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Capillary zone electrophoresis with electrochemical detection — a simple and effective method to analyze oxygen-consuming and peroxide-forming processes

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Abstract

Numerous biological, chemical and photochemical processes consume molecular oxygen, thereby forming peroxides, hydroperoxides and even hydrogen peroxide. This paper describes a new system for monitoring quasi simultaneously oxygen uptake and peroxide formation. For this purpose capillary electrophoresis with electrochemical detection was connected to a new automated injector that periodically injects a sample of the reaction mixture into the capillary electrophoresis system without any contact with the atmosphere. The device was tested with some pharmacologically relevant reactions such as enzymatic oxidation of glucose catalyzed by glucose oxidase, enzymatic lipid oxidation by lipoxygenase and the photodynamic reaction of the anti-inflammatory drug ketoprofen. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Injection methods; Instrumentation; Peroxides; Lipid hydroperoxides; Hydrogen peroxide; Enzymes; Ketoprofen; Profens

1. Introduction

Molecular oxygen is involved in many chemical and biochemical oxidation processes in nature. In most of these reactions electrons are transferred stepwise to the oxygen molecule, producing superoxide and subsequently peroxides [1]. The oxidation reactions are accelerated by heavy metals or through photochemical activation [2]. Moreover, many enzymes that catalyze the specific formation of various peroxides (hydrogen peroxide, lipid peroxides) exist in nature.

Generally, the reaction rate or the activity of such enzymes can be estimated by the oxygen consumption or the rate of the formation of the products. Oxygen uptake is determined in most cases by a Clark electrode [3]. The products are measured by photometry in simple cases. Various peroxides can be analyzed ‘off-line’ by HPLC after the reaction is frozen.

Since oxygen as well as many peroxides can be detected by an electrochemical detector in the reductive mode, both can be quantified after separation. Capillary electrophoresis with its high resolution power and short run times is very suitable [4] for this

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purpose and can easily be combined with electrochemical detection (ED). To utilize the oxygen peak as an analytical signal in capillary electrophoresis, it must be guaranteed that no oxygen from the ambient air is introduced into the reaction mixture or the capillary. Therefore, a device had to be constructed that periodically injects a sample from the reaction mixture into the capillary, without any contamination by the atmosphere. The reaction partners are immediately separated, so freezing of the reaction is not necessary. The very low injection volumes do not produce any perturbation of the reaction mixture. Compared to the indirect determination of reactive oxygen species (ROS) in capillary electrophoresis, based on the formation of a fluorescent dye [5], the ED method is straightforward and allows differentiating between the various peroxides. Molecular oxygen migrates with the electroosmotic flow (EOF), whereas hydrogen peroxide forms negatively charged complexes in the borate containing buffer and leaves the capillary well separated after the oxygen.

2. Experimental

2.1. Apparatus

The oxygen exchange between the reacting system and the ambient air was prevented by the device shown in Fig. 1. It essentially consists of two chambers manufactured from Perspex. The volume of each chamber is about 3 ml and the outer dimensions are 25×25×60 mm^3. The two chambers are coupled by a narrow bore connection with a septum in the middle. The capillary (uncoated fused silica, inner diameter 25 μm, length 50 cm (Supelco, Deisenhofen, Germany) can be moved by a stepper motor back and forth between the anode chamber, which contains the separation buffer, and the chamber containing the reaction mixture. The septum prevents undesired mixing of the two liquids. The separation electrolyte is purged with argon and connected to the high-voltage supply. The reaction chamber is plugged against the atmosphere. A hole in the plug (1 mm I.D.) enables bubble free filling and injection of reagents without affecting the oxygen concentration in the chamber.

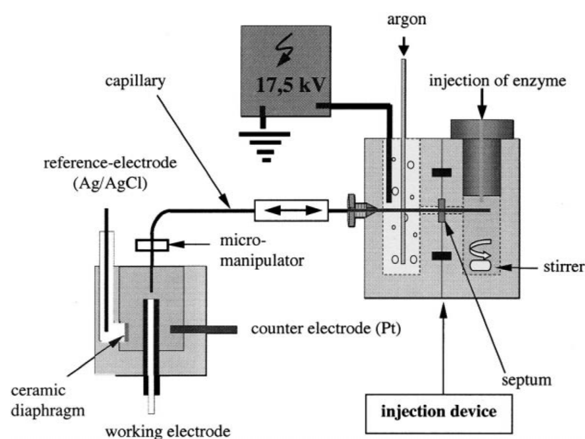


Fig. 1. Schematic view of the CE–ED system with the described injection device, detector cell and injection device are adjusted to the same liquid level.

All parts must be carefully isolated, since the injection device is connected to the high voltage. The two chambers are fixed on an isolating plate, and the movements of the stepper motor and the magnetic stirrer are transferred by isolating plastic parts to keep the metal parts at a minimal distance of about 10 cm from the high voltage. Except for the detector all parts are arranged in a Perspex housing (30×30×50 cm^3) to prevent electrical shock. The temperature inside the housing is kept constant in the range of 28–40±0.1°C by a Fuji PYZ 4controller (RS Components, Mörfelden, Germany) and an electrical heater (100 W) with a fan.

The electrochemical detection of oxygen and peroxides has already been described [6]. Briefly, the end of the capillary was fixed to a micro manipulator and adjusted to a gold microelectrode. The electrochemical detector was equipped with a Pt counter-electrode and an Ag/AgCl reference electrode. The potentiostat, an operational amplifier (CA 3140 from RS Components), keeps the potential of the reference electrode at the ground potential. The ground of the potentiostat is connected to the ground of the high-voltage generator (type HCN 7E-35000 from F.u.G. Rosenheim, Germany) and its counterelectrode feeds both the electrophoretic and the detector current to the ground. The current of the working electrode is connected to a current-to-voltage converter (OPA 110 from Burr-Brown, Filderstadt, Germany) equipped with a feed back resistor of 1 MΩ, resulting in a gain of 1 V/μA. Data acquisition and

control of the stepper motor were done by a microcontroller MC68HC11A8 from Motorola (MCT Paul & Scherer, Berlin, Germany) in connection with a personal computer. A scheme of the circuitry is given in [7].

2.2. Reagents

Chemicals and reagents were obtained from the following commercial sources: Sodium borate from Merck (Darmstadt, Germany); hydrogen peroxide and glucose from Fluka (Deisenhofen, Germany); linoleic acid, soya bean lipoxygenase (EC 1.13.11.12) (LOX), and glucose oxidase (EC 1.1.3.4) (GOD) from Sigma (Deisenhofen, Germany); 13(S)-hydroperoxy-octadeca-9Z,11E-dienoic acid (13-HPODE) solution in ethanol also from Sigma, and ketoprofen from Bayer (Leverkusen, Germany). The content of the freshly prepared stock solutions of peroxides was determined immediately before use by iodometric titration.

All samples, solutions and buffers were prepared with bidistilled water.

2.3. Injection procedure

The detection cell is filled with buffer electrolyte. After the working electrode is cleaned at oxidizing (+2 V) and subsequently reducing potentials (−2 V) for at least 1 min, the proper potential is applied and the capillary adjusted with the help of a stereo microscope and a micromanipulator. At a potential of −600 mV oxygen and most of the peroxides can be detected. After the capillary is filled with electrolyte with a syringe, the anodic chamber is purged continuously by argon to remove oxygen. The EOF due to the applied voltage is used to flush the capillary. The absence of oxygen is indicated by a drastic decrease in the cathodic current at −600 mV Ag/AgCl.

During the injection process the capillary is pushed through the septum from the anodic chamber to the reaction chamber. During this time the high voltage is switched off. Application of a short voltage impulse (1 s; 17.5 kV) electrokinetically injects the sample. The injection peak is caused by a change of the electroosmotic flow and a slight potential drift due to the switching of the high voltage. Then the capillary is pulled back into the anodic chamber and the high voltage is applied again. In accordance with the separation conditions for the reaction to be investigated, the injection procedure may be repeated till the injection peak of the last injection is just separated from the first oxygen peak.

Depending on the reaction rate and the conditions of the electrophoretic separation the sequence of the injection procedure must be adapted to avoid superposition of peaks and to allow a high analytical throughput.

There are different modes of injection to optimize the analytical performance. Fast reactions need high analyzing frequencies and short delay times. One possibility to realize these conditions is to apply a series of injections to a long capillary. The time between two injections must be greater than the difference between the migration times of the first and the last peak of the separation. The number of injections is limited by the time when the first peak appears (EOF), but after the last peak a new injection sequence can be started. Two examples are given in Figs. 3 and 5.

Sometimes the difference in migration times between the oxygen peak, which comes first with the EOF, and the oxidation product is great enough to fill this gap with the peaks from subsequent injections. This mode results in a number of oxygen peaks followed by the same number of product peaks. The number of injections in one sequence is limited by the time gap between EOF and product migration times (Fig. 4).

In the case of slow reactions there is enough time to wait between the injections till each separation procedure is completed and only then inject a new sample. The advantage of this regime is the exclusion of peak overlapping from different injections.

A short capillary and high separation voltage allow run times still short enough for monitoring of a chemical reaction in this mode.

2.4. Test of reproducibility

The reproducibility of the injected volume and the ED response are crucial for kinetic measurements. To estimate these influences the reproducibilities of the analytical responses of the analytes O_2, H_2O_2

and 13-HPODE were determined in the following experiments.

Both chambers were filled with 10 m*M* sodium borate solution. The solution in the reaction chamber was air-saturated and contained peroxides at different concentrations, see Table 1. All samples were injected repeatedly, resulting in 40 determinations for each analyte. Peaks were evaluated according to their area and height. The results are summarized in Table 1.

From previous investigations it was known that linoleic acid tends to inactivate the surface of the working electrode. This can be prevented by the addition of 50% methanol to the electrolyte in the detection cell.

2.5. Examples of application

2.5.1. Enzymatic oxidation of glucose

The glucoxidase reaction was chosen as an example for an enzyme-catalyzed oxygen-consuming process with formation of hydrogen peroxide. In accordance with the specification of the enzyme, a 1 m*M* solution of glucose in phosphate buffer pH 5.1 was used as substrate.

An aliquot of 4 ml of this solution was transferred to the sample chamber. The chamber was then sealed by the plug and the injection started.

First, the substrate solution was injected twice with a delay of 28 s in order to determine the initial oxygen concentration. The program was interrupted 37 s after the second injection to add the enzyme to the reaction chamber. Ten seconds after enzyme addition the registration of a new cycle was started with the injection of four samples with a delay of 55 s each. To pursue the further reaction the registration was continued with a new cycle comprising five injections at a distance of 55 s each. The detector signal in Fig. 3 shows four injection peaks (0–3 min), followed by two oxygen peaks from the first injection of the enzyme-free solution, then four oxygen peaks after the enzyme injection with the corresponding peaks of hydrogen peroxide. They are followed by the five last injection peaks (7.5–11.2 min). The registration continues with five oxygen peaks from the last injection series and the corresponding hydrogen peroxide peaks.

2.5.2. Enzymatic oxidation of linoleic acid

The reaction chamber was filled with 4 ml of a 0.2 m*M* solution of linoleic acid in 0.01 *M* sodium borate buffer. After the reaction chamber was sealed the following injection regime was started:

The first injection was applied to determine the starting concentration of oxygen in the substrate solution. The injection peak was not registered, but the oxygen peak appears in the subsequent registration. After 20 s the run was interrupted for the addition of the enzyme; subsequently, after a further delay of 20 s, a series of four injections, at distances of 23 s each, continued the measurement. After a run time of 150 s a further series of four injections was made. Thus the reaction could be registered over a range of about 3 min. Fig. 4 shows the complete registration. After the first oxygen peak from the previous injection of the enzyme free solution the four oxygen peaks elute, followed by four peaks of the 13-HPODE, then the next oxygen peaks, corre-

Table 1
Statistics of the determinations of O_2, H_2O_2 and 13-HPODE

Sample	Analyte		Mean ($n=40$)	SD	SD (%)
10 m*M* Borax	O_2	Area	697.77	6.28	0.9
1.2×10^{-4} *M* H_2O_2	O_2	under	675.26	11.78	1.74
	H_2O_2	the curve	93.85	6.08	6.48
2.5×10^{-4} *M* 13-HPODE	O_2	(AUC)	539.87	19.27	3.57
	13-HPODE		272.88	8.96	3.28
10 m*M* Borax	O_2	Peak	208.8	1.44	0.69
1.2×10^{-4} *M* H_2O_2	O_2	height	196.97	4.10	2.08
	H_2O_2	(h)	22.15	0.57	2.57
2.5×10^{-4} *M* 13-HPODE	O_2		186.20	2.29	1.23
	13-HPODE		74.44	3.43	4.61

sponding to the injection peaks from 0 to 80 s and the 13-HPODE peaks.

2.5.3. Photodynamic reaction

For photochemical measurements the reaction chamber was equipped with a quartz window. The reaction mixture was illuminated by a Philips UV-A lamp [CLEO Performance R UVA lamps (305–420 nm)] from Veith Import-Export (Westerau, Germany) mounted at a distance of 10 cm from the window. The photochemical decomposition of ketoprofen was chosen as an example for a photo-induced oxygen-consuming process. A solution of 1 m*M* ketoprofen in 10 m*M* sodium borate buffer was filled into the reaction chamber. After the chamber was sealed the oxygen content of the unradiated solution was determined by two injections. Then the UV-A lamp was switched on, and after an irradiation time of 10 s the automated procedure was continued with a delay time of 58 s (Fig. 5).

3. Results and discussion

3.1. Test of reproducibility

Fig. 2 demonstrates the high reproducibility of the device, that can be achieved in the measurement of, e.g., oxygen in borax buffer. The relative standard deviation reaches about 0.7% in the peak height as given in Table 1. The smaller peaks of hydrogen peroxide and the 13-HPODE exhibit greater variations due to the lower signal-to-noise ratio. On the other hand, the very dense sequence of peaks causes some overlap leading to an increase of the standard deviation of the oxygen signal in the presence of peroxides.

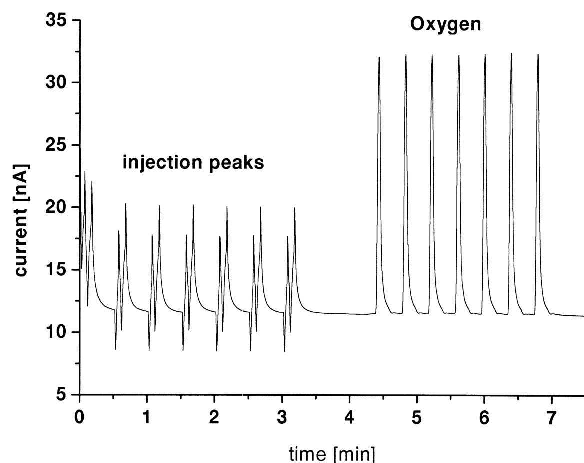


Fig. 2. Test of reproducibility — seven injections of buffer (separation voltage 17.5 kV; electrolyte 10 m*M* sodium tetraborate, electrokinetic injections 1 s; 17.5 kV).

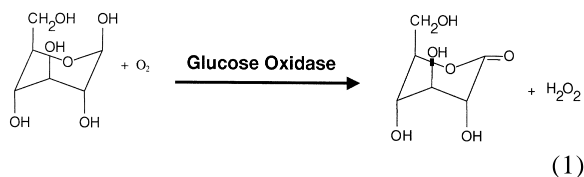
Generally, the peak heights appeared to be more reproducible than the peak areas. The deviations are attributed to uncertainties in fixing the limits of manual integration. Instead of areas, peak heights can directly be used to construct the concentration/time graph from the electropherograms by simply connecting the peaks of the same substances, because the migration times remain constant.

The detection signal for 13-HPODE was found to be linear between 10^{-5}–10^{-3} *M* with a detection limit of 6 μ*M*. The calibration function of hydrogen peroxide is linear in the concentration range between 10^{-5}–$2.5\cdot10^{-3}$ *M*.

During the measurements no clogging of the capillary occurred. However, clogging sometimes occurred with a new septum. Before mounting the septum was 'drilled' by a short piece of capillary. This is critically because the capillary bends and occasionally breaks, if too much force must be applied to push it through the septum. On the other hand the mixing of the fluid of the chambers must be avoided. We did not try to analyze enzyme suspensions, since all particles cause problems in CE. If they are not just clogging the capillary they tend to adsorb at the capillary wall.

3.2. Glucose oxidase reaction

The glucose oxidase is a flavoprotein occurring in plants and microorganisms (for example *aspargillus*) that catalyzes the specific oxidation of β-D-glucose to δ-D-gluconolactone and hydrogen peroxide [Eq. (1)]:



(1)

This reaction is the basis of all commercial test

strips for the determination of glucose in blood and urine [8].

In these test strips the hydrogen peroxide from the primary reaction forms a colored oxidation product of a suitable reagent (for example *o*-tolidine) under the influence of a peroxidase. The intensity of the color is evaluated to estimate the concentration of glucose. Therefore this reaction represents only an indirect determination. Beside the optical evaluation some test strips measure the hydrogen peroxide via electrooxidation at +600 mV [9]. Inactivation of the enzymes (for instance due to long or poor storage) would lead to wrong results.

The direct determination of both oxygen and hydrogen peroxide could avoid such false interpretations (Fig. 3).

3.3. Enzymatic oxidation of linoleic acid

Lipoxygenases belong to the family of dioxygenases and catalyze the specific reaction of fatty acids with a (1*Z*,4*Z*) pentadiene-system and molecular oxygen to form hydroperoxydienoic acids.

In the human organism the lipoxygenases 5-LOX, 12-LOX and 15-LOX (in addition to the cyclooxygenases COX1 and COX2) are decisively involved in the metabolism of arachidonic acid. The hydroperoxyeicosatetraenoic acids (HPOTEs) formed in these reactions are intermediates in the biosynthesis of a number of biologically highly active substances, the hydroxyeicosatetraenoic acids (HETEs), including the leucotrienes [10,11].

These messenger substances are involved in numerous physiological and pathophysiological processes, hence the medical modulation [12,13] of the arachidonic acid cascade has a high therapeutic value.

During the last decade a lot of new inhibitors [14] of the lipoxygenase reaction have been synthesized. The determination of the oxygen consumption by a Clark electrode [15] in the LOX-catalyzed oxidation of linoleic acid has been established as a convenient test system for estimation of LOX activity and their possible inhibition. Another possibility is the determination of the rate of peroxide formation by using the photometric determination of 13-HPODE at 234 nm [16].

CE–ED in combination with the device described allows the simultaneous quantification of oxygen and 13-HPODE [6], cf. Fig. 4. This is very useful to find side reactions and to interpret more complicated reaction mechanisms. Slow oxidation of an inhibitor, secondary decomposition of peroxides and other interesting information can be drawn from the electropherograms. Further, the separation of isomeric hydroperoxides (9-HPODE and 13-HPODE) is possible by the addition of 1 m*M* β-cyclodextrin [6].

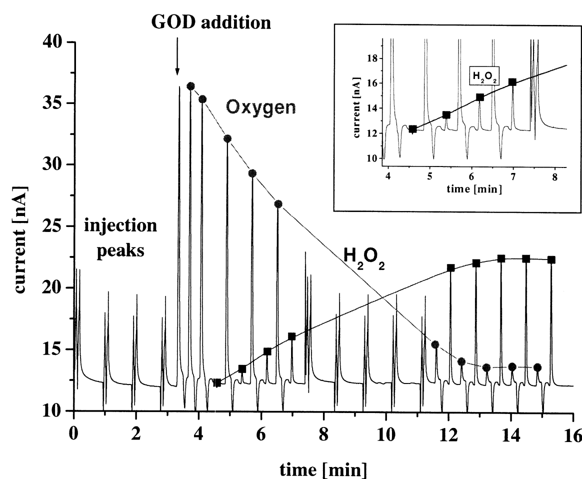


Fig. 3. Electrochemical monitoring of glucose oxidase reaction; conditions as in Fig. 2. The inset shows the enzymatic generation of H_2O_2 in detail.

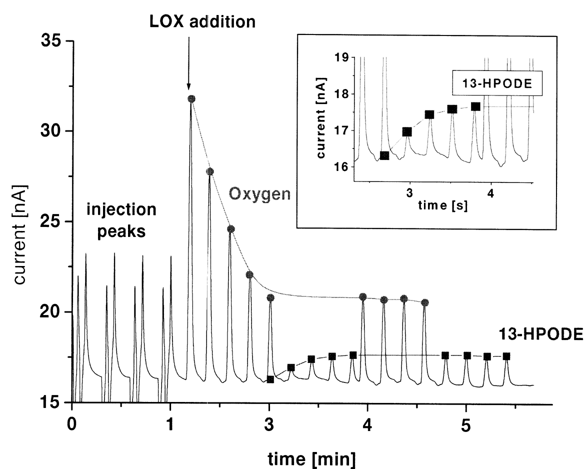


Fig. 4. Electrochemical monitoring of the enzymatic oxidation of linoleic acid; conditions as in Fig. 2. The inset shows the enzymatic generation of 13-HPODE.

Micellar electrochromatography with a sodium dodecylsulfate and Brij containing buffer is also described in literature [17]. In this way more information is obtained about the selectivity of the partly inhibited enzyme and the corresponding reaction mechanism.

3.4. Photodynamic reaction

Often reactive oxygen species (ROS) result from photochemically induced decomposition reactions of photolabile substances. Some widely used pharmaceuticals, e.g. tetracyclines and non-steroidal antirheumatics, belong to this group [18,19]. Beside exposure to light during storage, additional problems arise from the topical application of such drugs. Even at proper application, irradiation with sunlight causes a minor availability of the drug, and phototoxic and photosensitizing skin reactions after exposure must be taken into account [20].

Modern therapy concepts like PUVA therapy and light therapy (Photofrin, Methoxypsoralene) are based on the formation of reactive oxygen species through photochemical reactions. They are supposed to be very promising in the therapy of skin diseases and cancer [21–24].

Since all these reactions involve the consumption of oxygen, CE–ED offers a versatile and efficient method to get more insight into the basic processes.

An example of the photochemically induced consumption of oxygen is the decomposition of ketoprofen. This non-steroidal anti-rheumaticum of arylpropionic acid type has been applied successfully in the therapy of chronic polyarthritis and arthrosis since 1973. In 1995 the drug was admitted in Germany as a 2.5% gel for topical administration. Numerous investigations have shown that ketoprofen undergoes considerable structural changes during irradiation with UV-A light [25,26]. This photochemical reaction is accompanied by oxygen consumption and the formation of hydrogen peroxide [27,28].

Here CE–ED also enables the simultaneous determination of oxygen consumption and hydrogen peroxide formation (Fig. 5). Moreover, the kinetic parameters of the basic reaction can be determined as a function of the concentration of such additives as UV absorbers and radical scavengers, oxygen concentration, UV dose and other parameters with inexpensive instrumentation in a short time.

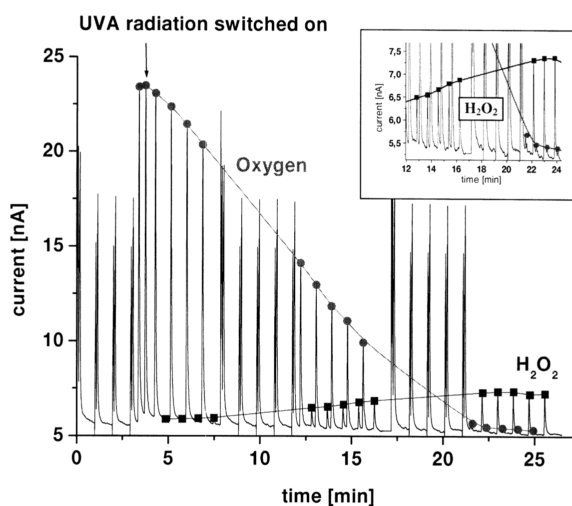


Fig. 5. Electrochemical detection of photodynamic reaction; conditions as in Fig. 2. The inset shows the photodynamic generation of H_2O_2.

4. Conclusions

The combination of capillary electrophoresis with reductive electrochemical detection represents a versatile method for the determination of easy reducible substances such as peroxides (hydrogen peroxide, 13-HPODE). With the injection device presented here it is possible to inject a reaction mixture periodically into the separating capillary without changing the oxygen concentration by gas exchange with the atmosphere. Thus, the oxygen content of the samples can be determined alongside the peroxides at minimal instrumental expenditure.

The examples described demonstrate the advantages of the device for the investigation of reactions which consume or produce oxygen.

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