Short Communications

Equilibrium constant for calcium ion and ascorbate ion

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Summary. The combination of calcium and ascorbic acid in water at 25 °C has been examined by measuring the change of free calcium ion concentration as ascorbate was added in small increment to a solution of calcium. The data show clearly that complex formation between calcium ion and ascorbate ion occurred. At ionic strength $\mu = 0.1$–0.2, the equilibrium constant of Ca^{++} and the singly-charged ascorbate ion has been measured to be 2.1 M^{-1}. The precision of the result is better than 5% and the accuracy is estimated to be better than 20%. The application of the equilibrium constants is discussed.

Since McLean and Hastings[2] demonstrated that ionic calcium is the physiologically active form of calcium in the body, there has been much concern about the effect of the binding of calcium by biological substances. It has been known that ascorbic acid forms relatively stable complexes with calcium ion[3] and the formation constant for the complex formed between calcium ion and ascorbate ion has been estimated by the equilibrium ion exchange technique.

The importance of vitamin C (ascorbic acid) in calcium metabolism and in bone formation and regeneration has been known for a long period of time, and osteoporotic patients usually show clinical or biological signs of ascorbic acid deficiency[4-9], but the role of ascorbic acid on calcium metabolism and the interaction between ascorbic acid and calcium in their metabolic processes are still largely unknown.

Because of our current research interest in the effect of large amounts of vitamin C on the absorption, bioavailability, and metabolism of calcium in humans, animals, and tissue culture, there is need for a reliable equilibrium constant for the combination of calcium and ascorbate, particularly data obtained under conditions resembling those in physiological systems. In this paper we report an equilibrium study of complex formation between calcium ion and the singly-charged ascorbate ion in water. Such a study can help to characterize the molecular environment, and thus to elucidate the bioavailability, of ionic calcium in the presence of large quantities of ascorbate in physiological systems.

In the equilibrium measurements a relatively high ionic strength background of 0.1–0.2 M NaCl was used because a) the ionic strengths in physiological solutions are in this neighborhood, b) the activity corrections change very little in the ionic range[10] between $\mu = 0.1$ and $\mu = 0.5$, c) the dissociation constants for ascorbic acid and for calcium hydroxide at high ionic strength are known[10,11], and d) ascorbic acid is more stable in solutions with high ionic strength. Calcium concentration of these solutions was chosen to be 0.01 M.

Experimental. Apparatus. The activity of free calcium ion, Ca^{++}, was measured using a calcium ion-selective electrode (Orion Research)[12-14]. Suitable pairs of electrodes were selected carefully to give stable and reproducible EMF readings. The potential of the solution was read with an Orion pH/mV meter (model 801A). The pH value of the solution was measured simultaneously with a combination electrode and a pH meter. The sample cell was a flat-bottomed cylindrical glass beaker equipped with a water jacket. A very slow stream of nitrogen was passed over the cell content throughout the measurement.

Procedure. A solution (100 ml) containing 0.01 M $CaCl_2$ and 0.1 M NaCl was titrated with 1 M sodium ascorbate solution. After each increment of ascorbate the solution was stirred for 1 min followed by a waiting period of 1 min before the EMF was read. The pH and total calcium concentration of the solution were maintained constant throughout the experiment by the addition of appropriate amounts of NaOH and $CaCl_2$.

Free Ca^{++} activity was calculated by comparison of the voltage elicited from experimental solutions with that obtained from solutions of known calcium concentrations. All experiments were carried out in duplicate or triplicate, and the producibility was excellent in all measurements.

Chemicals. All chemicals were of the highest purity commercially available. De-ionized water was distilled in all glass apparatus. Calcium standard was Orion Calcium Standard 92-20-06.

Results and discussion. Measurement of combination constants. In aqueous solution, ascorbic acid undergoes ionization to form the singly- and doubly-charged anions. Each of these anioic species may couple with calcium ion to form a complex of the 1:1 type. The individual reactions are shown as follows:

$$Ca^{++} + AH^- \leftrightharpoons CaAH^+ \qquad \text{eq. 1}$$

$$Ca^{++} + A^= \leftrightharpoons CaA \qquad \text{eq. 2}$$

Here, Ca^{++} is the free calcium ion, AH^- and $A^=$ are the singly- and doubly-charged ascorbate ion respectively and $CaAH^+$ and CaA are the 2 corresponding calcium complexes.

Following eqs 1 and 2, we define the equilibrium constants K_{CaAH^+} and K_{CaA} by eqs 3 and 4,

$$K_{CaAH^+} = [CaAH^+]/[Ca^{++}][AH^-] \qquad \text{eq. 3}$$

$$K_{CaA} = [CaA]/[Ca^{++}][A^=] \qquad \text{eq. 4}$$

where all the terms are molar concentration.

In the determination of the equilibrium constant of calcium ion and ascorbate ions, it was convenient to measure the calcium activities for a series of solutions containing a fixed

Table 1. Experimental values of calcium activity at various sodium ion concentrations (values are normalized to $[Na^+] = 100$ mM)

Na^+ (mM)	Calcium activity (%)
100	100
120	95.3
140	92.6
160	90.8
180	89.3
200	88.0
220	87.0
240	86.1
260	85.3
280	84.6
300	84.0

concentration of total calcium and various concentrations of added sodium ascorbate. The calcium ion activities were measured, at pH 7.5 at a background ionic strength $\mu = 0.1$–0.2 M NaCl, with a calcium ion-selective electrode as described in the experimental section.

Stability of ascorbate. In analyzing equilibrium reactions, we must consider the stability of each species in solution. The decomposition of ascorbate in dilute solution at neutral pH in the presence of oxygen is rapid[15]. An experiment was carried out in order to study the stability of ascorbate in a relatively concentrated solution under our experimental conditions.

A solution containing 0.1 M sodium ascorbate and 0.1 M NaCl at pH 7.5 was analyzed for ascorbate content immediately after preparation and 1 h later using high pressure liquid chromatography technique with electrochemical detection[16]. The loss in reducing power of ascorbic acid in one hour was found to be less than 3%.

Calculation of calcium ion concentration [Ca^{++}]. Sodium ion when present at high level is an electrode interference and will reduce calcium ion activity. Control experiments were conducted at pH 7.5 in order to establish the relationship between calcium concentration and calcium activity at various NaCl concentrations. Data in table 1 show that calcium activity decreases with increasing sodium ion concentration. Thus, calcium ion concentration at each titration point was calculated using the observed value of calcium activity and table 1.

Calcium hydroxide was known to be incompletely dissociated in water[11,17] and the dissociation constant for the reaction $CaOH^+ \rightleftharpoons Ca^{++} + OH^-$ is 4×10^{-2} at ionic strength $\mu = 0.1$ M. At pH 7.5, the concentration of $CaOH^+$ is negligibly small, therefore, no correction was needed in these calculations.

Calculation of K_{CaAH^+}. At pH 7.5, ascorbic acid has almost completely undergone a single ionization, with 99.9% of the ascorbate as AH^-. The principal equilibrium of ascorbate with calcium at that pH involves the formation of the singly-charged cationic complex, $CaAH^+$, that results from the combination of calcium ion and the singly-charged ascorbic acid. On this basis and neglecting the second dissociation of ascorbic acid, the combination constant for the calcium ion and the singly-charged ascorbate ion was calculated with data obtained at pH 7.5 and eq. 3.

To evaluate [AH^-], we used eqs 5 and 6.

$$[AH^-] = K_1[AH_2]/[H^+] \qquad \text{eq. 5}$$

$$[AA] = [AH_2] + [AH^-] + [CaAH^+] + [CaA] \qquad \text{eq. 6}$$

and the justified assumption that the amount of AH_2 or CaA is negligibly small. Thus, $[AA] = [AH^-] + [CaAH^+]$ where AH_2 is the un-ionized ascorbic acid, AA is the total amount of ascorbate added, H^+ is the hydrogen ion activity and the first dissociation constant of ascorbic acid, pK_1, is taken to be 4.05 at $\mu = 0.1$ and 25 °C[10].

The activity of calcium coupled with data for the relationship between Ca^{++} activity and concentration (table 1) enabled us to evaluate the concentration of calcium, [Ca^{++}]. Subtracting [Ca^{++}] from the amount of total calcium in the solution, we obtained [$CaAH^+$]. Details of the calculation of K_{CaAH^+} are listed in table 2.

It is of interest to note that the calculated K values in table 2 decreases with increasing sodium ascorbate and approaches a minimum of 1.9 M^{-1}. A reasonable explanation is that when the concentration of sodium ascorbate is very high, the ascorbic acid is probably not completely dissociated, therefore relatively less ascorbate ion is available for the combination with calcium.

Another equilibrium. A calcium-ascorbate complex of the formula $Ca(AH)_2$ might arise from the ionic association of a calcium ion and two singly-charged ascorbate ion (eq. 7) when the concentration of AH^- is very high.

$$Ca^{++} + 2AH^- \rightleftharpoons Ca(AH)_2 \qquad \text{eq. 7}$$

However, this theory must be rejected under the present experimental conditions because analysis of data in table 2 shows that the calculated values of K_{CaAH^+} is smaller when the concentration of AH^- is higher. This implies that excess of ascorbate does not tie up more calcium ion. Thus, it is very unlikely that the equilibrium of eq. 7 is important.

Theoretical calculation of bound calcium in body fluids. The calcium in the plasma is present in 3 different forms. Approximately 40% of the calcium is combined with the plasma proteins, 15% of the calcium is complexed with small molecules (for instance, citrate) and about 45% of the plasma calcium is ionized. This ionic calcium is important for most functions of calcium in the body[2].

Our data permit the estimation of the amounts of ascorbate bound calcium in biological systems. To take an example, the value of K_{CaAH^+} in table 2 in conjunction with knowledge of ascorbate concentration in blood and urine[18,19] enables us to obtain the amounts of calcium ion bound by ascorbate ion in these body fluids. At pH 7.5 and somewhat lower pH's, at which ascorbic acid has undergone a single ionization, the ratio of bound Ca^{++} to free Ca^{++} in blood or urine is simply the product of the concentration of ascorbate and the equilibrium constant, K_{CaAH^+}. In highly alkaline urine with pH values between 8.5 and 9, [AH^-] is still far greater than [$A^=$], only 0.06% of the ascorbate exists as [$A^=$], which can be neglected in the calculation.

Thus, in theory, a very small fraction of calcium would be bound by ascorbate in the blood and in urine containing a small amount of ascorbate. When the concentration of ascorbate in the blood is about 1.5 mg per 100 ml blood or 85 µM, about 0.02% of calcium would be bound by ascorbate. When the concentration of ascorbate in the urine is about 0.4 mM, about 0.1% of calcium would be bound by ascorbate ion. However, in the urine of people taking large amounts of vitamin C, 10 g per day or more, the concentration of ascorbate ion would be about 2 g/l or 11 mM. The theoretical amount of bound calcium in the urine would be 3% of total ionized calcium. Further, in the blood of people receiving large quantities of ascorbate by i.v. infusion, the concentration of calcium bound by ascorbate would be appreciably high.

Table 2. Data for the calculation of K_{CaAH^+} at pH 7.5 (all values are concentration)

AH^- (mM)	Ca^{++} (mM)	$CaAH^+$ (mM)	K_{CaAH^+} (M^{-1})
0	10	0	
10.5	9.68	0.32	3.1
19.6	9.56	0.44	2.3
30.3	9.33	0.67	2.4
46.5	9.16	0.84	2.0
56.6	8.96	1.04	2.1
69.0	8.83	1.17	1.9
80.2	8.66	1.34	1.9
90.9	8.53	1.47	1.9
102	8.37	1.63	1.9
111	8.23	1.77	1.9
123	8.08	1.92	1.9
		Mean ± SD	2.1 ± 0.4

1 Acknowledgments. The author wishes to thank Professor Linus Pauling for his advice during the course of this study.

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0014-4754/84/020168-03$1.50 + 0.20/0

The sterols of two hadromerida sponges[1]

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Summary. Sterols were extracted and identified from 2 marine sponges, *Aaptos aaptos* and *Suberites domuncula.* The sponges contained conventional C_{26}–C_{30} sterols with a saturated ring system. Minor amounts of cholest-7-en-3β-ol and cholesterol were also present. Cholestanol and 24-ethylcholestanol were the major components of the sterol mixtures.

Sponges are rich sources of complex mixtures of sterols and the components of the mixtures seem to be of dietary origin and products of chemical transformation subsequent to ingestion[2-4]. Stanols are found in sponges belonging to the family Suberitidae[5,6], Chondrosiidae[7], Stellettidae[8], Desmacidonidae[9], Hymeniacidonidae[10,11], Halichondriidae[11], Nepheliosphongiidae[12], Axinellidae[13,14] and Geodidae[15]. We report here the sterol composition of 2 marine sponges *Suberites domuncula* and *Aaptos aaptos* as a part of our work on the sterols from sponges[16].

Materials and methods. Sponges *Aaptos aaptos* (order Hadromerida, family Tethyidae) and *Suberites domuncula* (order Hadromerida, family Suberitidae) were collected in the Bay of Naples and supplied by the Zoological Station of Naples. Each sponge was extracted (3 times) with acetone for 3 days. Solvent was removed and the resulting suspension extracted with diethyl ether. After evaporation, the oily residue was chromatographed on a silica gel column using CH_2Cl_2 as eluent. The crude sterol fraction, after acetylation with Ac_2O-pyridine (1:1), was further purified by SiO_2 column using as eluent 40–70° light petroleum-C_6H_6 (7:3). Steryl acetates were separated by column chromatography on silica gel impregnated with $AgNO_3$ which was eluted with light petroleum containing increasing amounts of C_6H_6. The various column fractions were monitored by GLC and combined accordingly. Some fractions were further subjected to preparative silver nitrate-silica gel TLC. Each fraction was analyzed by capillary GLC, GC-MS, NMR and coinjection with previously identified sterols.

Combined GC-MS analysis was performed on a LKB 2091 S GC-MS instrument. An SE-30 (30 m) fused silica capillary column (J & W Scientific) programmed from 200 to 260 °C (6 °C/min) was used. Analytical GLC of steryl acetates was performed with a C. Erba Fractovap 4160 gas chromatograph using a DB-1 glass capillary column (30 m; J & W Scientific) at 270 °C. ^{1}H-NMR spectra were recorded with a Brucker WH-270 in $CDCl_3$ and TMS as internal reference.

Results and discussion. The table lists the sterols isolated

Sterol composition of sponges (%)

Sterol	RRT*	*S. domuncula*	*A. aaptos*
(22E)-24-nor-5α-Cholest-22-en-3β-ol	0.70	1.1	0.1
(22E)-27-nor-24-Methyl-5α-cholest-22-en-3β-ol	0.90	1.2	0.3
(22E)-5α-Cholest-22-en-3β-ol	0.94	4	0.9
5α-Cholestan-3β-ol	1.03	72.9	66.4
24-Methyl-5α-cholestan-3β-ol	1.31	2.3	2.1
(22E)-24-Methyl-5α-cholest-22-en-3β-ol	1.13	4	2
5α-Ergost-24(28)-en-3β-ol	1.26	3	0.3
24-Ethyl-5α-cholestan-3β-ol	1.59	6	25
(22E)-24-Ethyl-5α-cholest-22-en-3β-ol	1.41	0.8	0.4
(24E)-5α-Stigmast-24(28)-en-3β-ol	1.58	0.8	0.7
(24Z)-5α-Stigmast-24(28)-en-3β-ol	1.63	0.5	0.7
(24E)-24-Propylcholest-24(28)-en-3β-ol	1.78	0.1	0.1
(24Z)-24-Propylcholest-24(28)-en-3β-ol	1.88	0.3	0.2
5α-Cholest-7-en-3β-ol	1.12	2.5	0.6
Cholest-5-en-3β-ol (cholesterol)	1.00	0.4	0.1

* RRT, retention time of acetate derivatives relative to cholesteryl acetate.