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Polarographic measurement of ascorbate washout in isolated perfused rabbit hearts

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Abstract

To study the myocardial washout of ascorbate, the applicability of polarographic detection of ascorbate ions by a platinum electrode (sensitive area 0.03 mm²) was investigated, in both a calibration setup (sampling flow along the electrode: 100 $\mu\text{l}\cdot\text{s}^{-1}$) and isolated, retrogradely perfused rabbit hearts. In the calibration setup at pH 7.4, the sensitivity of the electrode was 70 $\mu\text{A}/\text{mol}$. This sensitivity increased moderately with increasing pH (13%/unit pH) and increasing sampling flow rate (14% at an increase from 100 to 150 $\mu\text{l}\cdot\text{s}^{-1}$). In the isolated hearts, ascorbate infused into the aorta was detected in a right ventricular drain by the electrode as well as by the use of ¹⁴C-labeled ascorbate. Both recorded time courses were similar except for a scaling factor dependent on flow velocity. During continuous infusion the arteriovenous difference of ascorbate was $2 \pm 2\%$ (SD), indicating a relatively low consumption of ascorbate by the isolated heart. We conclude that polarographic measurement of ascorbate in the coronary effluent of an isolated rabbit heart can be performed on-line and relatively easily.

Keywords

coronary circulation; electrode; radioactive label

DIFFUSION AND CONSUMPTION characteristics of substances within and the amount of flow through tissue can be estimated from venous outflow concentration-time curves following a bolus injection of the substance into the perfusing artery (2, 3). Within the tissue three regions (blood, interstitium, and parenchymal cells) separated by two barriers, (capillary wall and parenchymal cell membranes) can normally be distinguished (4, 15). Large molecules such as albumin transverse the capillary barrier so slowly that they serve as intravascular reference markers. Small molecules and ions diffuse through clefts between endothelial cells to enter the interstitial space. Lipid-soluble molecules can penetrate cell membranes, but most substrates and ions obtain entry via specialized transport sites. Very small lipid-soluble molecules (e.g., H₂, O₂, H₂O, Xe) diffuse so fast that counter-current exchange from artery to veins has to be considered (2, 5, 14).

Generally, most substances employed in tracer washout studies are detected by a sampling system using radioactive labeling. For some substances (e.g., indocyanine green, Evans blue) optical detection can be used, but in whole blood the sensitivity is usually limited due to the abundance of red blood cells disturbing the optical measurement. The use of

substances that can be detected directly in the venous effluent with sensitive electrodes may also overcome the necessity of sampling. In this respect ascorbate is worthwhile to consider because it can be detected continuously by a polarographic method, using a platinum or glassy carbon electrode. Ascorbate has been used as a tracer for the detection of left-to-right shunts in the human circulation (10) and for the measurement of cardiac output in piglets (16).

Our interest in the washout of ascorbate from cardiac muscle originates from the similarity between ascorbate and glucose, as far as molecular weight (glucose 180, ascorbate 176) and diffusion and consumption characteristics are concerned. Both D- and L-isomers of these substances will enter the interstitial space, but only D-glucose and L-ascorbate cross the cellular membrane rapidly. Their stereoisomers L-glucose (8) and D-ascorbate (12) lack specific transporters to facilitate crossing of the plasmalemma. Clinical application of glucose washout curves is hampered by the fact that the technique most commonly used to obtain glucose washout curves relies on the detection of a radioactive label in sampled volumes. The present study was initiated to investigate whether ascorbate rather than glucose can be used to obtain information about the physiologically important glucose transport system. In the clinical setting the use of ascorbate is advantageous because the dose needed is not toxic, whereas polarographic detection can be applied in patients.

In the first part of the present study the method for measuring ascorbate concentration by polarographic detection was evaluated in a calibration setup. Polarographic detection or, more precisely, amperometric detection of ascorbate using a platinum electrode is based on its property of easy oxidation by means of absorption of electrons by the platinum surface, causing an electric current through the electrode (9). In saline buffered to pH 7.4, ascorbic acid largely ionizes to ascorbate⁻ (pK 4.17), a small fraction of which ionizes further to ascorbate²⁻ (pK 11.57; Ref. 17). Free oxygen easily oxidizes ascorbate²⁻ to dehydroascorbate (1). Accordingly, oxidation of ascorbate by free oxygen strongly depends on the pH of the solution. Therefore, we investigated whether polarographic detection of ascorbate by a platinum electrode is also based on oxidation of ascorbate²⁻. If this is the case, at a fixed ascorbate concentration the electric current, as detected through the platinum electrode, would decrease considerably with decreasing pH. This was investigated in the present study.

The polarographic detection of ascorbate was also evaluated in experiments on Tyrode-perfused isolated rabbit hearts. The ascorbate concentration in the effluent was simultaneously measured by the polarographic method and by detection of the radioactivity of [¹⁴C]-ascorbate. This type of test is important because ascorbate is labile, and hence the effluent concentration-time curves of the ¹⁴C label will differ from the time course of polarographically detected, unchanged ascorbate, if the substance changes chemically during passage through the heart. Information about the possible loss of ascorbate in the myocardial tissue was obtained by measuring arteriovenous differences in ascorbic acid concentration during constant perfusion with ascorbic acid-containing perfusate.

METHODS

Polarographic detection of ascorbate

The platinum electrode (Fig. 1) consisted of a platinum wire (OD 0.2 mm) surrounded by shrink tubing. The sensitive area at the tip was made by cutting the tip with a scalpel. The Ag-AgCl reference electrode (Fig. 1) consisted of a silver wire (OD 0.3 mm, bare length 30 mm) in a tube (ID 1 mm; length 150 mm), filled with 0.1 N KCl solution. The silver electrode was coated with AgCl by directing a 1 mA DC current from the electrode into the KCl solution until the silver turned dark. The KCl solution was separated from the perfusate by a cotton wick at the end of the tube.

The current through the platinum electrode was measured with a current amplifier (Fig. 2) with a second-order, low-pass filter at the input (cutoff frequency 2 Hz, -6 dB, slope 12 dB/octave). The Ag-AgCl electrode was held 400 mV negative with respect to the platinum electrode, using a stainless steel auxiliary electrode for current feedback. In this way, a DC current through the AgCl electrode could be prevented. The preamplifier was completely insulated from the recording system by optocouplers for the signal and a low capacitance transformer for the power supply.

Experiments in the calibration setup

In the calibration setup a continuous flow ($0.1 \text{ ml}\cdot\text{s}^{-1}$) was withdrawn by a roller pump from a reservoir containing calibration fluid with known ascorbic acid concentration. This sampling flow passed a narrow tube (ID 1 mm; length 200 mm), which was connected to another, wider tube (ID 2 mm). Ten millimeters distal to the site of widening, the platinum electrode was positioned from the side through a hole in the wall of the tube so that the platinum tip was in contact with the flowing fluid. Thirty millimeters distal to the platinum electrode the tip of the tube of the Ag-AgCl electrode was in contact with the fluid via a T piece (ID 2 mm). Between T piece and roller pump a stainless steel needle serving as auxiliary electrode was inserted through the tubing wall into the fluid. The whole system was kept at 37°C by a water bath.

A stock solution of ascorbate (0.448 M) was made by dissolving 395 mg ascorbate and 248 mg NaHCO_3 in 3 ml of distilled water. The pH of this solution was adjusted to 7.4 by adding NaOH. Then $100 \mu\text{l}$ of a NaHSO_3 solution (50 mg/ml) and $100 \mu\text{l}$ of a Na_2EDTA (ethylenediaminetetraacetic acid) solution (0.25 mg/ml) were added. The pH was brought to 7.4 again, and the final volume of the stock solution was brought to 5 ml with distilled water. For calibrating the electrode this stock solution was first diluted 1:500 in 0.9% NaCl to give a 0.9 mM solution. Nine successive 1:2 dilutions provided calibrating solutions down to $1.8 \mu\text{M}$.

Variations in pH were obtained by mixing *solution A* ($9.08 \text{ g}\cdot\text{l}^{-1} \text{KH}_2\text{PO}_4$, $9.0 \text{ g}\cdot\text{l}^{-1} \text{NaCl}$) and *solution B* ($11.87 \text{ g}\cdot\text{l}^{-1} \text{Na}_2\text{HPO}_4$, $9.0 \text{ g}\cdot\text{l}^{-1} \text{NaCl}$) in different proportions. At an A-to-B ratio of 10/90, 25/75, 50/50, 75/25, and 90/10 the pH was measured to be 5.68, 6.20, 6.70, 7.20, and 7.72, respectively. For each of these values of pH the electrode current was measured at ascorbate concentrations of 0.896, 0.448, and 0.224 mM. These measurements

were performed immediately after the test fluids were mixed because ascorbate has limited stability, especially in the presence of oxygen at high pH values.

During each experiment the electrode background current at zero ascorbate concentration was measured. The current ascribed to ascorbate was obtained by subtracting the measured current from the background current. Generally the background current was in the order of 0.2 nA.

Experiments on isolated hearts

The experiments were performed on isolated Tyrode-perfused rabbit hearts (6). The Tyrode solution consisted of (in mM) Na^+ 147.43, K^+ 5.37, Ca^{2+} 1.80, Mg^{2+} 0.49, Cl^- 133.15, HCO_3^- 23.81, H_2PO_4^- 0.42, D-glucose 5.0, and EDTA 10 μM and was saturated with a mixture of 95% O_2 -5% CO_2 . The heart was beating spontaneously. Right and left ventricle were drained separately (Fig. 3). The ascorbate concentration was measured in the tube draining the right ventricle. The platinum electrode was put in position through the right atrium. The Ag-AgCl reference electrode was placed in the left ventricle through the left atrium. The stainless steel current feedback electrode was pierced through the wall of the right atrium. No external work was performed because of left ventricular drainage. Pressure in the aortic canula was measured using a Statham P23Db transducer. Retrograde aortic volume flow was controlled by a roller pump. Volume flow in the coronary arteries was measured by collecting the effluent from the right ventricle in a graduate cylinder.

At each of various levels of coronary arterial flow, a bolus of 0.1 ml of a 9.0 mM ascorbic acid solution (1:49 dilution of ascorbic acid stock solution in saline) was injected into the aortic canula. The platinum electrode current was recorded at a paper speed of 10 $\text{mm}\cdot\text{s}^{-1}$. In three experiments the response to radioactively labeled L-[1- ^{14}C]ascorbic acid (New England Nuclear) was measured in sequential samples from the right ventricular drainage for comparison with the simultaneously obtained polarographic response. The effluent samples were collected in test tubes in a rotating collector at intervals of 1.0 s (or at 0.4 s when coronary flow was high). After the first 30 samples, the outflow was sent to a second 30-tube rotating collector with the sampling frequency reduced to one-fifth of the original sampling frequency. After the experiments, 200 μl was pipetted from each sample into liquid scintillation vials, 10 ml of scintillation fluid was added, and the counts were obtained in a Nuclear Chicago Mark II counter using appropriate quench and efficiency corrections for aliquots of the injectate and for the samples.

In four separate experiments the possible loss of ascorbic acid in the heart was assessed during constant infusion (5.9 $\mu\text{l}\cdot\text{s}^{-1}$) of ascorbic acid solution (11 mM, 1:39 dilution of stock solution in saline) into the fluid perfusing the aorta. The ascorbic acid concentration was measured in the aorta and in the right ventricular drain, using the polarographic sampling system employed for calibration of the electrode. With the switch of a stopcock, samples were taken alternately from the inflow and the outflow.

RESULTS

Experiments in the calibration setup

At a pH of 7.4 the current through the platinum electrode appeared to be linearly related to the ascorbate concentration (Fig. 4) with a calibration factor of 68 ± 2 (SD) $\mu\text{A}/\text{M}$. The lowest measurable concentration was approximately 10^{-6} M. The sensitivity of the platinum electrode to an increase in pH was relatively small. An increase in pH of 1.0 results in an increase in sensitivity of $13 \pm 6\%$ (SD; $n = 6$). This change in sensitivity was opposite to what was expected on the basis of a proportionality of the polarographic current to the ascorbate $^{2-}$ concentration. In the calibration setup used, the electrode sensitivity to ascorbate increased with increasing flow through the measuring device (Fig. 5). Above a sample flow of $70 \mu\text{l}\cdot\text{s}^{-1}$, the sensitivity is only moderately dependent on flow. In terms of shear rate, with the assumption of a parabolic flow profile at a sample flow of $100 \mu\text{l}\cdot\text{s}^{-1}$, flow velocity in the center of the 2-mm measuring tube is $64 \text{ mm}\cdot\text{s}^{-1}$, and shear rate at the wall where the electrode is located is 128 s^{-1} .

Experiments on isolated hearts

Three illustrative curves from one heart at three different flow levels are shown in Fig. 6. The electrode curves (continuous lines) were scaled to the initial upslope and peak regions of the tracer curves (symbols) for presentation. The rationale for this was that in the first seconds no significant transformation of ascorbate into a form not detectable by the electrode was expected. Therefore, any difference between the tracer curves and the electrode curves was solely due to the dependence of the electrode calibration on the flow. The shape of the electrode and ^{14}C curves was found to be essentially identical during the first 10 s. The scaling factors for superimposition (given in the legends to Fig. 6) indicate an increase in electrode sensitivity with increasing flow, which is also suggested by the data obtained in the calibration setup (Fig. 5).

The electrode and tracer curves obtained at the higher flow levels are essentially similar, but the curves obtained at the lowest flow levels are dissimilar, the polarographic method indicating less ascorbate. At this flow of $39 \mu\text{l}\cdot\text{g}^{-1}\cdot\text{s}^{-1}$, the reduction in the tail amounts to about 8% of the area.

In the separate set of four experiments with flow levels in the range from 50 to $130 \mu\text{l}\cdot\text{g}^{-1}\cdot\text{s}^{-1}$, the loss of ascorbate in the heart was $2 \pm 2\%$ (SD, $n = 12$) when the ascorbate concentration in the perfusate was in the range of 100–500 μM . At lower concentrations of ascorbate in the perfusate (5–100 μM), obtained by lowering the ascorbate concentration of the infusion liquid, this loss slightly increased ($P = 0.05$) to $4 \pm 3\%$ ($n = 14$). At low flow levels, induced by perfusion pressures below 50 mmHg, no reliable data could be obtained because then the settling time for the concentration levels appeared to be so long that variations in perfusion pressure and flow occurred, probably due to induction of ischemia.

DISCUSSION

The ascorbate concentration in the effluent of a Tyrode-perfused isolated rabbit heart can be measured polarographically and on-line, using a platinum electrode with a surface area at

the tip of 0.03 mm². The sensitivity of the electrode to ascorbic acid concentration depends on temperature, pH, and flow velocity along the electrode.

The dependency on pH is opposite to and much less pronounced than what was expected, when postulating proportionality of the polarographic current to the concentration of ascorbate²⁻. The present measurements suggest that the polarographic current is proportional to the univalent ascorbate⁻ concentration because at pH 7.0 this ion is the most abundant form of ascorbate due to its low pK value (pK 4.17; Ref.17). This concentration is nearly independent of pH in the range of pH investigated. The dependency on pH is so small that a normally occurring arteriovenous pH difference of 0.03 is associated with a difference in ascorbate sensitivity of only 0.4%. Even in the Tyrode-perfused heart the arteriovenous difference in ascorbate sensitivity due to differences in pH are minor.

The enhancement in sensitivity of the ascorbate measuring system with an increase in fluid velocity along the electrode surface is inherent to the presence of convective transport and lateral diffusional transport close to the electrode surface. In the immediate neighborhood of the electrode surface, where flow velocity is low, ascorbate transport is diffusional rather than convective; at larger distances the opposite is the case. The thickness of the diffusional boundary layer diminishes with increased convective transport. The ascorbate concentration drop from the bulk to the electrode surface is proportional to the product of the boundary layer thickness and the ascorbate consumption rate at the surface. The higher this concentration drop the higher the sensitivity to changes in flow velocity will be.

Accordingly, Oeseburg and co-workers (13) found an increase of flow sensitivity at higher polarizing voltage, the latter being associated with a higher ascorbate consumption rate. A compromise between sufficiently high measuring sensitivity and low sensitivity to flow changes can be found in a polarizing voltage of 400 mV (13). In the Clark electrode commonly used for polarographic oxygen detection, the sensitivity to flow changes is suppressed by fixation of the diffusional boundary layer by covering the electrode surface with a porous membrane. To keep the electrode as small and simple as possible we have not used a membrane.

Under steady-state conditions in the isolated heart, temperature, pH, and flow are constant during the recording period of an ascorbic acid concentration curve, so the electrode current is proportional to the ascorbic acid concentration. When sampling with a constant flow and with the electrode positioned in the sampling tube, flow dependency is eliminated. At low flow, in the tail of the curve less ascorbic acid is detected polarographically than by the use of the radioactive label. This loss of ascorbic acid might result from oxidation to dehydroascorbic acid, which is probably more pronounced after a longer traveling time through the tissue. The ¹⁴C-label will always be detected because it remains within the molecule during oxidation. The decrease in ascorbate as detected in the venous effluent cannot be explained by a change in pH. At low flow, after all, the pH of the perfusate decreases during passage through the coronary circulation, resulting in an increase in sensitivity of the polarographic detection. Then the ascorbate concentration as detected would have been increased rather than reduced.

Peak ascorbate concentrations as measured (4×10^{-5} M) are within the physiological range of plasma concentrations (2×10^{-6} to 10^{-4} M; Refs. 6, 7, 10). In the present setup no changes in perfusion pressure and flow were detected, indicating that no important changes in coronary vascular resistance were induced at this high level of chemical activity.

In conclusion, polarographic measurement of ascorbic acid in the coronary effluent of an isolated perfused rabbit heart can be performed on-line and relatively easily. The electric current through the ascorbic acid-sensitive platinum electrode represents the ascorbic acid concentration except for an unknown scaling factor depending on temperature and flow velocity close to the electrode. Under physiological circumstances, changes in sensitivity due to changes in pH can be neglected. At normal perfusion pressures loss of ascorbic acid from the coronary circulation of the isolated heart is approximately 2%. With the use of a sampling system with constant sampling flow, the flow dependency of the polarographic method to detect ascorbic acid can be eliminated.

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FIG. 1.

Design of the electrodes. *Upper panel:* platinum electrode. A, platinum wire; B, heat shrink insulation; C, wire connection. *Lower panel:* Ag-AgCl reference electrode. D, cotton wick; E, 0.1 N KCl solution; F, chlorided silver wire; G, wire connection; H, tubing filled with KCl solution; J, stopcock.

FIG. 2.

Current amplifier circuit for electrodes. Pt, platinum electrode; Ag-AgCl, silver chloride reference electrode; FB, auxiliary current feedback electrode.

FIG. 3.

Schematic representation of the experimental setup for obtaining ascorbate outflow dilution curves via platinum electrode detection and sequential sampling for measurement of tracer concentration-time curves. Indicator is injected or infused into cannulated aorta of isolated, perfused rabbit hearts. Sample collection tubes are translated by a rotating collector.

FIG. 4.

Calibration of electrode system for ascorbic acid in calibration setup. Electrode current as a function of ascorbic acid concentration.

FIG. 5. Calibration of electrode system for ascorbic acid in calibration setup. Sensitivity of electrode current to changes in sampling flow velocity.

FIG. 6.

Ascorbate outflow dilution curves from an isolated perfused rabbit heart following aortic injection at 3 different flows. *Continuous lines*, polarographic detection. Symbols: [^{14}C]ascorbate at flows of 39 ($\circ$), 103 ($\bullet$), and 147 ($\square$) $\mu\text{l}\cdot\text{g}^{-1}\cdot\text{s}^{-1}$. Scale factors for multiplying each electrode curve to superimpose [^{14}C]ascorbate curves were 5.3, 5.4, and 3.7 counts/pA, respectively. Perfusion pressures at various flow levels were 35, 85, and 125 mmHg. Lower apparent concentration by Pt electrode in tail portion of curve obtained at lowest flow suggests a small degree of chemical transformation.