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The entrapment of [^{14}C]ascorbic acid in human erythrocytes

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Radioactively labelled ascorbic acid and dehydroascorbic acid, when incubated with human blood, migrate irreversibly into human red blood cells. Isolation and characterization of the moieties trapped within the cells via infrared spectroscopy established both their identities as L-ascorbic acid. Evidence in the form of the degree of in vitro entrapment of ascorbic acid as a function of the times of incubation and the effect of incubation temperature, anion recognition site inhibitor, and active transport inhibitor on the rate of entrapment support the hypothesis that ascorbic acid is oxidized on or near the surface of the red blood cell to dehydroascorbic acid which migrates through the lipid portion of the cell wall and is reduced back to ascorbic acid within the cell. The resulting L-ascorbic acid can not pass through the cell wall and is therefore entrapped.

Many studies [1–4] indicate that dehydroascorbic acid penetrates the erythrocyte both in vivo and in vitro. Inconsistent results were obtained by Golden and Sargent [1], who showed ascorbic acid either passed into or moved out of erythrocytes when these cells were suspended in media containing ascorbic acid, and by Borsook et al. [5], who concluded that red cells were absolutely impermeable to ascorbic acid. The lack of agreement in the studies concerning ascorbic acid probably depends to a great extent on the analytical methods used for the determination of ascorbic acid. Hornig et al. [6] tried to avoid these difficulties by undertaking their in vitro studies with guinea pig erythrocytes using radioactively labelled ascorbic acid and dehydroascorbic acid. Their results [6] strongly suggest that the erythrocyte membrane is permeable to both ascorbic acid and dehydroascorbic acid in either direction. Most recently, Okamura [7] found that ascorbic acid was taken up very slowly by human erythrocytes and that dehydroascorbic acid was taken up very rapidly by human erythrocytes to establish equilibrium after one minute.

In the present study the interaction of radioactively labelled ascorbic acid and dehydroascorbic acid with human erythrocytes was examined over a period of time of hours rather than minutes. 4-ml samples of whole blood in EDTA-treated tubes were incubated with 1.0 ml of a mixture of 1.2 mg L-[1-^{14}C]ascorbic acid containing 50 μCi activity and 10.8 mg L-ascorbic acid dissolved in 12 ml isotonic phosphate buffer solution prepared from 0.15 M KH_2PO_4 and Na_2HPO_4 (pH 7.2). The samples were incubated at 37 °C for 3 h. The whole blood was then centrifuged at 1300 × *g* via a clinical centrifuge for 5 min and the plasma was separated from the erythrocytes. The erythrocytes were washed several times with an equal quantity of isotonic phosphate buffer solution with samples of the supernatant removed for liquid scintillation counting. When the supernatant count was equal to the background count the washed red cells were

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quantitatively transferred to a dialysis casing (length: 6 inches; open diameter: 7/16 inch; Molecular Weight Cutoff range 3500) and exhaustively dialyzed against 50 ml isotonic buffer solution at 4° C. Samples were removed from the dialysate for liquid scintillation counting until background count was reached which was an indication that reversibly bound ascorbic acid was dialyzed away from the red cells. Deionized water was added to the dialysis bag containing the red cells and the resulting solution was exhaustively dialyzed against 50 ml deionized water at 4° C. The red cells were osmotically lyzed and any ascorbic acid trapped within them was dialyzed away. Samples were removed from the dialysates until background count was reached. These latter dialysates were pooled and lyophilized. The percentage entrapment was calculated by comparing the amount of radioactivity entrapped to the total amount used in the incubation mixture. All samples for counting were prepared in 5 ml aquasol (New England Nuclear) and were counted three times against a Cesium external standard channel to 0.3% error using a Beckman Liquid Scintillation Counter (Model LS-3801). This procedure was repeated using radioactively labelled dehydroascorbic acid prepared immediately before use by oxidation of L-[1-^{14}C]ascorbic acid with bromine [8].

We found that with an incubation temperature of 37° C and an incubation time of 3 h 16.3 ± 1.1% of the original radioactively labelled ascorbic acid and 16.2 ± 1.2% of the original radioactively labelled dehydroascorbic acid were entrapped within the red cells. Since the amount of entrapment was virtually the same, whether the oxidized or reduced form of the vitamin was used, the entrapped residues from the pooled and lyophilized final dialysates were characterized in the form of KBr pellets via their infrared (Nicolet FTIR) spectra. The total lyophilized residues were used to produce the specific infrared spectra in Figs. 1 and 2. Note that the spectra are identical. This means that the entrapped moieties are struct-

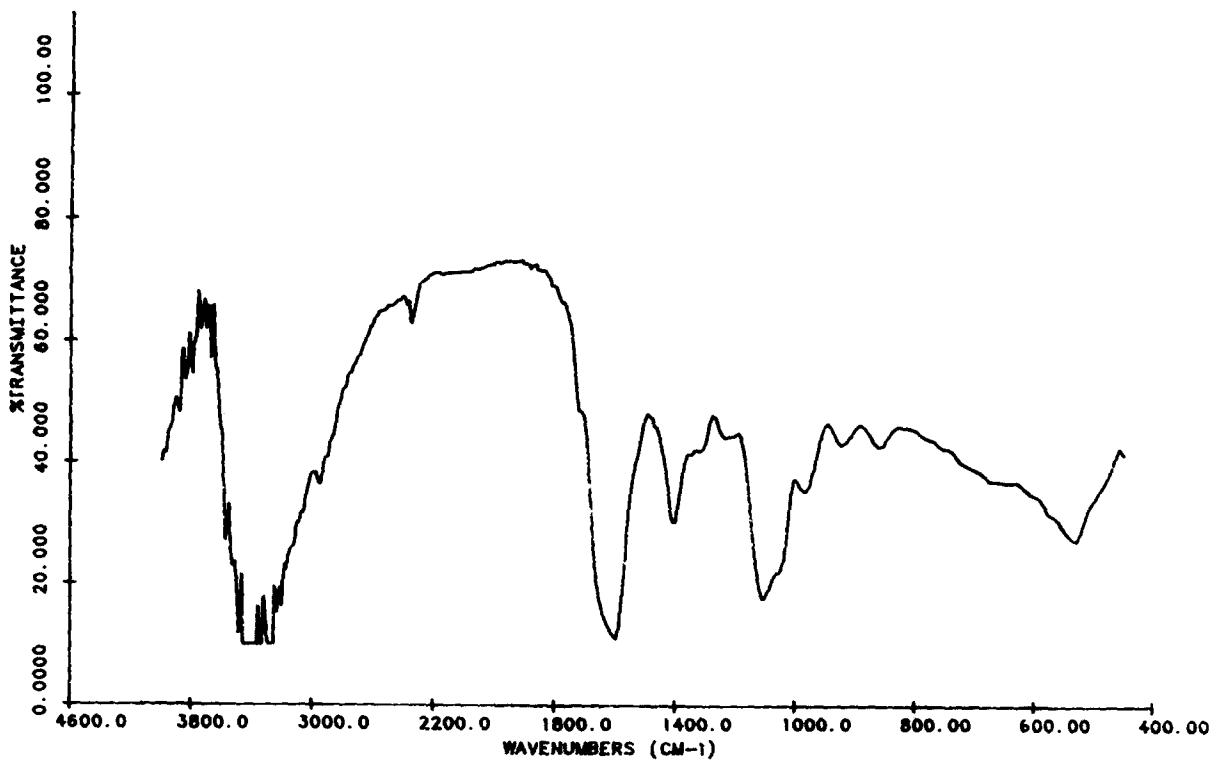

Fig. 1. Infrared spectrum of the unknown entrapped residue in the red blood cells from the pooled and lyophilized dialysates obtained after 3 h incubation of ascorbic acid with human blood at 37° C.

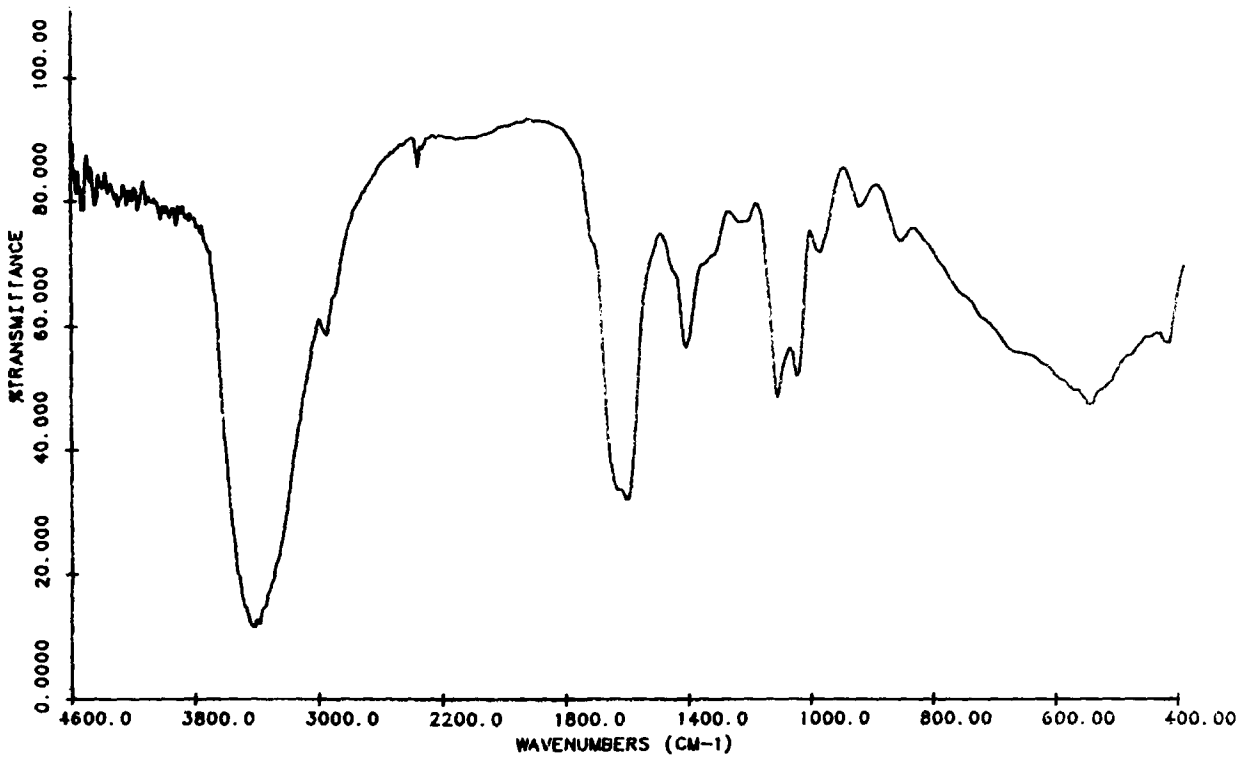

Fig. 2. Infrared spectrum of the unknown entrapped residue in the red blood cells from the pooled and lyophilized dialysates obtained after 3 h incubation of dehydroascorbic acid with human blood at 37 ° C.

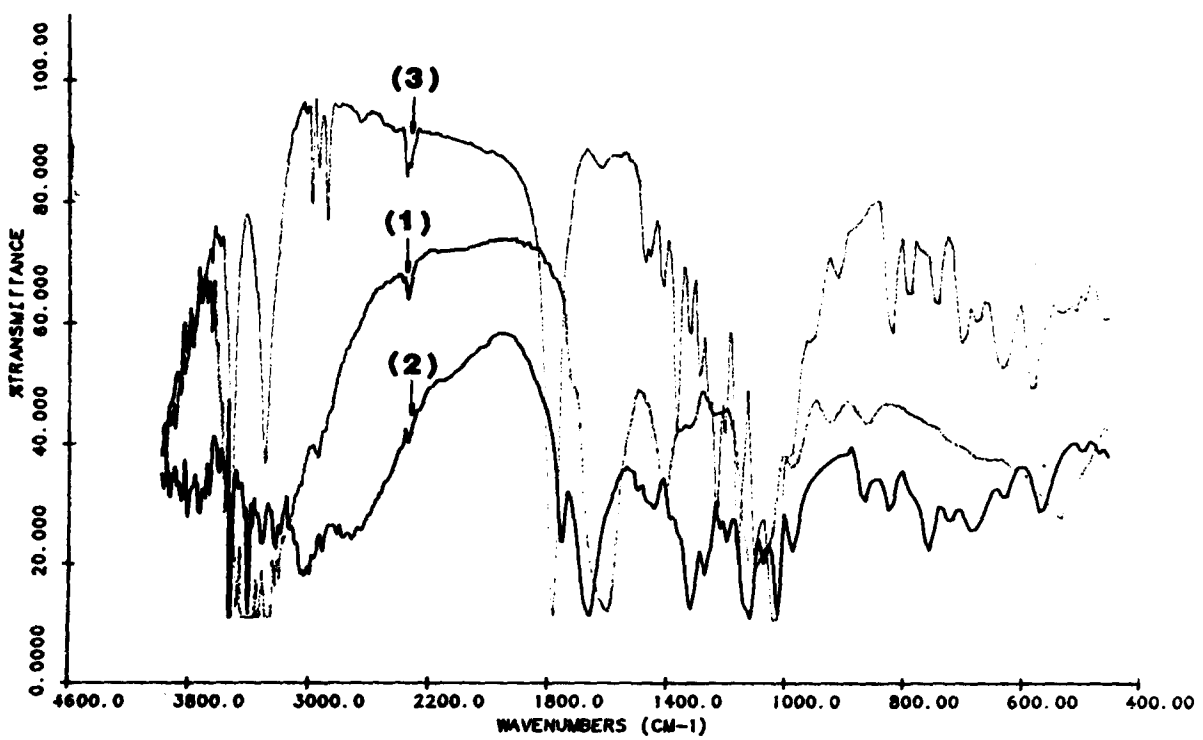

Fig. 3. Comparison of the infrared spectra of pure dehydroascorbic acid (3), pure ascorbic acid (2), and the unknown entrapped residue (1) in the red blood cells from the pooled and lyophilized dialysates obtained after 3 h incubation of ascorbic acid with human blood at 37 ° C.

urally the same no matter in which form the vitamin is initially present in the incubation mixture. A comparison of the infrared spectra of ascorbic acid, dehydroascorbic acid, and the entrapped residue from the ascorbic acid incubation mixture, as presented in Fig. 3, identified the entrapped residue as ascorbic acid.

Further experiments using radioactively labelled ascorbic acid and varying the time of incubation at 37 °C to 2 and 4 h resulted in observed entrapments of $10.2 \pm 0.9\%$ and $24.4 \pm 1.8\%$, respectively. Varying the incubation temperature to 27 °C and 10 °C for 3 h incubation time resulted in observed entrapments of $10.0 \pm 0.8\%$ and $5.6 \pm 0.6\%$, respectively. Varying the concentration of ascorbic acid in the 3 h incubation mixture by diluting the radioactive ascorbate with 2- and 4-fold excess unlabelled ascorbate did not alter the amount of labelled ascorbate entrapped; this means the rate of ascorbate entrapment was directly proportional to the amount of ascorbate present in the incubation mixture. Incubation in the presence of oubain, an active transport inhibitor, and 4,4′-dinitro-2,2′-stilbenedisulfonic acid, an anion recognition site inhibitor, had no effect on the percent entrapment of radioactive ascorbic acid.

It is concluded from the isolation and characterization data presented that ascorbic acid is entrapped in human erythrocytes in its native form, the reduced state. The degree of entrapment of the vitamin is time dependent and is not affected by active transport or anion recognition site inhibitors. This suggests that the uptake of ascorbic acid, in whatever form, is most probably a process of diffusion. Finally, the observation that the entrapment is temperature sensitive could mean that the entrapment phenomenon is enzymatically mediated.

The results of the experiments presented lead to the hypothesis that L-ascorbic acid is enzymatically oxidized on or near the surface of the red blood cell to dehydroascorbic acid which diffuses through the lipid portion of the cell wall. Once in the cell, dehydroascorbic acid is reduced back to ascorbic acid which lacks a transporter within the red blood cell membrane. It is unclear whether the oxidizing or reducing enzyme controls the rate of entrapment of ascorbic acid. Experiments are in progress to further elucidate this hypothesis.

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