Effect of Graded Doses of Erythorbic Acid on Activities of Drug Metabolic Enzyme and Phosphatases in Guinea Pigs

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Summary The enzyme activities which depended on the ascorbic acid (AsA) tissue levels were assayed to investigate the effect of erythorbic acid (ErA) administration on the AsA availability in the guinea pigs administered 5 mg of AsA/day or 1 mg of AsA/day. The guinea pigs were given 5 mg of AsA and 1, 5, 20, 100 mg of ErA/day, or 1 mg of AsA and 1 or 20 mg of ErA/day for 16 days. The animals were sacrificed, blood was collected, and their livers were removed. The activities of liver aniline hydroxylase, liver acid phosphatase, and serum alkaline phosphatase, as well as the liver cytochrome P-450 content were measured. These enzyme activities and the liver cytochrome P-450 content of animals administered 5 mg of AsA seemed to show no change regardless of ErA supplement. Animals administered 1 mg of AsA showed different activities of liver aniline hydroxylase and liver acid phosphatase compared with those of animals administered 5 mg of AsA; however, the enzyme activities in animals administered 20 mg of ErA together with 1 mg of AsA were similar to those of the animals administered only 5 mg of AsA. These results indicated that ErA administration had no effect on the enzyme activities and the liver cytochrome P-450 content in the 5 mg AsA-supplemented animals, but administration of 20 mg of ErA was effective to maintain at normal levels the activities of liver aniline hydroxylase and liver acid phosphatase in the 1 mg AsA-supplemented animals.

Key Words erythorbic acid, ascorbic acid, guinea pig, liver aniline hydroxylase, liver acid phosphatase, serum alkaline phosphatase, liver cytochrome P-450
In our previous papers (1, 2), we reported that administration of a large amount of erythorbic acid (ErA) reduced the ascorbic acid (AsA) content of the tissues in guinea pigs administered AsA, but the ErA-supplemented animals showed no decrease in body weight. It was not clear whether the decrease in AsA tissue content due to ErA administration had any influence on the enzyme activities which depended on the AsA dosage.

It is known that the activities of aniline hydroxylase, serum alkaline phosphatase, and of acid phosphatase, and the liver cytochrome P-450 content are influenced by the vitamin C tissue level. For instance, in the case of guinea pigs, the activity of the liver mixed-function oxygenase (3, 4) and the liver cytochrome P-450 content (5, 6) were reported to be dependent on the AsA tissue content, which was dependent on the AsA dosage in guinea pigs. The activity of mixed-function oxygenase and the cytochrome P-450 content of the vitamin C-deficient animals were apparently lower than those of the guinea pigs administered a sufficient amount of AsA (3–6), and the hydroxylations of aniline, hexabarbital, and zoxazolamine were markedly reduced (7). Furthermore, the activity of serum alkaline phosphatase showed the same decreasing tendency as that of the mixed-function oxygenase. In the case of AsA-deficient guinea pigs, this enzyme activity decreased though they did not show apparent scurvy symptom and their body weights continued to rise (8). On the other hand, the activities of various lysosomal enzymes, β-N-acetylhexosaminidase, β-D-glucuronidase, α-D-galactosidase, α-D-mannosidase, and acid phosphatase increased in the scurvy guinea pigs (9), but the biochemical role of AsA in these enzyme reactions is still uncertain.

Therefore, we assayed the liver cytochrome P-450 content, the activities of liver aniline hydroxylase, liver acid phosphatase, and serum alkaline phosphatase in order to study the effect of ErA administration on the AsA availability in the guinea pigs administered adequate or marginal levels of AsA.

**MATERIALS AND METHODS**

*Animals and diet.* Male Hartley guinea pigs with initial body weight of about 220 g were fed *ad libitum* the AsA-deficient diet described in the previous paper (1). The animals were housed individually in wire cages and their body weights were measured daily. All animals were randomly divided into eight groups according to the following experimental plan.

*Experimental plan.* Animals were divided into eight groups. The adequate-AsA-supplemented groups were given 5 mg of AsA and the marginal-AsA-supplemented groups were given 1 mg of AsA.

Group A was orally supplemented with 5 mg of AsA/day, group B with 5 mg of AsA and 1 mg of ErA/day, group C with 5 mg of AsA and 5 mg of ErA/day, group D with 5 mg of AsA and 20 mg of ErA/day, group E with 5 mg of AsA and 100 mg of ErA/day, group F with 1 mg of AsA/day, group G with 1 mg of AsA and 1 mg of ErA/day, and group H with 1 mg of AsA and 20 mg of ErA/day. AsA and ErA were...
dissolved in water immediately before use. The experimental feeding period was 16 days. At the end of this period, the animals were sacrificed after 24-h fasting and blood was collected from aorta abdominalis and the liver was removed.

**Assay of the enzymes**

**Preparation of crude enzyme solution.** The liver was homogenized with 4 volumes of 1.15% KCl solution. A part of the resulting crude liver homogenate was centrifuged at 9,000 × g for 30 min, and the supernatant fraction was used for the assay of aniline hydroxylase.

Three volumes of 1.15% KCl solution containing Triton X-100 were added to the remaining liver homogenate and rehomogenized. The resulting homogenate was used as crude enzyme solution for the assay of acid phosphatase.

**Assay of aniline hydroxylase.** The assay of aniline hydroxylase was carried out aerobically by passing oxygen gas into the reaction mixture at 37°C for 30 min. The reaction mixture in final volume of 2.5 ml contained 10 mM aniline, 1 mM NADPH, 5 mM MgCl₂, 0.1 M Tris-HCl buffer (pH 7.4), and 1 ml of crude enzyme solution. Aniline hydroxylase activity was determined according to the method described by Imai *et al.* (10) measuring the amount of p-aminophenol produced.

**Assay of acid phosphatase.** The reaction mixture containing 0.5 ml of liver homogenate treated with Triton X-100, 12.5 mM p-nitrophenylphosphate, and 50 mM acetate buffer (pH 5.0) in a final volume of 1 ml, was incubated at 37°C for 5 min. Acid phosphatase activity was determined by the method of Desai (11).

**Assay of serum alkaline phosphatase.** The serum alkaline phosphatase activity was determined by using Alkaline Phospha B-Test (Wako Pure Chemical Industries, Osaka, Japan) with p-nitrophenylphosphate as substrate (12).

**Preparation of microsomes.** The liver was homogenized with 4 volumes of 1.15% KCl solution. After this homogenate was centrifuged at 9,000 × g for 30 min, the supernatant fraction was centrifuged at 105,000 × g for 60 min. The microsomes were washed once by resuspension in 1.15% KCl solution and again centrifuged at 105,000 × g for 30 min. The washed microsomes were finally suspended in 1/15 M phosphate buffer (pH 7.2). All procedures were done at 0–4°C.

**Determination of liver cytochrome P-450.** The liver cytochrome P-450 content in microsomes was determined by the method of Omura and Sato (13), measuring the carbon monoxide difference spectra of dithionite-reduced microsomes.

**Determination of protein.** Protein was measured by the method of Lowry (14) using bovine serum albumin as a standard.

**Statistical tests.** The significant difference between the mean of two groups was statistically analyzed by Student's *t*-test for equal variance or the Cochran-Cox test for different variance.

**RESULTS**

Table 1 shows the liver aniline hydroxylase activity of the guinea pigs administered various levels of ErA at 5 mg AsA administration. The activity of
Table 1. Effect of graded doses of ErA on liver aniline hydroxylase activity of guinea pigs administered an adequate amount of AsA.

| Group  | Activity (nmol p-aminophenol formed/min·mg prot) |
|--------|-----------------------------------------------|
| A      | 0.240 ± 0.014*                                |
| B      | 0.236 ± 0.014                                 |
| C      | 0.243 ± 0.034                                 |
| D      | 0.247 ± 0.025                                 |
| E      | 0.219 ± 0.025                                 |

A, 5 mg AsA-supplemented group; B, 5 mg AsA + 1 mg ErA-supplemented group; C, 5 mg AsA + 5 mg ErA-supplemented group; D, 5 mg AsA + 20 mg ErA-supplemented group; E, 5 mg AsA + 100 mg ErA-supplemented group. *Values are mean ± SE, n=6–8 (except A, n=13).

Group A was not significantly different from that of groups B, C, D, and E, suggesting that ErA administration had no effect on the aniline hydroxylase activity at an adequate AsA dosage.

Tables 2, 3, 4 show the liver cytochrome P-450 content, the liver acid phosphatase activity, and the serum alkaline phosphatase activity, respectively, of the guinea pigs administered various levels of ErA at an adequate amount of AsA. The content of liver cytochrome P-450 of group A was similar to that of groups B, C, D, and E, indicating that the liver cytochrome P-450 content did not change with increasing amounts of ErA administered. Also, the activities of liver acid phosphatase and serum alkaline phosphatase showed the same tendency as that of liver aniline hydroxylase. The activities were not significantly different among these five groups (groups A, B, C, D, and E).

Table 5 shows the activity of liver aniline hydroxylase of the guinea pigs administered various levels of ErA at 1 mg AsA dosage. The activity of liver aniline hydroxylase of group H was higher than those of groups F and G, and increased to the level similar to those of the animals administered 5 mg AsA. The increase of liver aniline hydroxylase activity seemed to be due to the administration of 20 mg ErA at a marginal AsA dosage.

Tables 6, 7, 8 show the liver cytochrome P-450 content, the activity of liver acid phosphatase, and the activity of serum alkaline phosphatase, respectively. The liver cytochrome P-450 content of group F was higher than that of group H, which was almost the same as that of group A, but the difference was not significant. In group G the content of liver cytochrome P-450 was not significantly different from groups F and H.

The activity of liver acid phosphatase of group H was similar to that of group A, and that of group H was lower than those of groups F and G. This enzyme activity seemed to decrease with increasing ErA dosage at a marginal amount of AsA administered to the guinea pigs.
Table 2. Effect of graded doses of ErA on liver cytochrome P-450 content of guinea pigs administered an adequate amount of AsA.

| Group | Content (nmol/mg prot) |
|-------|------------------------|
| A     | 0.86 ± 0.04*           |
| B     | 0.91 ± 0.05            |
| C     | 0.81 ± 0.05            |
| D     | 0.80 ± 0.10            |
| E     | 0.87 ± 0.10            |

A–E, see Table 1 legend. * Values are mean ± SE, n=7–9 (except A, n=13).

Table 3. Effect of graded doses of ErA on liver acid phosphatase activity of guinea pigs administered an adequate amount of AsA.

| Group | Activity (nmol p-nitrophenol liberated/min·mg prot) |
|-------|---------------------------------------------------|
| A     | 42.1 ± 2.3*                                        |
| B     | 43.2 ± 1.7                                         |
| C     | 50.9 ± 4.3                                         |
| D     | 48.7 ± 3.8                                         |
| E     | 44.6 ± 1.4                                         |

A–E, see Table 1 legend. * Values are mean ± SE, n=6–8 (except A, n=13).

Table 4. Effect of graded doses of ErA on serum alkaline phosphatase activity of guinea pigs administered an adequate amount of AsA.

| Group | Activity (mmol p-nitrophenol liberated/h·liter) |
|-------|-------------------------------------------------|
| A     | 5.70 ± 0.49*                                    |
| B     | 5.45 ± 0.72                                      |
| C     | 5.56 ± 0.67                                      |
| D     | 7.45 ± 1.15                                      |
| E     | 5.04 ± 0.56                                      |

A–E, see Table 1 legend. * Values are mean ± SE, n=5–8 (except A, n=11).

The serum alkaline phosphatase activity of group F was similar to that of group A and was not significantly different with groups G and H, in spite of administration of AsA together with ErA.

Table 9 shows the final body weight gains of the experimental groups. The guinea pigs administered both an adequate and a marginal amount of AsA...
Table 5. Effect of graded doses of ErA on liver aniline hydroxylase activity of guinea pigs administered a marginal amount of AsA.

| Group | Activity (nmol p-aminophenol formed/min•mg prot) |
|-------|--------------------------------------------------|
| F     | $0.197 \pm 0.019^*$                              |
| G     | $0.170 \pm 0.019^a$                              |
| H     | $0.252 \pm 0.021^a$                              |

F, 1 mg AsA-supplemented group; G, 1 mg AsA + 1 mg ErA-supplemented group; H, 1 mg AsA + 20 mg ErA-supplemented group. *Values are mean ± SE, n=9–11. Means with a common superscript letter are significantly different. $^a p<0.05$.

Table 6. Effect of graded doses of ErA on cytochrome P-450 content of guinea pigs administered a marginal amount of AsA.

| Group | Content (nmol/mg prot) |
|-------|------------------------|
| F     | $1.02 \pm 0.05^*$      |
| G     | $0.99 \pm 0.11$        |
| H     | $0.88 \pm 0.06$        |

F–H, see Table 5 legend. *Values are mean ± SE, n=9–13.

Table 7. Effect of graded doses of ErA on liver acid phosphatase activity of guinea pigs administered a marginal amount of AsA.

| Group | Activity (nmol p-nitrophenol liberated/min•mg prot) |
|-------|----------------------------------------------------|
| F     | $55.7 \pm 2.5^a$                                   |
| G     | $48.1 \pm 3.7$                                     |
| H     | $43.5 \pm 2.9^a$                                   |

F–H, see Table 5 legend. *Values are mean ± SE, n=9–13. Means with a common superscript letter are significantly different. $^a p<0.05$.

continued to show a gain in body weight. The body weight gain of group F was lowest among these experimental groups.

DISCUSSION

In the guinea pigs administered an adequate amount of AsA, i.e. 5 mg AsA, the administration of ErA did not alter the activities of liver aniline hydroxylase, liver acid phosphatase, and of serum alkaline phosphatase, and the content of liver cytochrome P-450, which apparently changed in the scorbutic guinea pigs (3–9).
Table 8. Effect of graded doses of ErA on serum alkaline phosphatase activity of guinea pigs administered a marginal amount of AsA.

| Group | Activity (mmol p-nitrophenol liberated/h·liter) |
|-------|-----------------------------------------------|
| F     | 6.02 ± 0.42*                                  |
| G     | 7.07 ± 1.17                                   |
| H     | 6.19 ± 0.48                                   |

F–H, see Table 5 legend. * Values are mean ± SE, n=8–13.

Table 9. Final body weight gains of guinea pigs supplemented with AsA or with AsA and ErA.

| Group | Relative body weight gain |
|-------|---------------------------|
| A     | 123 ± 4*                  |
| B     | 131 ± 3                   |
| C     | 131 ± 3                   |
| D     | 127 ± 4                   |
| E     | 126 ± 3                   |
| F     | 118 ± 3*                  |
| G     | 129 ± 4*                  |
| H     | 123 ± 5                   |

A–E, see Table 1 legend; F–H, see Table 5 legend. * Values are mean ± SE, n=7–18. Means with a common superscript letter are significantly different. *p < 0.05.

Moreover, the body weight gains in these five groups supplemented with an adequate amount of AsA (groups A, B, C, D, and E) were similar (Table 9). But, in the animals administered both 5 mg of AsA and 100 mg of ErA, the content of AsA in the liver was about 50% lower than that of the animals administered only 5 mg of AsA (1, 2), although, the ErA administration did not affect both the enzyme activities and cytochrome P-450 content (Tables 1–4). Thus, the animals remained healthy.

In the guinea pigs administered a marginal amount of AsA (1 mg of AsA), the liver acid phosphatase activity was significantly higher than that of the animals administered 5 mg of AsA, and the AsA tissue content was much lower than that of 5 mg AsA-supplemented animals described in our previous paper (2). Also, the body weight gain of the animals administered 1 mg of AsA (group F) was lower than that of the animals administered 5 mg of AsA (group A). Furthermore, the liver aniline hydroxylase activity of group F was lower than that of group A. However, the activity of serum alkaline phosphatase of group F was similar to that of group A. Our observations indicated that the administration of 1 mg AsA might be insufficient to maintain the activities of acid phosphatase and aniline hydroxylase at
normal levels. On the contrary, the addition of 20 mg ErA to a marginal dosage of AsA maintained these enzyme activities at a normal level; thus, ErA was effective in the maintenance of enzyme activities, and ErA administration did not seem to reduce the AsA utilization in the guinea pigs.

The liver cytochrome P-450 content of group F showed a higher tendency than that of group H but their values were not significantly different.

In the guinea pigs administered an adequate amount of AsA, ErA administration had no effect on the enzyme activities described above; moreover, in the animals administered a marginal amount of AsA, ErA was effective to maintain the enzyme activities and the body weight gain at a normal level. These observations described above seemed to indicate that ErA administration did not reduce the availability of AsA in the guinea pigs administered AsA; furthermore, ErA administration was beneficial in terms of the enzyme activities, and this suggested that ErA might have vitamin C activity which may not be the same as that of AsA. In the previous papers (1, 2), we observed that the content of AsA in the tissues of the AsA-supplemented guinea pigs was higher than that of AsA in the both AsA plus ErA-supplemented animals. Our observations suggested that ErA may not reduce the availability of AsA in the guinea pigs which were simultaneously supplemented with AsA and ErA, although a large amount of ErA supplementation decreased the AsA tissue content as also reported by Hornig and Weiser (15), and ErA may act the same as AsA on the activity of enzymes that depended on AsA level of the tissues.

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