Structure of Ascorbic Acid and Its Biological Function: V. Transport of Ascorbate and Isoascorbate across Artificial Membranes as Studied by the Spin Label Technique

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It could be shown by the reduction of the spin label (1,14) located within DPPC vesicles, that Na-ascorbate and K-isoascorbate can permeate membranes. At physiologic pH value, these two compounds exist as electroneutral radicals with a cyclic side chain structure. Ascorbic acid and isoascorbic acid, on the contrary, can hardly permeate such an artificial membrane. Since the radical will cause lipid peroxidation, it must be modified prior to permeation. This can be done by GSH which changes the radical state but maintains the electroneutral bicyclic configuration.

Introduction

The permeation of ascorbic acid across natural and artificial membranes is still questionable [1]. Most of the experimental results deny such a transport for the reduced and semi-oxidized forms, at least at temperatures lower than the phase transition temperature [2]. In some other reports, a permeation has been observed above the phase transition temperature, e.g. $> 40\,^{\circ}C$ [3]. While these findings could not be explained yet, the inability of the semi-oxidized form of vitamin C, the ascorbyl radical, to permeate membranes was thought to be due to its negative charge.

Recently we could show [4] that, at physiologic pH value, the side chain of ascorbic acid forms a cyclic structure. Since the resulting complex, the Na-Ascorbate radical, is electroneutral, it was believed that, in this configuration, it might be able to permeate the membrane even at room temperature. Therefore, the effect of ascorbic acid, isoascorbic acid, and of their radicals, which are present at physiologic pH, on artificial membranes has been investigated at room temperature. For these studies, DPPC vesicles were labelled with the spin label I (1,14) which is located at the apolar end of the CH_2 chain. This lable can be reduced by ascorbic acid [5] and can be used, therefore, as an indicator for the permeation of vitamin C.

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Material and Methods

150 mg of DPPC (dipalmitoylphosphatidylcholine; Fluka, Neu-Ulm) and 1.8 mg of spin label I (1,14) (2-(14-carboxytetradecyl)-2-ethyl-4, 4-dimethyl-3-oxazolidinoxyl; Syva Corp., Palo Alto, California) were dissolved in chloroform. After evaporation of the solvent under vacuum the dry lipid was suspended in 5 ml of 0.2 M Tris/HCl (pH 7.4), transferred to small test tubes, and sonicated at about 45 °C in a bath sonicator (Bransonic 12) until the suspension became clear, which occurred usually after about 90 min.

Ascorbate solutions (pH 7.4) were prepared immediately before the electron spin resonance (ESR) measurements by dissolving appropriate amounts of ascorbic acid (ASC, Merck, Darmstadt) or isoascorbic acid (Iso-ASC, Fluka, Buchs) in NaOH or KOH, resp. The reaction started when 10 µl of different concentrations of Na-ascorbate (Na-ASC) or K-isoascorbate (K-Iso-ASC) solution were added to a 100 µl vesicle suspension. Right after this, the ESR spectra of these samples were measured with a Varian E-9, 100-kHz modulation X-band spectrometer. The modulation amplitude was 1 mT and the microwave power 5 mW for all samples investigated at 22 °C. The decay of the peak-to-peak height (spin concentration) of the center line of the spin probe spectrum was used to monitor the kinetics of ascorbate transport. This procedure is justified as long as neither the ascorbate free radical interferes significantly with the spin probe spectrum nor the line shape of the signal changes. Both of

these effects could not be observed. Red. glutathione (GSH) was purchased from Sigma, St. Louis, and $FeSO_4$ from Merck, Darmstadt.

Results and Discussion

The effect of ascorbate and isoascorbate on the reduction of the spin label I (1,14) incorporated into DPPC vesicles is shown in Fig. 1. This spin label is located at the end of the hydrophobic side chain of the lipids. Any decrease in peak height (≙ spin concentration) of the spin label is caused by the reducing ability of ascorbic acid. This occurs, of course, only if vitamin C is able to permeate the membrane.

As can be seen in Fig. 1, the reduction of the spin label increases with increasing concentrations of Na-ASC. It is interesting to note that the reduction occurs biphasicly. An initial steep decrease ("fast reaction" rate) is followed by a slow decrease ("slow reaction" rate) depending on the concentrations used.

There was no change in lipid configuration, since the same suspension could be used for at least 5 hours with identical results in the inactivation of the spin label.

It should be emphasized that K-Iso-ASC exhibits the same permeation rate as Na-ASC. The only difference could be observed in the spin concentration of the "slow reaction" phase which was consistently lower in the case of K-Iso-ASC: the reaction rate, however, was unchanged. This is shown in Fig. 1 for the case of 100 mM of K-Iso-ASC only: the "fast reaction" phase is identical in spin concentration and rate for both of the compounds used. Similar results were obtained for the other concentrations (not shown).

An explanation cannot be given yet neither for the biphasic behavior nor for the difference in the "slow reaction" phase between Na-ASC and K-Iso-ASC.

The linear dependence of the slope, being proportional to the permeation rate, of the "fast reaction" phase on the ascorbate concentration is shown in Fig. 2. It could not be determined if a plateau will occur above the concentrations used. Unfortunately, a faster determination of the peak height was impossible with the procedure applied.

From the results obtained one might conclude that the ascorbyl radical is able to permeate the membrane even at room temperature. It has been proposed that the reduction in spin concentration by Na-ASC is based on flip-flop events of the lipids [6]. Since the spin label (12,3) which is located near the polar head groups is reduced faster by Na-ASC [5, 7], the effect might be rather due to the different diffusion distance within the hydrophobic environment. For such a diffusion, an electroneutral bicyclic radical configuration, as is the case with Na-ASC, is an assumption. When 100 mM of ASC were added to a labelled lipid suspension, the pH of which had

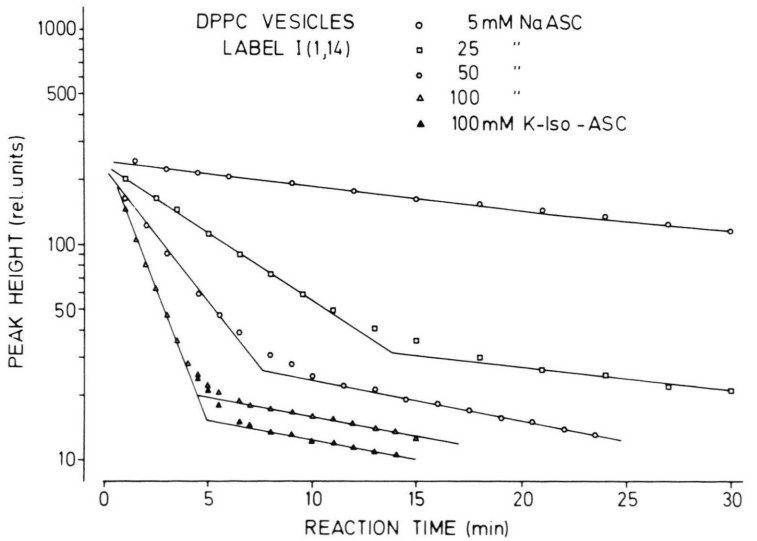

Fig. 1. The dependence of the spin concentration of spin label I (1,14) located at the apolar end of the CH_2 chain of DPPC vesicles on Na-ascorbate (Na-ASC) and K-isoascorbate (K-Iso-ASC). S.D. ≦ 5%.

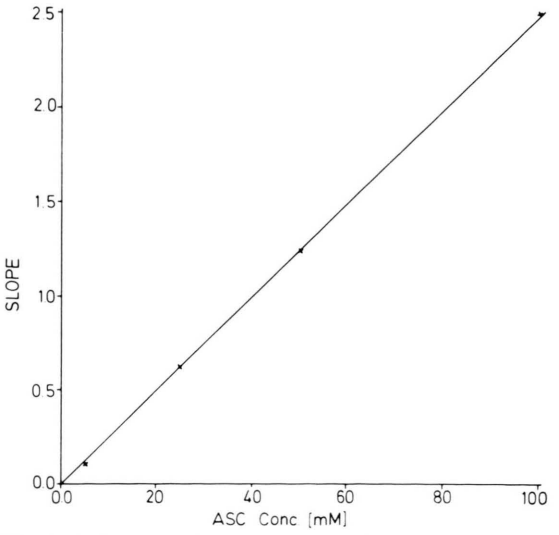

Fig. 2. Influence of the concentration of Na-ascorbate (Na-ASC) and K-isoascorbate (K-Iso-ASC) on the "fast reaction" rate of the inactivation of the spin label I (1,14) located within DPPC vesicles. Data taken from Fig. 1. S.D. ≦ 5%.

been lowered to pH 3.5 by addition of HCl, there was almost no reduction of the spin label.

The radical configuration causes, however, lipid peroxidation and, thus, destruction of the membrane [8]. For this reason, the ascorbyl radical must be modified prior to permeation. This can be done by red. glutathione (GSH) which causes the disappearance of the radical state and yet maintains the electroneutral bicyclic configuration. The effect of different concentrations (up to 50 mM) of GSH on the permeation of vesicles by a 100 mM solution of Na-ASC has been studied. GSH and Na-ASC were mixed prior to adding to the lipid suspension. At 50 mM of GSH, radicals could not be detected any more. If a labelled lipid suspension was treated with such a Na-ASC-GSH mixture, the effect was the same as for Na-ASC alone. When a 50 mM GSH solution, the pH of which had been adjusted to pH 7.4 by adding NaOH, was added only, a reduction in spin label could not be observed indicating its inability to permeate the membrane.

Similar results were obtained when in addition to GSH $FeSO_4$ was used: the reduction in spin label was the same for 100 mM of Na-ASC with or without addition of 50 mM GSH + 50 mM $FeSO_4$. Again, a reduction was not observed when a solution containing only 50 mM GSH + 50 mM $FeSO_4$ was added. The latter solution when mixed, however, with a TEMPO label reduced it within a very short period of time (< 1 min). From this it can be concluded that GSH or GSH + $FeSO_4$ can reduce a spin label indeed, but cannot permeate a membrane and reduce, thus, the spin label I (1,14) located within the membrane. Therefore, the reduction of the internal spin label I (1,14) is solely due to the effect exerted by ascorbic acid/isoascorbic acid, which are present in an electroneutral bicyclic configuration and the radical state of which has been changed by GSH.

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