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EFFECT OF LARGE DOSES OF ASCORBIC ACID ON THE MIXED FUNCTION OXIDASE SYSTEM IN GUINEA PIG LIVER

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Abstract—Reports of the beneficial effects of large doses of ascorbic acid have stressed its water solubility and non-toxic properties. In this study male guinea pigs, dosed with 150 mg twice daily. ascorbic acid, demonstrated no differences in effect on liver weight, body weight or hepatic total protein when compared with controls. The activities of NADPH-dependent cytochrome *c* reductase, *N*-demethylase (Type I) and *O*-de-ethylase enzymes (Type II) remained unaffected, but the activity of the Type I hydroxylating enzyme, biphenyl-4-hydroxylase, and the amounts of cytochromes P-450 and b_5 were significantly reduced. Total microsomal haem proteins were reduced and mirrored the effects in cytochromes P-450 and b_5. The rate-limiting enzyme in haem synthesis, δ-amino-laevulinic acid synthetase, rose in the ascorbic acid group and this was associated with a fall in activity of the haem degrading enzyme, microsomal haem oxygenase. Thus, large amounts of ascorbic acid have similar effects to those found in scorbutic animals and appear to interfere with the construction of the cytochrome P-450 molecule.

Reports of the beneficial effects of large doses of ascorbic acid have stressed its water solubility and consequent non-toxic properties, and have resulted in doses of several grams of the vitamin being taken on a regular basis. The effects of ascorbic acid deficiency on hepatic drug metabolism have been investigated by a number of workers [1–4], but few investigations have been made of the effects of administering large doses of the vitamin.

Sulphate transfer is important in the biotransformation of phenolic compounds [5] and in the metabolism of ascorbic acid to ascorbate-2-sulphate [6]. This pathway is dependent on the availability of sulphate and is of limited capacity in man. It is therefore subject to competitive inhibition and indeed, Houston and Levy [7] provided evidence of competitive inhibition when 2 g ascorbic acid and 150 mg salicylamide were administered concomitantly to healthy volunteers. Due to this competition, the fraction of the dose of salicylamide excreted as the sulphate was significantly reduced whilst that excreted as the glucuronide was increased. This study suggests that large doses of ascorbic acid could affect the metabolism of drugs by virtue of effects on hepatic microsomal enzymes.

The present study was undertaken to investigate the effect of large doses of ascorbic acid on the hepatic drug-metabolism enzyme system in guinea pigs. It would seem to be of paramount importance to evaluate these effects for long term treatment with drugs may well coincide with taking large doses of the vitamin.

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MATERIALS AND METHODS

Male Dunkin–Hartley guinea pigs (250–300 g), maintained on stock diet (FDI pellets, Labsure Ltd., Poole, U.K.) plus water *ad lib.*, were divided into 2 groups paired according to body weight, following a period of equilibration in metabolic cages (Forth–Tech Services Ltd., Dalkeith, U.K.). One group received 150 mg ascorbic acid twice daily in 1 ml of buffered 20% sucrose, orally, for four successive days. The second group received an equal volume of sucrose solution, and served as controls. The animals were pair-fed and killed by cervical fracture on the fifth day. The livers were quickly removed, chilled, weighed and homogenised in ice-cold KCl (1.15%) in Tris buffer (20 mM) at pH 7.6, using a glass Potter–Elvehjem homogeniser with a loose fitting Teflon pestle, to produce a 25% homogenate.

Homogenates were spun at 10,000 *g* for 20 min at 4°. Aliquots of the supernatant were centrifuged at 105,000 *g* for 1 hr and the microsomal pellet retained and resuspended in the original volume of buffer.

Washed microsomes were produced by a second 105,000 *g* centrifugation of the resuspended pellet. When storage of the microsomes was required, the pellet was resuspended at a concentration of 1 g original tissue per ml, and frozen at −40°. Biphenyl-4-hydroxylase was measured by the method of Creaven *et al.* [8]. The cytochrome P-450 content of microsomes was determined from the carbon monoxide difference spectra of dithionate reduced samples using an extinction coefficient between 450 and 490 nm of 91 $cm^{-1}\,mM^{-1}$ [9]. The cytochrome b_5 content of microsomes was calculated from the difference spectrum between NADH-reduced and oxidised samples using an SP1800 spec-

Table 1. Effect of oral administration of ascorbic acid (300 mg/day for four successive days) on the activity of the hepatic drug metabolising enzyme system in the guinea pig

	No. of animals	Control	Test	Significance
Cytochrome P-450 (nmole)	16	0.88 ± 0.07	0.67 ± 0.006	$P < 0.001$
Cytochrome *b* (nmole)	16	0.54 ± 0.05	0.40 ± 0.03	$P < 0.001$
NADPH cytochrome *c* reductase (nmole/min)	18	87.84 ± 5.91	82.81 ± 3.4	N.S.
Biphenyl-4-hydroxylase (nmole/min)	10	12.3 ± 1.8	5.3 ± 1.7	$P < 0.001$
Ethylmorphine-*N*-demethylase (nmole/min)	15	2.63 ± 0.32	2.72 ± 0.21	N.S.
Ethoxyresorufin-*O*-de-ethylase (pmole/min)	12	6.66 ± 0.93	6.56 ± 0.29	N.S.

Values for all compounds expressed per mg microsomal protein.
Each value is the mean of number of animals shown ± S.E.M.
Significance was measured by a paired Student's *t*-test.

trophotometer [10]. An extinction coefficient increment between 424 and 409 nm of 185 cm^{-1} mM^{-1} was used to calculate the concentration of the haemoprotein.

Cytochrome *c* reductase activity was measured by monitoring the rate of reduction of cytochrome *c* at ambient temperature at 550 nm [11]. Protein content was estimated by the method of Lowry *et al.* [12]. Ethylmorphine-*N*-demethylase was measured in terms of rate of formaldehyde formation [13]. Ethoxyresorufin-*O*-de-ethylase was measured by the method of Burke and Mayer [14]. The total phospholipid content of the liver and microsomes was determined using a modification of the method of Naito [15]. Individual phospholipids were separated by thin layer chromatography [16] and quantitated in terms of phosphate content. Glucose-6-phosphatase was measured in the total liver homogenate [17]. Glucose-6-phosphate dehydrogenase activity was determined in the 10,000 *g* fraction [18]. Total haem was determined by formation of the pyridine haemochromogen [19]. Lipid peroxidation was measured by the method of Tappel and Zalkin [20]. Haem oxygenase was assayed in the 10,000 *g* fraction using the method of Tenhunen *et al.* [21]. δ-Amino-laevulinic acid synthetase was assayed in a 5% total homogenate employing [2,3-^{14}C]succinate (50 mCi/mmole) as substrate and 5-amino [4-^{14}C]laevulinic acid hydrochloride (42 mCi/mmole) as standard by an adaptation [22] of the method of Ebert [23]. In view of criticisms of the original Ebert technique, appropriate steps were taken to ensure that the substance measured was indeed δ-amino-laevulinic acid.

RESULTS

Oral administration of ascorbic acid (300 mg/day) to guinea pigs for four successive days had no apparent effect on liver weight, body weight, and the hepatic content of total protein. Table 1 shows that while the activities of the NADPH-dependent cytochrome *c* reductase, the *N*-demethylase (Type I), and *O*-de-ethylase enzymes (Type II) remained unaffected in these animals, the Type I hydroxylating enzyme, biphenyl-4-hydroxylase, was reduced in activity by 50%, compared with that of the control

Table 2. Effect of oral administration of ascorbic acid (300 mg/day) on components of the haem system in the guinea pig

	No.	Control	Test	Significance
Total hepatic haem (nmole)	6	0.85 ± 0.12	0.80 ± 0.09	N.S.
Microsomal haem (nmole)	10	1.13 ± 0.08	0.62 ± 0.07	$P < 0.001$
Microsomal haem oxygenase (pmole)	8	64.9 ± 10.3	32.7 ± 6.9	$P < 0.01$
δ-Amino-laevulinic acid synthetase (pmole)	8	1.26 ± 0.12	2.70 ± 0.43	$P < 0.01$

Values expressed per mg of microsomal or total protein, as appropriate.
Each value is the mean of number of animals shown ± S.E.M.
Significance was measured by a paired Student's *t*-test.

Table 3. Effect of oral administration of ascorbic acid (300 mg/day) on total and microsomal phospholipids of guinea pig liver

	No.	Control	Test	Significance
Total phospholipids	12	13.90 ± 1.92	13.16 ± 1.18	N.S.
Microsomal phospholipids	12	22.52 ± 2.18	21.10 ± 1.60	N.S.
Microsomal phosphatidyl choline	20	12.36 ± 1.08	9.27 ± 0.65	$P < 0.01$
Microsomal phosphatidyl ethanolamine	20	8.30 ± 0.79	6.76 ± 0.48	$P < 0.05$

All values are expressed as μg phosphate/mg protein.
Each value is the mean of number of animals shown ± S.E.M.
Significance was measured by a paired Student's *t*-test.

animals. Cytochromes P-450 and b_5 tended to be similar to biphenyl-4-hydroxylase in that ascorbic acid administration resulted in a significantly decreased hepatic concentration.

Microsomal haem concentrations (Table 2) mirrored the effects seen in the cytochromes, for there was a decrease in concentration of 43% in the group of animals receiving ascorbic acid. However, the concentration of total hepatic haem remained unaltered, and this suggests that there was an increase in non-microsomal haem. The rate limiting enzyme in haem synthesis, δ-amino-laevulinic acid synthetase (δ-ALA), showed a two-fold increase in activity in the ascorbic acid group and this was associated with a 45% fall in activity of the haem degradating enzyme, microsomal haem oxygenase.

Lipid peroxidation, another pathway for haem catabolism, was investigated and found to be unaffected by ascorbic acid. In addition there was no significant difference in the activities of the two marker enzymes, glucose-6-phosphate dehydrogenase, and glucose-6-phosphatase, suggesting that hepatic damage had not been incurred. Phospholipid, an essential component in the hepatic drug metabolising system, also remained unaltered in concentration when measured in both total liver homogenates and in the microsomal suspension (Table 3). However, when the individual phospholipids were isolated by thin layer chromatography, both phosphatidylcholine and its precursor, phosphatidyl ethanolamine, were significantly decreased.

DISCUSSION

In scorbutic guinea pigs, the hepatic concentration of cytochromes P-450 and b_5 and activities of various drug-metabolising enzymes are decreased [1, 24–26]. In the present study large oral doses of ascorbic acid have been found to have similar effects. Hence, it appears that there may be a biphasic effect of ascorbic acid on drug metabolising enzymes with an inhibitory effect at both high and low concentrations.

However, Sato and Zannoni [27] described a quantitative relationship of 2 μmole of ascorbic acid to 1 μmole cytochrome P-450 in the drug metabolising tissues of scorbutic animals. From our observations it would seem that such a relationship no longer exists in animals given 300 mg/day, or the equivalent of 5 g ascorbic acid per day for man [28].

Neither ascorbic acid deficiency [27] nor excess, as in the present study, affected the concentration of total phospholipid in the liver, but high doses did reduce the concentration of phosphatidyl choline, important in drug metabolism as an electron transport component, and its precursor, phosphatidyl ethanolamine. There was no effect of the high doses on lipid peroxidation. The fact that the marker enzymes, glucose-6-phosphate dehydrogenase and glucose-6-phosphatase remained unaffected as also did the histological appearance suggests that the liver was not damaged.

Ascorbic acid treatment caused a two-fold increase in δ-ALA synthetase and a 45% decrease in activity of microsomal haem oxygenase activity. This suggests an attempt by the liver to conserve haem and hence the vitamin seems to be exerting its effect either directly or indirectly following the formation of α-ALA but before the production of a complete cytochrome P-450 molecule. A similar decrease in microsomal haem oxygenase activity also occurs in ascorbic acid deficient guinea pigs [29]. Thus both ascorbic acid excess and deficiency appear to interfere with the construction of the cytochrome P-450 molecule from its component parts, possibly at the level of iron incorporation or via effects on the apoprotein synthesis or its incorporation with haem.

It is of interest that ascorbic acid deficiency has in fact been reported to affect the formation of the apoprotein moiety, unlike the work presented here, and not to affect haem synthesis [2].

With regard to a possible effect via iron incorporation, this could be at the level of incorporation of iron by ferrochelatase. Sato and Zannoni [27] presented evidence for an association of ascorbic acid with the ferrous iron of cytochrome P-450 on the basis of studies with metal chelators with a high affinity for ferrous iron, in which a protective action of the vitamin on the chelating binding sites was suggested.

Omaye and Turnbull [4] proposed that ascorbic acid can indirectly affect the availability of ferrous iron for incorporation into the haemoproteins. Following pharmacological doses of the vitamin (25 mg/100 g body weight/day), a two- to three-fold decrease in serum copper and caeruloplasmin and in liver copper was seen [29]. In ascorbic acid deficient animals, a corresponding increase in these parameters was seen and, in the dose range of ascorbic

acid used, serum and hepatic iron, haemoproteins, haemoglobin and haematocrit increased with increasing intake of ascorbic acid. Similar trends were also seen in monkeys receiving marginal intakes of copper and two different levels of the vitamin, suggesting that an inter-relationship between ascorbic acid, copper and iron may affect the availability of ferrous iron for incorporation into the haemoproteins. It would be of interest to discover whether at very high intakes of the vitamin, as with deficiency, ascorbic acid causes an increase in caeruloplasmin concentrations, like that which occurs with deficiency, and a shift in the equilibrium of the hepatic iron pool from the ferrous to the ferric form, hence rendering Fe^{2+} less available.

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