In Vitro and In Vivo Prolonged Biological Activities of Novel Vitamin C Derivative, 2-O-α-D-Glucopyranosyl-L-Ascorbic Acid (AA-2G), in Cosmetic Fields

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Summary The biological activity of the novel vitamin C derivative, 2-O-α-D-glucopyranosyl-L-ascorbic acid (AA-2G), was evaluated in vitro and in vivo. The percutaneous absorption of AA-2G was determined in five Japanese males. The excretion of ascorbic acid (AA) in the subjects administered AA-2G was sustained for a longer period than in the subjects administered ascorbic acid 2-phosphate (AA-2P), which is a conventional vitamin C derivative. An analysis of the distribution of AA in the skin showed that small black specks assumed to be AA were observed in the epidermis even 3d after applying AA-2G. The melanin synthesis in B16 melanoma cells was inhibited more by AA-2G than by AA-2P, and AA-2G also prevented more UV-induced damage of human skin keratinocytes and fibroblasts than AA-2P did. From these in vivo and in vitro results, it is supposed that the conversion of AA-2G to AA is sustained for a long time compared with that of AA-2P, and that AA-2G is an effective and available compound having vitamin C activity in human subjects.

Key Words L-ascorbic acid, 2-O-α-D-glucopyranosyl-L-ascorbic acid, vitamin C activity, human subject

L-Ascorbic acid (AA) functions in many biological processes, such as collagen synthesis, antioxidation, intestinal absorption of iron, and metabolism of some amino acids (1, 2). An essential function of L-ascorbic acid is to act as a cofactor for the hydroxylation of proline residues in collagen synthesis. However, various inexplicable phenomena in the action of AA have recently emerged. Because of its in vivo inhibitory action on melanin synthesis, AA is widely used as a whitening agent for cosmetics in Japan. However, AA is less resistant to oxidative conditions than other vitamins are and is easily decomposed.

To solve these problems, Yamamoto et al have tried to develop a vitamin
C derivative having (1) a stability in cosmetics, (2) an activity to be converted to AA in the skin, and (3) a sustained AA efficacy in the skin. They proposed a new vitamin C derivative, ascorbic acid 2-glucoside (2-O-β-D-glucopyranosyl-L-ascorbate) (AA-2G) (3–5), because it has these characteristics. In the present study, the biological activities of AA-2G and its application in cosmetics were examined.

MATERIALS AND METHODS

Materials. The materials used in this work were obtained from the following sources: The AA-2G was synthesized in an enzymic reaction by Hayashibara Biochemical Research Institute Co., Ltd. (6). The chemical structure is shown in Fig. 1. AA-2P (ascorbic acid 2-phosphate) was obtained from Showa Denko Co. (Tokyo, Japan); Eagle’s minimum essential medium (MEM) from Nissui Pharmaceutical Co. (Osaka, Japan); and fetal bovine serum from GIBCO Laboratories (USA). All other reagents were of analytical grade and commercially available.

Measurement of the percutaneous absorption of AA-2G and its distribution in the skin. The percutaneous absorptions of AA-2G and AA-2P were measured according to the method of Miya et al (7). Two creams containing vitamin C derivatives were prepared (Table 1), one containing 2% AA-2G and the other 3% AA-2P. AA-2G cream (7.5 g) was applied to the legs of five males (Japanese, 31–45 y), and the AA-2P cream (also 7.5 g) was applied after an interval of at least one week. A cream containing no vitamin C derivative (placebo) was applied to one other male subject (Japanese, 48 y). Informed consent was given by each volunteer, and the study was approved by the ethical committee of our laboratory.

The areas to which the test creams were applied were covered for 14 h with cling film, and a bandage was applied on top of it. Urine samples were taken every 2 h until the 26th h. The samples were diluted with an equal volume of 10% metaphosphoric acid and stored at −20°C. The amount of AA in the urine samples was measured by the 2,4-dinitrophenylhydrazine method (8).

The distribution of AA in the skin was measured by the method of Imai et al (9). The AA-2G cream was applied to an area on the inside of the forearm of the
Table 1. Compositions of the test cream.

|                     | AA-2G cream | AA-2P cream | AA cream | Placebo cream |
|---------------------|-------------|-------------|----------|--------------|
| AA-2G               | 2%          | —           | —        | —            |
| AA-2P               | —           | 3%          | —        | —            |
| AA                  | —           | —           | 1%       | —            |
| Stearyl alcohol    | 4           | 4           | 4        | 4%           |
| Behenyl alcohol    | 1.8         | 1.8         | 1.8      | 1.8          |
| Glycerin           | 1           | 1           | 1        | 1            |
| 1,3-Butylene glycol | 5           | 5           | 5        | 5            |
| Hydrogenated oil   | 3           | 3           | 3        | 3            |
| Jojoba oil         | 5           | 5           | 5        | 5            |
| Squalane           | 3           | 3           | 3        | 3            |
| Polyoxyethylene cetyl ether | 2    | 2           | 2        | 2            |
| Glyceryl monostearate | 2      | 2           | 2        | 2            |
| Potassium hydroxide | 0.384       | 0.384       | 0.384    | 0.384        |
| Methyl parahydroxybenzoate | 0.15    | 0.15        | 0.15     | 0.15         |
| Ethyl parahydroxybenzoate | 0.1    | 0.1         | 0.1      | 0.1          |
| Trisodium edetate  | 0.1         | 0.1         | 0.1      | 0.1          |
| Compound perfume   | Trace       | Trace       | Trace    | Trace        |
| Water              | To 100.0%   | To 100.0%   | To 100.0%| To 100.0%    |

male subject (Japanese, 50y), and this area was covered with cling film and a bandage for 12 h. Samples were taken from the area by punch biopsy 12 h and 3 d after the bandage and the cling film had been removed. The same procedure was carried out for the AA-2P cream and the cream containing no vitamin C derivative. But in the latter, the punch biopsy was done 3 d after the cover was removed.

The skin samples obtained were fixed with formalin, and sections were prepared in the normal manner and stained by the Reticulin silver impregnation method (10).

Quantification of melanin synthesis. B-16 melanoma cells were cultured in Eagle’s MEM containing 10% fetal bovine serum and 0.013 mol/L NaHCO₃ (MEM-10) at 37°C in a 5% CO₂–air atmosphere for a certain period in the presence of 2 mmol/L AA-2G or without AA-2G. At 24 h before the cells were harvested, 0.5 mmol/L theophylline was added. The cells were then treated with trypsin (0.25%), harvested, and the number of viable cells counted by the trypan blue exclusion method.

The amount of melanin in the cells was measured by using the method of Oikawa and Nakayama (11).

Efficacy of AA-2G against UV damage. Human epidermal keratinocytes purchased from Iwaki Glass Co. were cultured at 37°C in 2.0 mL of Medium 154 containing human keratinocyte growth supplement (1%, Cascade Biologies, Inc.) and 0.2 mmol/L AA-2G in a 5% CO₂–air atmosphere. After 24 h of incubation, the cells were irradiated with UVB at 17 mJ/cm², then cultured for a further 24 h. The cells, which had been treated with trypsin-blue, were then harvested by trypsin
digest, and the number of viable cells was determined by using a hematocytometer.

Another experiment used normal human foreskin fibroblasts from a healthy child (male, 3 d old). The fibroblasts were purchased from RIKEN Cell Bank and cultured at 37°C in Eagle’s MEM supplemented with 10% fetal bovine serum and 0.013 mol/L NaHCO₃ (MEM-10) in a 5% CO₂-air atmosphere. After becoming confluent, the cells were irradiated with UVB at 292 mJ/cm², and 0.2 mmol/L AA-2G was then added to the culture medium. After 7 d of culturing, the number of surviving cells was determined by the DNA assay method (12).

**Counting sunburn cells.** Seven healthy males (Caucasian, 21–46 y) were chosen as the subjects. The AA-2G cream was applied to the inside of the upper part of one arm once a day for 20 d, and a cream not containing AA-2G (placebo) was applied to the other arm under the same conditions. Five hours after the last application of the cream, the subjects’ arms were irradiated with UVB at a dose of twice the minimal erythema dose (MED). After 24 h, skin samples were taken from the irradiated areas by shave biopsy. The samples were fixed in 2% formalin and processed for routine histology. To count the number of sunburn cells (basal keratinocytes having pyknotic nuclei and eosinophilic cytoplasm), 3 biopsy sections were taken from each experiment site and stained with duplicate haematoxylin and eosin. The number of sunburn cells per given condition was calculated under a microscope (13).

**Statistical analysis.** The means and standard deviations of all data were calculated, and comparison was done by the paired or unpaired t-test with significant probability levels of less than 0.01.

**RESULTS**

**Percutaneous absorption of AA-2G and its distribution in the skin**

The percutaneous absorption of the vitamin C derivatives and its conversion to AA in the skin and other parts of the body were demonstrated by applying creams containing AA-2G or AA-2P to the legs of the subjects and measuring the amount of AA excreted in their urine.

Figure 2 shows the average for five subjects of the difference between the total amount of AA excreted into the urine after application and the amount of AA in the urine before the cream was applied. In the case of the cream containing AA-2P, the amount of AA excreted into the urine was at a maximum 4 h after application and showed a decreasing trend thereafter. After 16 h, it had dropped to the same level as before the application.

In the case of the cream containing AA-2G, although there was no sharp increase like that with the cream containing AA-2P, the amount of AA excreted into the urine had increased over the amount measured before application. Moreover, the amount of AA excreted into the urine was sustained at virtually the same level for 10 h and peaked at 14th h. Even after the cream was removed (16th h following application), AA was still being excreted into the urine, and the excretion...
Fig. 2. Measurement of AA-2G’s percutaneous absorption and metabolism to AA. We applied 7.5 g of the AA-2G cream (○) to the legs of five males, and AA-2P cream (■) was applied at least one week later. A cream containing no vitamin C derivative (□) was applied to one other male subject. The areas to which the test creams were applied were covered for 14 h with cling film, and a bandage was applied on top of this. Urine samples were taken every 2 h until the 26th h. The amount of AA in the urine samples was measured by the 2,4-dinitrophenylhydrazine method. Each point represents the mean ± SE of 5 males. * p < 0.01 compared by paired t-test with the AA excreted into the urine before application.

was observed until the 26th h.

In regard to the cream containing no vitamin C derivative (placebo cream), which was applied to only one subject, the amount of AA excreted into the urine did not change.

The increase in AA concentration in the urine seemed to originate solely from the cream because the subjects were not allowed to take vitamin C orally. Furthermore, this study clearly shows that the AA-2G and AA-2P in the creams were absorbed percutaneously and converted into AA through their metabolism in the skin and other parts of the body.

The total amounts of AA-2P and AA-2G absorbed percutaneously were virtually the same (data not shown), suggesting differences in the rates at which they are converted to AA in the skin and in other parts of the body. These results further suggest that the efficacy of the AA in the AA-2G cream was prolonged because the conversion of AA-2G to AA was sustained at a constant level.

Figure 3 shows micrographs of skin samples from areas to which the AA-2G or AA-2P cream had been applied taken 1 d and 3 d after application. Small black silver particles indicate the presence of AA in the skin because they were not
Fig. 3

AA-2G cream After 1 d
AA-2G cream After 3 d
AA-2P cream After 1 d
AA-2P cream After 3 d
Placebo After 3 d

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observed in the case of the cream containing no vitamin C derivative. In the case of AA-2P, silver particles were observed between the epidermal cells after one d, but there were almost none after 3 d, whereas in the case of AA-2G, silver particles could still be observed after 3 d.

These results show that AA-2G releases AA in the skin over a longer period of time than AA-2P does, demonstrating that it is a sustained release type of vitamin C derivative.

\textit{Effect of AA-2G on melanin synthesis}

We took the amount of melanin in B16 melanoma cells to which no vitamin C derivative had been added as a baseline (Fig. 4). When AA-2P or AA was added, we were able to observe an inhibitory effect on melanin synthesis over a period of
Fig. 5. Reducing action of AA-2G on existing melanin. AA-2G (2 mmol/L), AA-2P (2 mmol/L), or AA (2 mmol/L) was added after the B16 melanoma cells were cultured until confluent. One day before the end of the culture period, 0.5 mmol/L theophylline was added. Cells were recovered after trypsin digest, and the quantity of melanin in cells was measured by using the method of Oikawa et al. The number of those still living was counted by means of the trypan blue method. Each value is the mean of triplicate cultures. *p < 0.01 compared by unpaired t-test with AA-2P.

After culturing B16 melanoma cells for 48 h, we added the vitamin C derivatives and monitored the change in pigmentation amount in them. The result is shown in Fig. 5. When AA-2P or AA was added, the amount of melanin pigmentation dropped to 50% during the first day of incubation, but the rate of decrease tapered 15–20 h, but after this, melanin synthesis rapidly increased, and after 30 h of culturing, the melanin amount was virtually the same as the baseline level.

On the other hand, when AA-2G was added to the culture, its inhibitory effect on melanin synthesis lasted for the longest period.

Because it is known that AA and its derivatives lighten the color of existing melanin (reducing action on melanin), we decided to determine if AA-2G had this action.

After culturing B16 melanoma cells for 48 h, we added the vitamin C derivatives and monitored the change in pigmentation amount in them. The result is shown in Fig. 5. When AA-2P or AA was added, the amount of melanin pigmentation dropped to 50% during the first day of incubation, but the rate of decrease tapered...
Fig. 6. Effect of AA-2G in preventing UV-induced skin damage. Human skin keratinocytes were incubated in 2.0 mL of fresh Medium 154 supplemented with keratinocyte growth factor (1%) and 0.2 mmol/L AA-2G, AA-2P, or AA. After 24 h, the culture was irradiated with UVB at 17 mJ/cm² and cultured for 24 h. Trypan blue treated cells were recovered with trypsin digest, and the number of living cells was determined by using a hematocytometer. Each value is the mean ± SD of triplicate cultures. * p < 0.01 compared by unpaired t-test with the UV irradiation control (None).

Effect of AA-2G on UV damage in tissues

Figure 6 shows the result of irradiating human keratinocytes after they were treated with AA-2G, AA-2P, or AA. When there was no vitamin C derivative (none), the number of viable cells after irradiation with UVB had dropped about 44% over the number before irradiation. When AA-2G or AA-2P was added to the culture medium, after irradiation, the number of living cells was about 65% of the number before the addition, showing that more cells survived when AA-2G or AA-2P was added. This indicates that AA-2G and AA-2P help to prevent cell death because of UVB exposure.

Figure 7 shows the extent that AA-2G repaired UV damage, which was determined by using the DNA method to learn the viability of cells after UV irradiation. When no ascorbic acid or vitamin C derivative was added (none), the DNA amount had plummeted to 20% of the preirradiation amount 7 d after irradiation, showing that a large number of cells had died. However, the addition of AA-2G increased the DNA amount to about 50% of the preirradiation level,
Fig. 7. Effect of AA-2G in aiding recovery from UV-induced skin damage. A culture of human skin fibroblasts was made confluent in an MEM-10. Cells were irradiated with UVB at 292 mJ/cm², and AA-2G (0.2 mmol/L), AA-2P (0.2 mmol/L), or AA (0.2 mmol/L) was then added to the culture medium. After 7 d of culturing, the number of surviving cells was determined by the DNA assay method. Each value is the mean ± SD of triplicate cultures. *p < 0.01 compared by unpaired t-test with the UV irradiation control (None).

indicating a reduction in the number of dead cells. In contrast, the addition of AA-2P or AA had no effect at all in this respect.

*Mitigating effect of AA-2G on sunburn cell formation*

Because the appearance of characteristic sunburn cells in the epidermis is proportional to the dose of the UVB to which the skin is exposed, sunburn cells have been suggested as a measure of skin damage resulting from UV (14).

Inasmuch as AA-2G prevents and repairs UV damage at the cellular level, we monitored changes in the numbers of sunburn cells in human subjects to see if the effect of AA-2G would be demonstrated in this regard too. When a cream containing AA-2G (AA-2G cream) was applied to the skin, fewer sunburn cells appeared in the area of application (Fig. 8), suggesting that ultraviolet rays would cause little damage to the skin if an AA-2G containing cream were applied to it.

*Inhibitory effect of AA-2G on peroxidation of lipids*

When human keratinocytes were irradiated with UVB at a dose of 3 J/cm², the intracellular amount of peroxidized lipids doubled (Fig. 9). However, when AA-2G was added to make a final concentration of 3 mmol/L before the keratinocyte culture was irradiated with UVB, the amount of intracellular peroxidized lipids decreased significantly. Moreover, when we added both AA-2G and 3 mmol/L DL-α-tocopherol acetate to the keratinocyte culture before irradiation with UVB, the intracellular peroxidized lipid amount was at the normal level, i.e., the level without irradiation.

*Effect of AA-2G on hydroxy radical production*

The results of electron spin resonance (ESR) measurements shown in Fig. 10...
Fig. 8. Inhibitory effect of AA-2G cream on formation of sunburn cells. Seven healthy males were chosen as the subjects. The AA-2G cream was applied to the inside of the upper part of one arm once a day for 20 d, and a cream containing no AA-2G was applied to the other arm under the same conditions. Five hours after the last application of the cream, the subjects' arms were irradiated with UVB at a dose of 2MED. After 24 h, skin samples were taken from the irradiated areas by shave biopsy. The samples were fixed in 2% formalin and processed for routine histology. For counting the number of sunburn cells, 3 biopsy sections were taken from each experiment site and stained with duplicate haematoxylin and eosin. The number of sunburn cells per given condition was calculated under a microscope. Each value is the mean ± SE of seven subjects. *p < 0.01 compared by paired t-test with the nonradiation.

indicate that AA reduced hydroxy radical production, whereas there was no reduction with AA-2G. Moreover, we assume that AA-2G would be able to reduce hydroxy radical production after being converted to AA by the α-glucosidase present in cells and biological systems (15).

The reason why AA-2G inhibited the formation of peroxidized lipids because of irradiation with UVB is assumed to have been the direct capture of free radicals arising in the aqueous phase by AA derived from AA-2G. It is further assumed that damage to biological membranes through their oxidation in this manner would be prevented by AA, thereby preventing cell death.

DISCUSSION

It is considered that the major reason the physiological function of AA has
Fig. 9. Inhibitory effect of AA-2G on peroxidation of lipids. Keratinocytes recovered after trypsin digest was minced and made into a suspension in PBS (−), to which AA-2G (3 mmol/L) or AA-2G (3 mmol/L) + DL-α-tocopherol acetate (3 mmol/L) was added. After incubation for 1 h, the suspension was irradiated with UVB at 3 J/cm² and centrifuged. The quantity of peroxidized lipids was then determined by the phenol indoleamine method. Each value is the mean ± SD of triplicate cultures. *p < 0.01 compared by unpaired t-test with the no-drug control.

Fig. 10. Capture of hydroxy radicals by AA. A culture of human skin fibroblasts was made confluent. Then AA or AA-2G (0.2 mmol/L) was added to the culture medium. After 24 h, cells were minced by using the ultrasonic method. ESR spectra were obtained at room temperature by using a Bruker ESP 300 spectrometer operating at a 9.73 GHz modulation frequency. ESR instrument settings for the spin trapping experiment were microwave power, 40 mW; modulation amplitude, 0.75 G; time constant, 0.3 s; scan rate, 60 G/41.9 s; receiver gain, 1 × 10⁶. Each value is the mean of three separate experiments. *p < 0.01 compared by unpaired t-test with the no-drug control.
not yet been fully clarified is because of its instability in in vitro culture systems. AA-2G was shown to be very stable under oxidative conditions and to release AA adequately through enzymatic hydrolysis (16).

Yamamoto et al have compared the physiological actions of AA-2G with AA on the promoting activity of collagen synthesis and cytotoxicity (17, 18). They discovered that AA-2G showed physiological actions unseen with AA because, unlike AA, AA-2G had no inhibitory effect on the cell level and was much more stable.

Because of the instability of ascorbic acid, it is impossible to incorporate it directly into cosmetics other than powder products. The stable vitamin C derivative AA-2P has been used for many years to avoid this problem. In consideration of using the novel vitamin C derivative AA-2G in cosmetics, it was considered necessary to compare it with AA-2P to see how their physiological actions differed. So the object of the present study was to compare AA-2G, AA-2P, and AA to learn their abilities to provide the physiological actions required by cosmetics.

With the exception of special cases, the active ingredients of cosmetics must be absorbed percutaneously to manifest their physiological action in the skin. Therefore we first tested the creams to see if they were percutaneously absorbed or not. We used human subjects for the test, and creams containing AA-2G and AA-2P were applied to their legs. The test was carried out under the conditions of strict dietary restrictions, and all subjects followed a similar lifestyle pattern. Measures of the increase of ascorbic acid excreted into the urine showed that vitamin C and vitamin C derivatives were all percutaneously absorbed and that the derivatives were converted to ascorbic acid. The distinguishing feature of AA-2G was that ascorbic acid was excreted into the urine for a longer period than with AA-2P. This was assumed to be because AA-2G is converted to ascorbic acid at a slower rate than AA-2P is. This was also borne out by an experiment in which the distribution of ascorbic acid in the skin was measured; small black specks assumed to be ascorbic acid were observed in the epidermis even 3 d the AA-2G cream was applied. From this we supposed that the activity of α-glucosidase, which hydrolyzes AA-2G in the human body, is lower than that of alkaline phosphatase, which hydrolyzes AA-2P. It has been reported, however, that when the conversion of AA-2G to ascorbic acid was measured in human fibroblasts, the rate for AA-2G was 9.6 times higher than that for AA-2P, and the activity of α-glucosidase was 1.6 times higher than alkaline phosphatase (17), which is contrary to what we had speculated. Although the reason for this is still not clear, because our experiment employed percutaneous absorption in the human body, the vitamin C conversion rate observed may reflect the activity of enzymes in epidermal keratinocytes.

Vitamin C derivatives are being used in whitening cosmetics. These products are designed to inhibit the activity of tyrosinase and the formation of melanin because they are considered to be the cause of skin becoming darker when it is exposed to ultraviolet radiation. We thus decided to test whether AA-2G would be effective in inhibiting melanin synthesis and preventing UV-induced damage at the
cellular level. For the most part, the results showed these effects of AA-2G to be sustained. When the action was measured for a short culture period, AA-2G, AA-2P, and AA showed no differences in terms of efficacy, but for a long culture period, the results showed that AA-2G was much more effective. From this observation, we concluded that the effect of the novel vitamin C derivative AA-2G is sustained over a long period.

Sunburn cells are thought to arise from the apoptosis of epidermal keratinocytes that have been damaged by UV radiation. In the clinical research, sunburn cells are used as a phototoxic reaction indicator (19). Because AA-2G had acted to prevent UV-induced damage at the cellular level, we thought it might also inhibit sunburn cell formation. The experiment was carried out at Ivy Laboratories in the United States on five subjects who had signed the consent form (20). The results showed that AA-2G prevented UV-induced damage in the human subjects as it had done at the cellular level. Although it is quite easy to detect the effect of ascorbic acid at the cellular level, this has been difficult to do in humans. In the present study, however, its efficacy in preventing UV-induced damage in human subjects was clearly demonstrated through measurements made on sunburn cells. This is assumed to have been possible as a result of the stability of AA-2G and its lack of the cytotoxicity found with AA, and because it is steadily converted to ascorbic acid in the skin (17, 18).

In the present study on the biological activity on the novel vitamin C derivative, AA-2G, we have demonstrated that its conversion to ascorbic acid is sustained, unlike that of AA-2P, the vitamin C derivative that has been in use until now.

REFERENCES

1) Burns JJ, Rivers JM, Machin LJ, eds. 1987. Third conference on vitamin C. Ann NY Acad Sci 498: 1–533.
2) Tajima S, Pinnell RS. 1982. Regulation of collagen synthesis by ascorbic acid. Ascorbic acid increases type I procollagen mRNA. Biochem Biophys Res Commun 106: 632–637.
3) Yamamoto I, Muto N, Nagata E, Nakamura T, Suzuki Y. 1990. Formation of a stable L-ascorbic acid z-glucoside by mammalian z-glucosidase-catalyzed transglucosylation. Biochim Biophys Acta 1035: 44–50.
4) Muto N, Nakamura T, Yamamoto I. 1990. Enzymatic formation of a non-reducing L-ascorbic acid z-glucoside: Purification and properties of z-glucosidases catalyzing site-specific transglucosylation from rat small intestine. J Biochem 107: 222–227.
5) Muto N, Suga S, Fujii K, Goto K, Yamamoto I. 1990. Formation of a stable ascorbic acid 2-glucoside by specific transglucosylation with rice seed z-glucosidase. Agric Biol Chem 54: 1697–1703.
6) Aga H, Yoneyama M, Sakai S, Yamamoto I. 1991. Synthesis of 2-O-2-glucopyranosyl L-ascorbic acid by cyclomaltodextrin glucanotransferase from Bacillus steareothermophilus. Agric Biol Chem 55: 1751–1756.
7) Miya H, Nomura H, Imai Y, Takashima H. 1970. Chemistry and application of ascorbic
acids phosphate. *Bitamins (Vitamins*) **41**: 387–398.

8) Okumura M, Arakawa N. 1989. Chapter 16. Vitamin C. In: Vitamin Handbook 3 (the Vitamin Society of Japan, ed), p 135–143. Kagaku Dojin, Kyoto.

9) Imai Y, Usui T, Matsuzaki T, Yokotani H, Mima H, Aramaki Y. 1967. The antiscorbutic activity of l-ascorbic acid phosphate given orally and percutaneously in Guinea pigs. *Jpn J Pharmacol* **17**: 317–324.

10) Maeda A, Sasaki K. 1988. Manual of Histologic Staining Methods (in Japanese), p 54–56. Ishiyaku Publishers, Tokyo.

11) Oikawa A, Nakayama M. 1973. Quantitative measurement of melanin as tyrosine equivalent and as weight of purified melanin. *J Biol Med* **46**: 500–507.

12) Rango R, Mitchen J, Wilding G. 1990. DNA fluorometric assay in 96-well tissue culture plate using Hoechst 33258 after cell lysis. *Anal Biochem* **191**: 31–34.

13) Darr D, Combs S, Dunston S, Manning T, Pinnell S. 1992. Topical vitamin C protects porcine skin from ultraviolet radiation-induced damage. *Br J Dermatol* **127**: 247–253.

14) Johnson BE, Mandell G, Daniels F. 1972. Melanin and cellular reactions to ultraviolet radiation. *Nature N Biol* **235**: 147–149.

15) Yamamoto I, Suga S, Mitoh Y, Tanaka M, Muto N. 1990. Antiscorbutic activity of L-ascorbic acid 2-glucoside and its availability as a vitamin C supplement in normal rats and Guinea pigs. *J Pharmacobiodyn* **13**: 688–695.

16) Muto N, Terasawa K, Yamamoto I. 1992. Evaluation of ascorbic acid 2-O-d-glucoside as vitamin C source: Mode of intestinal hydrolysis and absorption following oral administration. *Internat J Vit Nutr Res* **62**: 318–323.

17) Yamamoto I, Muto N, Murakami K, Akiyama J. 1992. Collagen synthesis in human skin fibroblasts is stimulated by a stable form of ascorbate, 2-O-d-glucopyranosyl-L-ascorbic acid. *J Nutr* **122**: 871–877.

18) Yamamoto I, Tanaka M, Muto N. 1993. Enhancement of in vitro antibody production of murine splenocyte by ascorbic acid 2-O-d-glucoside. *Int J Immunopharmac* **15**: 319–325.

19) Danno K, Horio T. 1987. Sunburn cell: Factors involved in its formation. *Photochem Photobiol* **45**: 683–690.

20) Allen ME, ed. 1991. Good Clinical Practice in Europe (Investigator’s Handbook), p Ap.11: 1–2:4. IBRD-Rostrum Global, Romford.