholesterol administration. The time-course of the curves confirms an accelerated oxidation of labelled cholesterol in both groups resaturated with high doses of ascorbic acid. The difference in the amount of expired $^{14}CO_2$ between the vitamin C-deficient group and the resaturated *ad lib.*-fed group was significant from the 1st day (1st day $P < 0.05$, 2nd day $P < 0.01$, all remaining intervals $P < 0.001$), between the vitamin C-deficient and the resaturated pair-fed group from the 2nd day of the experiment (2nd day $P < 0.05$, 3rd–5th day $P < 0.02$–$0.01$, all remaining intervals $P < 0.001$). [26-$^{14}$C]-cholesterol oxidation in the pair-fed groups slightly decreased when compared with that of the *ad lib.*-fed group; however, this difference was significant only between the 3rd and the 6th day of the experiment ($P < 0.05$).

As most of the cholesterol is probably catabolized in the liver, we have correlated the percentage of [26-$^{14}$C]cholesterol oxidized to $^{14}CO_2$ in 10 d with the ascorbic acid concentration in liver for all three groups. Results are presented in Fig. 3 and they show a very close correlation between these two measurements ($P < 0.001$).

From the values for the specific activities of blood serum or liver cholesterol and from the amount of $^{14}CO_2$ excreted during the 24 h period before the animals were killed, the rate of cholesterol breakdown was calculated according to the method of Myant & Lewis (1966). In the vitamin C-deficient group the mean value was about 8 mg cholesterol per animal per d, in the resaturated *ad lib.*-fed group about 14 mg per animal per d, and in the resaturated pair-fed group about 17 mg per animal per d. These values are only approximate, as isotopic equilibrium was probably not achieved and the results for cholesterol turnover to bile acids obtained by the [26-$^{14}$C]cholesterol method are underestimated (Chevallier & Lutton, 1966).

## DISCUSSION

Oxidation of the side-chain of cholesterol is implicated in the synthesis of bile acids which play the quantitatively most important part in the elimination of cholesterol from the body. Results obtained from this study together with our demonstration of retarded transformation of [4-$^{14}$C]cholesterol to bile acids and decreased [26-$^{14}$C]cholesterol oxidation to $^{14}CO_2$ in guinea-pigs with latent chronic vitamin C deficiency (Ginter *et al.* 1971) suggest that ascorbic acid plays an important part in this catabolic process. In this connexion, it is already known that ascorbic acid stimulates 7-dehydrocholesterol oxidation in vitro (Kandutsch, 1966) and cholesterol transformation to bile acids in liver mitochondria from scorbutic guinea-pigs (Guchhait, Guha & Ganguli, 1963). Shimizu (1970) demonstrated stimulation of the cholesterol side-chain cleavage reaction in bovine and porcine adrenal mitochondrial preparations by low concentrations of ascorbic acid. The hypocholesterolemic effect of ascorbic acid observed by some authors in humans (Myasnikova, 1947; Ginter, Kajaba & Nizner, 1970; Spittle, 1971) is probably connected with the stimulation of cholesterol oxidation described in this work.

When bile acids are formed in the liver by the degradation of the cholesterol side-chain, the three terminal carbon atoms are removed as propionyl-CoA (Suld, Staple & Gurin, 1962), which is quickly oxidized to $CO_2$. It is improbable that labelled intermediates from oxidized [26-$^{14}$C]cholesterol accumulate during the period allowed for partial equilibration and that resaturation by vitamin C increases the excretion of $^{14}CO_2$ merely by causing a 'sweeping out' of labelled pools. In some experimental animals total $^{14}$C activity in tissues was compared with the activity of the digitonin-precipitable fraction. Nearly all the $^{14}$C activity was found in the digitonin-precipitable fraction. It would seem that, under our conditions, there is no significant accumulation of metabolites of the propionyl fragment.

Ascorbic acid is an important cofactor in the hydroxylation of many substances, for example proline, aromatic amino acids, tryptophan and dopamine. It is therefore possible that vitamin C stimulates also the hydroxylation reactions of cholesterol, by means of which cholesterol is transformed to bile acids in the liver. The mechanism of ascorbic acid intervention in cholesterol catabolism remains an open question.

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