THE ANTISCORBUTIC ACTIVITY OF L-ASCORBIC ACID PHOSPHATE GIVEN ORALLY AND PERCU- TANEOUSLY IN GUINEA PIGS*

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Cutolo and Larraza (1) synthesized 3-phosphoryl-L-ascorbic acid, and they noted that some amount of the compound was excreted as ascorbic acid (AsA) in the guinea pig urine after oral or intraperitoneal administration, however, its antiscorbutic activity is not yet estimated.

The authors studied the antiscorbutic activity of the compound as well as its percutaneous absorption in guinea pigs.

MATERIALS

L-AsA phosphate, synthesized by the method of Cutolo and Larraza, was supplied as Mg salt by Messrs. H. Nomura and K. Sugimoto of this laboratory. This compound was a mixture of monophosphoryl esters of the endiol group of L-AsA, though the mixing ratio was not determined, and did not consume any iodine in the aqueous state at 25°C within 24 hours. The cream base of O/W type was composed of cetyl alcohol 3%, hydrogenated lanoline 4%, vegetable oil such as olive oil 3%, isopropyl myristate 6%, polyethylene glycol 6%, non-ionic surface-active agents such as polyoxyethylene stearate and glycerol monostearate 16% and preservatives. L-AsA or L-AsA phosphate-Mg were mixed in the cream base after dissolved in water, separately. All these creams; namely, cream base, VC-cream (1.4% L-AsA) and VC-P-cream (3% L-AsA phosphate-Mg), were supplied by Mr. H. Takashima and Miss M. Tabata of this laboratory.

METHODS

Male guinea pigs weighing about 250–300 g were used for the evaluation of the antiscorbutic activity. They were housed individually in clean metal cages and fed on a stock diet, Oriental GC-5 ***, or a scorbutogenic diet and water ad libitum. The scorbutogenic

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*** Oriental Kōbo Kōgyō Co., Ltd., Tokyo.
diet and criteria of scurvy were described previously (2). That is, as briefly described, guinea pigs were divided into groups after 10 day-feeding of the scorbutogenic diet, consisted of bran 45 g, crushed oats 25 g, skim milk 30 g and vitamin A palmitate 2,000 i.u. in 100 g and mixed with 1/4 volume of water before use. Then, the experimental animals were orally or percutaneously given the samples for following 10 days. In the study of percutaneous administration, the cream was applied on the clipped skin (about 2.5×3 cm) of the back just posterior to the neck. The body weight gain, subcutaneous hemorrhage and plasma alkaline phosphatase activity were used as criteria after 21 days from the start. The plasma alkaline phosphatase activity was measured according to a modification of the method of King and King (3) by means of "Technicon Auto Analyzer"*. The methods of Fujita (4) and of Ohnacker (5) were used for the chemical and histochemical determination of tissue AsA, respectively.

RESULTS

1. Antiscorbutic activity of oral L-AsA phosphate-Mg

The antiscorbutic activity of L-AsA phosphate-Mg was compared with L-AsA as shown in Table 1. In spite of the positive weight gain, the administration of 0.5 mg/animal/day of L-AsA still did not prevent the development of scurvy as sufficiently as the dose of 1 mg/animal/day. L-AsA phosphate-Mg was also shown to have a considerable effect to prevent the scorbutic syndrome at the dosage of 2.1 mg/animal/day (equimolar to 1 mg of L-AsA). All these criteria, i.e. body weight gain, prevention of hemorrhage and recovery of plasma alkaline phosphatase activity, were not fully coincide with each other, but there was a close relationship among them. Therefore, the antiscorbutic activity of L-AsA

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of guinea pigs</th>
<th>Initial body weight (g)</th>
<th>Body weight gain (g)</th>
<th>Subcutaneous hemorrhage</th>
<th>Plasma alkaline phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal*1</td>
<td>21</td>
<td>273</td>
<td>70±4*2</td>
<td>0/21</td>
<td>22.1±1.4*3</td>
</tr>
<tr>
<td>Deficient</td>
<td>22</td>
<td>288</td>
<td>-72±7</td>
<td>21/22</td>
<td>3.5±0.6</td>
</tr>
<tr>
<td><strong>L-AsA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 mg/day</td>
<td>9</td>
<td>274</td>
<td>23±14</td>
<td>3/9</td>
<td>17.1±2.3</td>
</tr>
<tr>
<td>1.0</td>
<td>13</td>
<td>278</td>
<td>35±5</td>
<td>1/13</td>
<td>17.1±1.9</td>
</tr>
<tr>
<td>2.5</td>
<td>13</td>
<td>292</td>
<td>33±5</td>
<td>0/13</td>
<td>18.8±1.8</td>
</tr>
<tr>
<td>5.0</td>
<td>15</td>
<td>276</td>
<td>34±7</td>
<td>0/15</td>
<td>22.8±2.6</td>
</tr>
<tr>
<td><strong>L-AsA phosphate-Mg</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.05 mg/day*3</td>
<td>9</td>
<td>314</td>
<td>-8±13</td>
<td>3/9</td>
<td>10.4±0.5</td>
</tr>
<tr>
<td>2.1</td>
<td>7</td>
<td>302</td>
<td>9±9</td>
<td>1/7</td>
<td>15.7±1.1</td>
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<tr>
<td>5.25</td>
<td>4</td>
<td>308</td>
<td>39±6</td>
<td>0/4</td>
<td>20.1±1.2</td>
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<tr>
<td>10.5</td>
<td>4</td>
<td>284</td>
<td>21±7</td>
<td>0/4</td>
<td>23.0±2.5</td>
</tr>
</tbody>
</table>

* Oriental GC-5, *2 During the 11-21st day, *3 King-Armstrong unit, *4 Equimole to 0.5 mg of L-AsA. Mean±standard error.

* Technicon Instruments Corp., Chauncey, New York, U.S.A.
phosphate-Mg might be concluded to be about the same or a little weaker than L-AsA.

2. Antiscorbutic activity of percutaneous L-AsA phosphate-Mg (VC-P-cream)

In this experiment, the liver AsA content was measured in addition to the criteria mentioned above. Each one-third of the daily dose of the cream was applied at 8 A.M., and 0 and 4 P.M. After application of the cream the guinea pigs which did not lick it were observed. As shown in Table 2, 7 mg/animal/day of L-AsA (0.5 g cream/day) or 15 mg/animal/day of L-AsA phosphate-Mg (0.5 g cream/day) was shown to prevent efficiently the development of scurvy, though the activity of the latter might be somewhat weaker than that of the former. The liver AsA content of the VC-P-cream group clearly exhibited the accumulation of AsA in comparison with the control group. At the end of experiment, 16 hours after the last application of cream, the skin of the back (cream-applied) and of the abdomen (non-applied) were examined histochemically at the following groups, the deficient control, VC-cream 1 g (L-AsA 14 mg)/animal/day and VC-P-cream 19 (L-AsA phosphate-Mg 30 mg)/animal/day groups. As shown in Figs. 1, 2 and 3, the applied skin of the back in the VC-cream and VC-P-cream groups indicated the existence of AsA in the intercellular space of the epithelium, contrasting with the absence of AsA in the control group and in the skin of the abdomen of all these groups.

3. Elevation of blood AsA level after oral administration of L-AsA phosphate-Mg

About 300–350 g male guinea pigs fed on the stock diet, Oriental GC-5, for over a week were fasted for 16 hours prior to use. Then, they were orally given 25 mg/animal of L-AsA or 54 mg/animal of L-AsA phosphate-Mg dissolved in 1 ml of water at 9 A.M. To the control group 1 ml of water was given. About 0.6 ml of blood were taken by the heart puncture with a small amount of heparin under no anesthesia at various intervals.
Fig. 1. Negative identification of AsA in the skin of the control group.

Fig. 2. Distribution of AsA in the skin of the VC-cream group. AsA was visualized as Ag particle in the intercellular space of epithelium of the back.
Fig. 3. Distribution of AsA in the skin of the VC-P-cream group.
AsA was visualized as Ag particle in the intercellular space of epithelium of the back.

Fig. 4. Blood AsA level after oral administration of L-AsA phosphate-Mg.
Five guinea pigs, fasted for 24 hours, mean ± standard error.

Fig. 4, the blood AsA level showed to elevate rapidly within 1 hour and begin to decline thereafter, but to be high enough even after 8 hours. There was, however, found no significant difference in the change of the blood AsA level between the L-AsA and L-AsA phosphate-Mg groups throughout the experimental period.

4. Hydrolysis of L-AsA phosphate-Mg in vitro
After the incubation of L-AsA phosphate-Mg with the homogenates of small intestine and liver or with the crude extracted solution of skin of guinea pigs, the amount of liberated AsA, reacted with 2,4-dinitrophenylhydrazine, indicated that L-AsA phosphate was more inclined to undergo the hydrolysis in an acidic medium than in an alkaline as shown in Figs. 5 and 6. In contrast, only a slight (about 5%) or negligible hydrolysis of L-AsA phosphate was observed in the incubation with the N/20-HCl or pancreatin solution, respectively, at 37°C during 1 hour.

**Fig. 5.** Hydrolysis of L-AsA phosphate-Mg in the homogenates of small intestine and liver. 37°C, 3.70 mg/g tissue/10 ml, buffer solution: 0.05 M, pH 4.8: acetate, 7.4: Krebs-Ringer phosphate, 10.0: carbonate-bicarbonate buffer.

**Fig. 6.** Hydrolysis of L-AsA phosphate-Mg in the crude extract of skin of guinea pigs. 37°C, 9.23 mg/2 g tissue/15 ml, pH 4.7: acetate, 7.2 and 9.4: veronal buffer.
DISCUSSION

In the study for obtaining stable derivatives of L-AsA, some acyl derivatives such as 2-0-benzoyl-L-AsA (2), 6-0-stearoyl-L-AsA (6) and 3,6-di-0-palmitoyl-L-AsA (7) have been shown to be active antiscorbutically in guinea pigs. These compounds were, however, found to be somewhat unstable in the medium containing water, e.g. in a cosmetic cream, and darken it as time passed.

L-AsA phosphate, relatively stable even in an aqueous state, was shown to have a strong activity to prevent the development of scurvy in guinea pigs when it was administered orally or percutaneously. It is of interest to note that L-AsA phosphate showed a strong antiscorbutic activity in spite of having an ester linkage at the endiol group, since 3-methyl-L-AsA (8, 9), 2,3-diphenacyl-L-AsA (10) and 2,3,5,6-tetraacetyl-L-AsA (11) were reported to show little or no antiscorbutic activity. On the other hand, the strong activity of 2-0-benzoyl-L-AsA seems to be, at least partly, ascribed to its unstability in an aqueous state releasing non-esterified endiol group (2), while L-AsA phosphate remains to be stable as described above. Therefore, the strong antiscorbutic activity of L-AsA phosphate might be ascribed to the reactivity to undergo the hydrolysis easily in vivo, as deduced from the result of in vitro experiment. This deduction is further supported by the facts that the plasma AsA level rose rapidly after the oral administration of L-AsA phosphate-Mg and that there was the accumulation of the liver AsA content in the VC-P-cream group as well as VC-cream group. Since L-AsA-phosphate itself did not react with 2,4-dinitrophenylhydrazine, the rise of AsA values in the blood and liver undoubtedly originated from the hydrolysis of L-AsA phosphate. From the experiment using VC-cream and VC-P-cream, the apparent existence of AsA in the intercellular space of the epithelium not in the skin of the abdomen (cream non-applied), but in the skin of the back (applied) and the negative identification in both skin of the control group strongly suggest the direct absorption of L-AsA phosphate through the skin and its hydrolysis in situ. The plentiful distribution of acid phosphatase in the skin (12) should be noted in connect with the above findings.

SUMMARY

1. The antiscorbutic activity of L-AsA phosphate was studied in about 300 g male guinea pigs. By the oral administration, 2.1 mg/animal /day of L-AsA phosphate-Mg might by effective, though not fully, to prevent the development of scurvy, while L-AsA seems to be somewhat more active than the equimole of L-AsA phosphate-Mg.

2. By the percutaneous application such as a cosmetic cream, L-AsA phosphate-Mg was shown to be as active antiscorbutically as L-AsA. The direct absorption of L-AsA phosphate through the skin and the hydrolysis in situ were suggested by the histochemical identification of AsA.

3. The blood AsA level rapidly rose after the oral administration of L-AsA phosphate-Mg in normal guinea pigs fasted for 24 hours.

4. L-AsA phosphate-Mg was shown easily to undergo the hydrolysis in vivo by the
homogenates of small intestine and liver or by the crude extracted solution of skin of guinea pigs.

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