SPECTROPHOTOMETRIC DETERMINATION WITH HEMOGLOBIN OF THE RATE OF OXYGEN CONSUMPTION IN MITOCHONDRIA

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1. Introduction

The study of respiratory processes and energy transduction in mitochondria or other respiring preparations often requires analysis of the kinetics of oxygen consumption and of the quantitative relationship between the rates of oxygen utilization, ATP synthesis and ion transport [1–3].

Accurate determination of the rate of oxygen consumption presents technical difficulties when dealing with rapid, transient changes of the respiratory activity, as induced by addition to the respiring material of oxygen, reductants, ADP, P_i and cations. In fact the polarographic method which is generally used to measure oxygen consumption may be inadequate to measure accurately rapid changes of respiratory rates [2,4], as judged from its intrinsic response-time characteristics [5,6].

This paper reports the application and suitability of a spectrophotometric method with hemoglobin [7,8] for accurate determination of initial rates of oxygen consumption during rapid functional transitions of respiratory systems. Oxyhemoglobin is used both as oxygen donor and as indicator of respiration, taking advantage of the specific absorbance changes which it undergoes upon deoxygenation.

2. Methods

Hemoglobin was prepared from whole human blood as in [9]. The concentration of HbO_2 was estimated, on the heme basis, with an ϵ_{mM} at 577 nm of 15.4 [7]. Samples of 10 mM HbO_2 were stored frozen for a maximum of 10 days. The difference spectrum of HbO_2 versus Hb (produced by addition of Na-dithionite) was recorded at room temperature with a Varian (model 634) double-beam spectrophotometer, scanning speed 25 nm/min. Mitochondria from rat-liver or beef-heart [10] were suspended in the reaction mixtures (see fig.3,4) and aliquots of the same suspension transferred to a glass cell equipped with oxygen electrode and a spectrophotometer cuvette (1 cm lightpath), both thermostatically controlled at $25 \pm 0.01^{\circ}C$. When desired, the concentration of dissolved oxygen was reduced by blowing argon (or nitrogen) onto the surface of the incubation mixture in the measuring cells under vigorous magnetic stirring. The cells were then sealed with glass plugs with 3 cm long, thin channels, filled with the incubation mixture, for insertion of microsyringe needles. These cells excluded detectable O_2 diffusion as shown by the polarographic and absorbance traces of incubation mixtures where O_2 was lowered to 27 μM O_2 with argon (fig.4).

Polarographic measurement of respiration was carried out with a Clark electrode (4004 YSI, Yellow Spring, OH) coated with a standard membrane (YSI 5775) or a high sensitivity membrane (YSI 5776). O_2 concentration was calibrated by setting the null point after dissolving $Na_2S_2O_4$ to the reaction mixture and taking the signal displayed at equilibrium with atmospheric oxygen at $25^{\circ}C$ and 10^5 Pa as corresponding to 250 μM in 150 mM LiCl [11]. Calibration was also checked by aerobic oxidation of duroquinol by mitochondria (see fig.4).

Deoxygenation of HbO_2 added to respiring mitochondrial suspension was monitored as decrease in $A_{577-568}$ with a Johnson Foundation dual wavelength

Abbreviations: HbO_2, oxyhemoglobin; Hb, deoxyhemoglobin

spectrophotometer (Univ. Pennsylvania, PA). The $A_{577-568}$ was linear with HbO_2 from 5–35 μM, in H_2O or mitochondrial suspension (1–3 mg protein/ml). The $\Delta\epsilon_{mM}$ at 577–568 nm was 4.8.

Rates of oxygen consumption in ng atoms oxygen/min (V) were obtained from:

$$V = Vy \cdot 2 \cdot f$$

where Vy is the deoxygenation rate of hemoglobin (nmol heme/min) and f is a correction factor which depends on hemoglobin concentration and the relative affinity for oxygen of hemoglobin and the respiratory system [8]. The correction factor was calculated by two independent methods [7,8]:

(i) Spectrophotometric estimation of the rate of HbO_2 deoxygenation caused by respiration at different HbO_2 concentrations;

(ii) Polarographic estimation of O_2 consumption in the presence of HbO_2 (fig.3).

Duroquinol oxidation was monitored at 270–285 nm with dual wavelength spectrophotometer (Mercury Arc and a liquid Ni–Co sulfate filter).

3. Results and discussion

The difference spectrum of human hemoglobin (HbO_2 minus Hb) is shown in fig.1. HbO_2 deoxygenation can be followed by monitoring ΔA at one of the positive 538 and 577 nm or negative 558 and

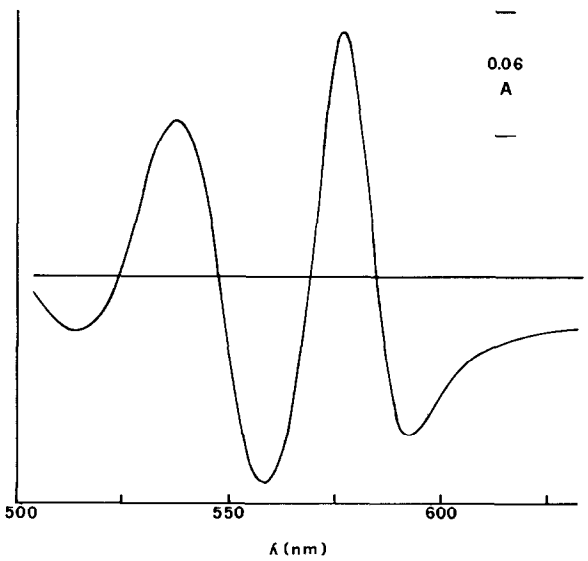

Fig.1. Difference spectrum of human hemoglobin. Both the measure and reference spectrophotometric cells (1 cm light-path) contained 25 μM HbO_2 in H_2O. Deoxygenation was obtained by adding few grains of solid Na-dithionite to the reference sample For details see section 2.

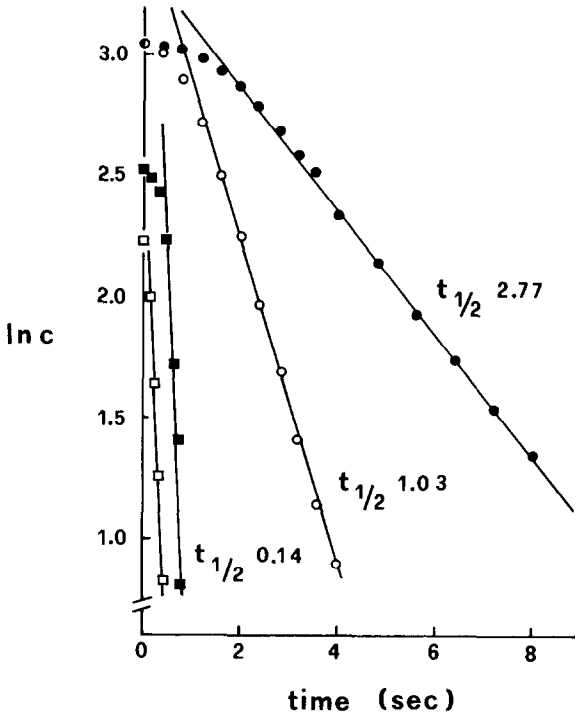

Fig.2. First-order plots of spectrophotometric recording of HbO_2 deoxygenation and potentiometric recording of consumption of dissolved O_2 caused by Na-dithionite. Few grains of solid Na-dithionite were added to a reaction medium consisting of: 130 mM LiCl, 1 mM KCl and 3 mM Hepes buffer (pH 7.2). In the spectrophotometric assay the concentration of hemoglobin was 25 μM. Circles: polarographic recording of oxygen consumption; O_2-electrode coated with standard membrane (●) and (○) high sensitivity membrane. Squares: spectrophotometric assay of HbO_2 deoxygenation caused by addition of Na-dithionite to air-saturated hemoglobin solution (■) and to 25% deoxygenated hemoglobin solution (□). The concentrations of dissolved O_2 and HbO_2 are expressed in arbitrary units.

436 nm (not shown) absorbance peaks [7,8]. However, when the process is used to determine the respiratory activity of turbid biological suspensions, which present serious unrelated ΔA and when, in addition, high sensitivity is required, it is convenient to use dual wavelength spectrophotometry [12].

After direct exploration of the suitability of the various possible wavelength combinations (see fig.1) the couple 577–568 nm was chosen to monitor HbO_2 deoxygenation.

(i) Redox transitions of cytochromes, caused by aerobic–anaerobic transition or addition of solid Na-dithionite to aerobic mitochondria did not cause detectable ΔA at this couple (see fig.3);
(ii) The two wavelengths are close enough to avoid significant absorbance artifacts by light-scattering changes.

Fig.2 illustrates the response-time characteristics for spectrophotometric determination at 577–568 nm of HbO_2 deoxygenation caused by addition of solid dithionite to the hemoglobin solution kept in the spectrophotometric cell under vigorous magnetic stirring. The absorbance decrease associated to HbO_2 deoxygenation was recorded as a first-order process with $t_{1/2}$ of 140 ms (this comprehends the overall response time of the spectrophotometric recording system). The absorbance decrease was preceded by a lag of 500 ms when dithionite was added to the hemoglobin solution equilibrated with atmospheric oxygen (hemoglobin initially 100% oxygenated). There was practically no lag, when the oxygen concentration was pre-lowered with argon to cause 25% deoxygenation of hemoglobin. Deoxygenation of HbO_2 proceeds with a sigmoidal curve, linear from 25–85% deoxygenation [7,8]. As comparison the response-time characteristics of the oxygen polarograph is shown. The oxygen electrode coated with a standard membrane responded to immediate oxygen consumption by dithionite after a lag of 3 s with a first-order process with $t_{1/2}$ of 3 s. When the electrode was coated with high sensitivity membrane the lag was 1.5 s and $t_{1/2}$, 1 s (cf. [6]).

Fig.3 illustrates the time course of HbO_2 deoxygenation and respiration in succinate-supplemented mitochondrial suspension. Deoxygenation started when dissolved O_2 was lowered to 90 μM and exhibited a sigmoidal curve with a linear tract from 25–85% deoxygenation, which corresponds to an effective 50–10 μM O_2 (dissolved O_2 plus O_2 contributed by HbO_2). In this region, and with a respiratory system like mitochondria which have an affinity for oxygen 3 orders of magnitude higher than that of hemoglobin [7,8], part of the oxygen consumed by respiration is donated by HbO_2, as shown by the decline in the rate of consumption of dissolved O_2 monitored polarographically (see fig.3), and there is, at a given hemoglobin concentration, a fixed relationship between the rate of oxygen consumption by the respiratory system (V) and the rate of HbO_2 deoxygenation (Vy).

Experimental determination of the V/Vy ratio, or correction factor f, allows V to be obtained from spectrophotometric measurement of Vy. Fig.3 illustrates the calculation of f from:

(i) The rates of Vy at various HbO_2 concentrations;
(ii) The polarographic rates of consumption of dissolved O_2 at high O_2 concentrations, where HbO_2 remains fully oxygenated, and low O_2 concentrations, where HbO_2 donates O_2 for respiration.

The two methods give exactly the same f values.

Fig.3 shows that the values of respiratory rates obtained from deoxygenation of HbO_2, at 20–30 μM, were all the same and equal to that measured polarographically in the absence of hemoglobin.

The hemoglobin method can be adopted to provide accurate determination of rapid and transient changes of respiratory activity, as they ensue immediately upon addition of reactants, like respiratory substrates, ADP and Ca^{2+}. In this case the oxygen is pre-lowered to 50–25 μM with argon or nitrogen, where further deoxygenation of partially deoxygenated hemoglobin proceeds per se with a linear slope (until dissolved O_2 is ~8 μM).

Fig.4A shows that respiration elicited by addition of succinate to a suspension of rat-liver mitochondria, whose O_2 concentration had been pre-lowered by argon to cause 50% deoxygenation of HbO_2, resulted in immediate further deoxygenation of HbO_2. The Clark electrode, on the contrary, responded to respiration elicited by succinate with a lag of 3 s, when the electrode was coated with the conventional membrane, and 1.5 s with the high sensitivity membrane. The respiratory rate measured either polarographically or spectrophotometrically was non-constant and declined with time. Furthermore the initial respiratory rate estimated spectrophotometrically was twice as high as that measured polarographically with the conventional membrane and still 30% higher than that measured with the high sensitivity membrane. The mean values ± SEM for 20 determinations of respiratory rate in ng atoms oxygen . min^{-1} . mg $protein^{-1}$ were: (a) Polarographic assay with standard membrane, 53.2 ± 2.7; (b) polarographic assay with high sensitivity membrane, 82 ± 7; spectrophotometric assay with hemoglobin 106 ± 4.5 (see [13]).

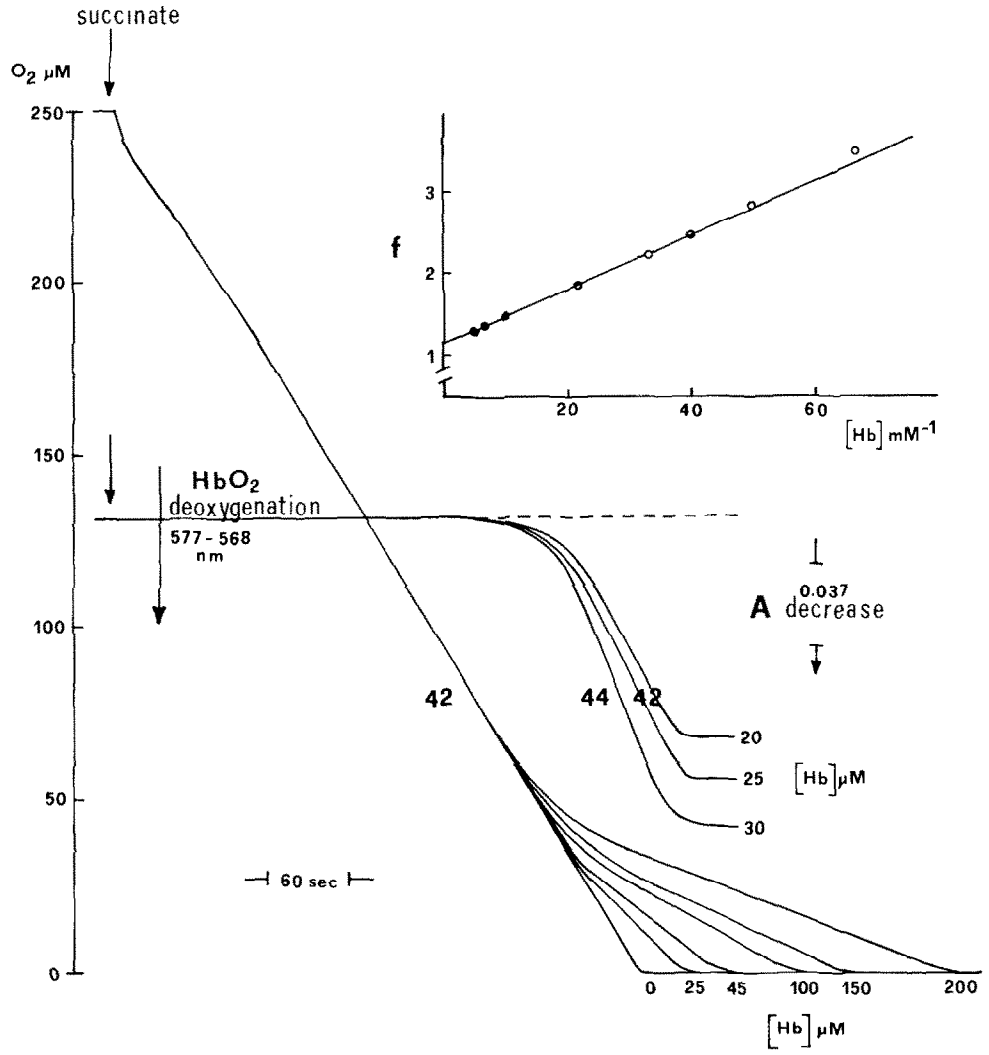

Fig.3 Polarographic monitoring of consumption of dissolved O_2 and spectrophotometric recording of HbO_2 deoxygenation in suspensions of respiring rat-liver mitochondria. Determination of *f*. Mitochondria (2 mg protein/ml) were suspended in 130 mM LiCl, 3 mM Hepes buffer (pH 7.2), rotenone (0.5 μg/mg protein) and valinomycin (0.1 μg/mg protein). The mitochondrial suspension, supplemented with HbO_2 at the concentrations indicated, was transferred to the measuring cells and after 5 min for thermoequilibration at 25 ± 0.01°C, 10 mM Li-succinate was added. It can be noted that in the absence of HbO_2 no $\Delta A_{577-568}$ was observed when mitochondria became anaerobic (dashed trace). The number on the traces are rates expressed as ng atoms oxygen consumed . min^{-1} mg protein^{-1}. In the presence of hemoglobin oxygen consumption entered a phase of reduced rate which coincided with HbO_2 deoxygenation. The correction factor was calculated from the polarographic trace of oxygen consumption dividing the slope of the linear region preceding HbO_2 deoxygenation by the difference between this and the slope of the linear region coincident with HbO_2 deoxygenation [7,8]. Spectrophotometric determination of *f* was obtained from Lineweaver-Burk plot of the rate of HbO_2 deoxygenation as in [7]. For details see section 2.

Insert: Plot of the correction factor (*f*) as a function of 1/[Hemoglobin]. (•) Polarographic measurements; (○) spectrophotometric measurements

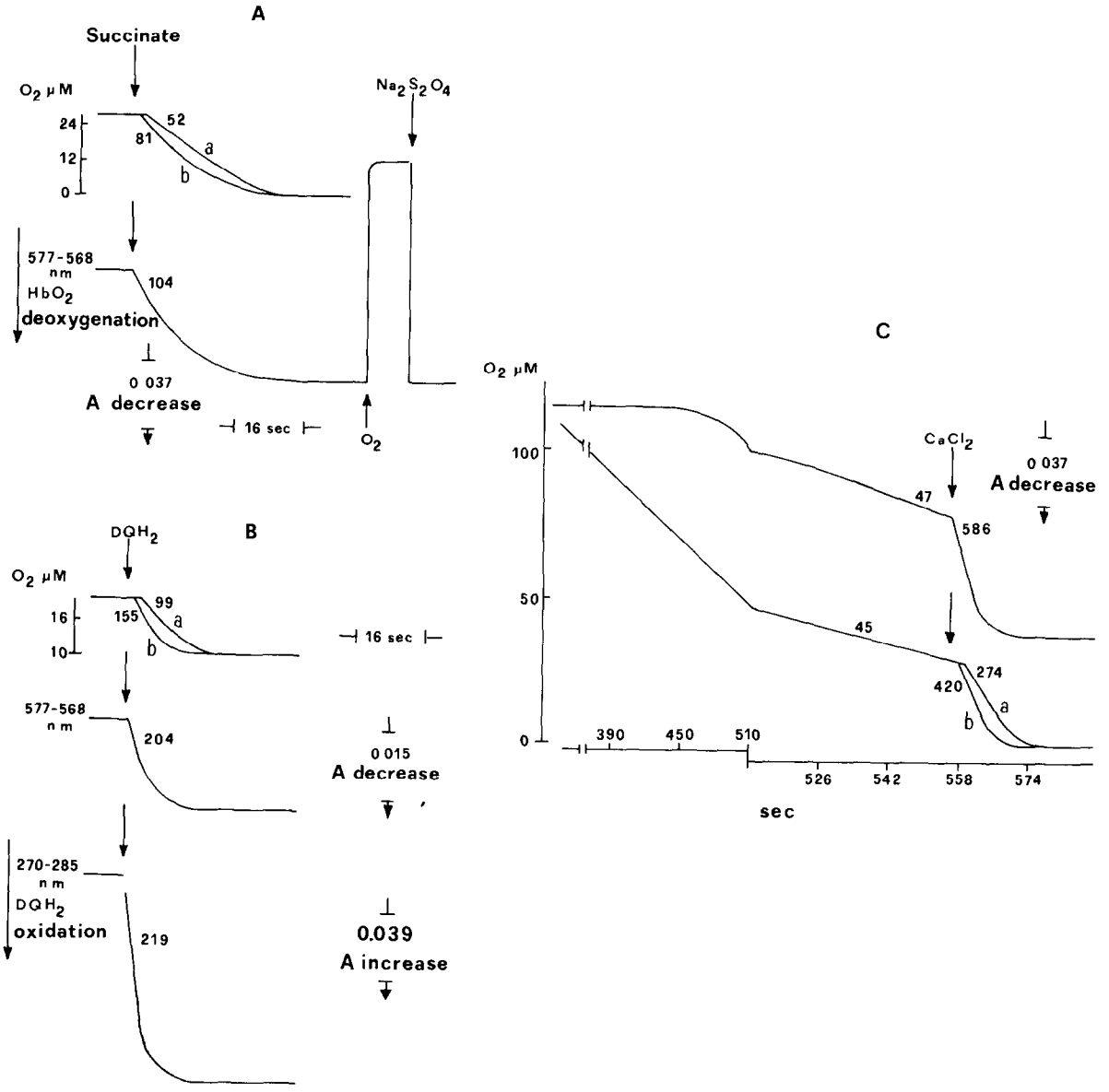

Fig.4. Polarographic and spectrophotometric determination of the initial rate of O_2 consumption during rapid changes of respiratory activity in mitochondria.

(A) Rat-liver mitochondria (2.5 mg protein/ml) were suspended in the reaction mixture to fig.3. Argon was blown onto the surface of the mitochondrial suspension for 4 min, until dissolved O_2 in the polarograph cell sample was reduced to 27 μM and HbO_2, added at 25 μM to the spectrophotometric cell sample, was 50% deoxygenated. After 1 min further preincubation for temperature equilibration at 25°C, 1 mM succinate was rapidly injected.

(B) Beef-heart mitochondria (1 mg protein/ml) were suspended and treated as in (A). Duroquinol (19 μM) was rapidly injected instead of succinate as respiratory substrate. Malonate was 5 mM.

(C) Rat-liver mitochondria (1 mg protein/ml) were suspended in the reaction mixture of fig.3, with the addition of 2 mM K-phosphate buffer (pH 7.2) and omission of valinomycin. Once dissolved O_2 was reduced to 50 μM in the polarographic cell sample, no hemoglobin added, and 25 μM HbO_2, added to the spectrophotometric cell sample, was 50% deoxygenated, 690 nmol $CaCl_2$ were rapidly added. The numbers on the traces are rates expressed as ng atoms O_2 consumed and nmol duroquinol oxidized . min^{-1} . mg $protein^{-1}$. (a) Standard O_2 membrane; (b) high sensitivity membrane.

Fig.4B shows a similar experiment where 19 μM duroquinol was added to beef-heart mitochondria supplemented with rotenone and malonate at reduced O_2 concentration. The value for initial quinol respiration calculated from the rate of HbO_2 deoxygenation coincided with that measured by monitoring directly duroquinol oxidation at 270–285 nm but was much higher than that estimated polarographically. The mean values for 5 determinations of the Respiratory rate were: (a) 95 ± 6; (b) 148 ± 7; spectrophotometric assay with hemoglobin, 210 ± 9; duroquinol oxidation (nmol . min^{-1} . mg $protein^{-1}$) 207 ± 12.

Fig.4C documents the adequacy of the spectrophotometric method to estimate transient respiratory burst induced by addition of Ca^{2+} to mitochondria respiring with succinate in state 4. Also in this case the rates measured polarographically were, with both membranes, much lower than that measured spectrophotometrically. The mean values for 5 determinations of the respiratory rate elicited by $CaCl_2$ were: (a) 296 ± 20; (b) 415 ± 23; spectrophotometric assay with hemoglobin 616 ± 35.

In conclusion the data show that the Clark electrode is inadequate to estimate accurately initial respiratory rates in rapid functional transitions of respiratory systems. However, it is shown that the spectrophotometric method with hemoglobin is perfectly suitable for kinetic analysis of oxygen consumption and estimates of transient respiratory bursts. The spectrophotometric method is the choice for this type of measurement.

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