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High Time Resolution

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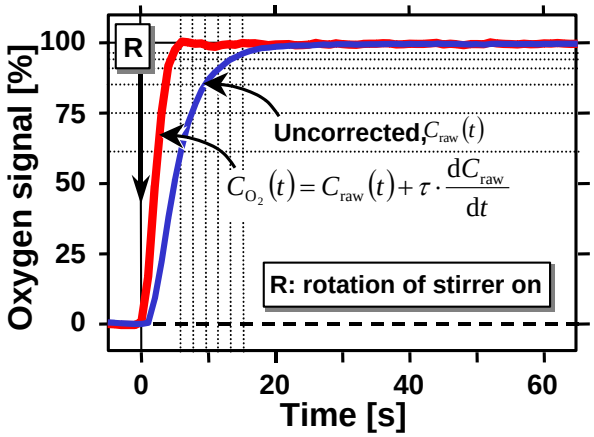

From Gnaiger (2001) *Respir. Physiol.* 128: 277-297

Summary: The signal of polaroc
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oxygen in the medium. This delay
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Section Page

1 Static and dynamic calibration

Static calibration involves the determination of the constant signal of the polarographic oxygen sensor (POS) at 0 % and 100 % air saturation (R_0 and R_1)

under the particular experimental conditions (temperature, stirring speed, medium). Dynamic calibration requires the determination of the exponential time constant, τ. τ can then be used for the time correction (deconvolution) of the Oxygraph signal. τ can be experimentally determined by pulse-titration of anoxic into air-saturated medium or by turning the stirrer off and on. The response is fitted to an exponential function which yields the value of τ [s].

τ critically depends on experimental temperature, with a Q_{10} of c. 0.69. In contrast, neither the stirring speed (100 to 700 rpm) nor the presence of 10 % dextran 15,000-20,000 or 10 % dextran 70,000 significantly affected τ.

While time correction is unnecessary for steady-state experiments (during periods of constant oxygen flux), it may critically influence the quantitative results in non-steady-state experiments, typical of many kinetic applications (ADP pulse titrations, oxygen kinetics; Gnaiger 2001; Gnaiger et al. 1995; Gnaiger et al. 2000; Gnaiger and Kuznetsov 2002).

2 The exponential time constant, τ

2.1 The physical basis of τ

Rapid changes of oxygen partial pressure, p_{O_2}, of the experimental medium in the Oxygraph chamber are detected by the polarographic oxygen sensor only with a time delay. This convolution of the signal is due to the separation of the oxygen sensor from the experimental medium by a membrane and an electrolyte layer. Consequently, the signal at the cathode responds to a change in oxygen only after oxygen diffusion has taken place through the membrane to the cathode (Fig. 1).

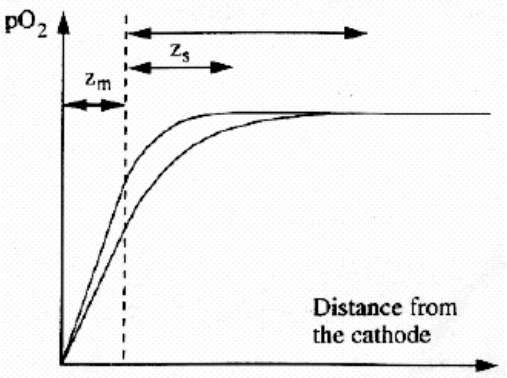


Fig. 1 p_{O_2} as a function of distance from the cathode, z. The oxygen sensor continuously consumes all oxygen that diffuses to the cathode surface where oxygen concentration is zero. Under steady-state conditions, there is a linear oxygen pressure gradient through the membrane. z_m, thickness of the sensor membrane; z_s, thickness of the unstirred boundary layer.

The time response to changes of p_{O_2} depends mainly on the thickness of the sensor membrane (z_m), the oxygen permeability of the membrane, temperature, and the unstirred boundary layer of the experimental solution, z_s (Hale 1983). For simplicity, diffusion

through the electrolyte layer between cathode and sensor membrane is neglected in Fig. 1.

The effect of the time constant is illustrated by the signal response to a step change of oxygen concentration (Fig. 2).

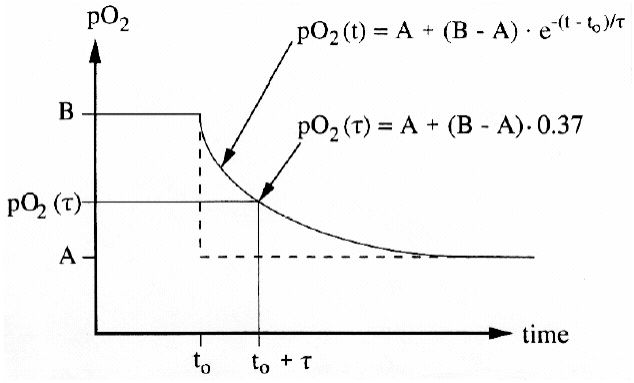


Fig. 2: Exponential response of the oxygen sensor (full line) to a step change of p_{O_2} in pulse titration of anoxic water at time t_o (dashed line).

At time t_o, the partial pressure of oxygen in the Oxygraph chamber is instantaneously reduced. The recorded oxygen signal can be described by the exponential equation,

Eq. 1. $$p_{O_2}(t) = A + (B\text{-}A)\cdot e^{-(t\text{-}to)/\tau}$$

τ is the time interval in which the signal decreases by 63 % of the full response, i.e. one *e*-th of the signal difference (*B*-*A*),

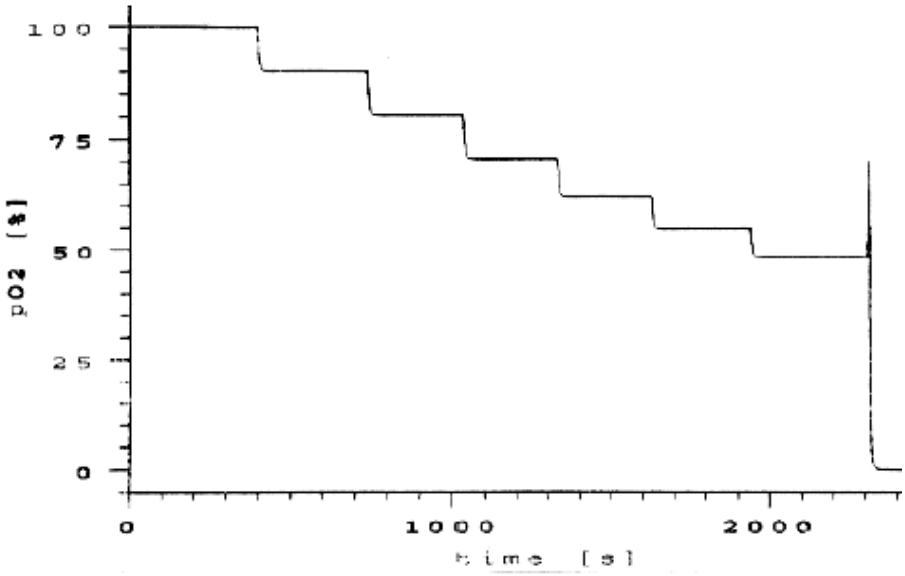
Eq. 2. $$p_{O_2}(\tau) = A + (B\text{-}A)\cdot e^{-1} = A + (B\text{-}A)\cdot 0.37$$

When plotting the experimental data, $p_{O_2}(t)$, in a semi-logarithmic graph versus time, the exponential curve (Eq. 1) transforms into a straight line with the slope -1/τ.

2.2 Experimental determination of τ

τ can be determined experimentally by *(1)* the "stirrer test", turning the stirrer of the Oxygraph chamber on after it has been switched off for some time (Fig. 3); *(2)* pulse-titration of anoxic medium into air-saturated medium (Fig. 4); and *(3)* pulse-titration of air-saturated medium into anoxic medium. Control experiments demonstrated that the stirrer test and titrations yield identical results. The stirrer test presents a simple routine procedure, although marginally less precise than the titration method. An initial slow start of the magnetic stirrer must be accounted for a few seconds in the stirrer test. The titration test may involve disturbances introduced by insertion and removal of the syringe, by temperature differences between experimental and injection media, and by the time required for homogeneous mixing of oxygen in the chamber immediately after the pulse titration.

Figures 3B and 9 show typical experiments for the determination of τ, with uncorrected (right curves) and time-corrected (left curves) Oxygraph traces.



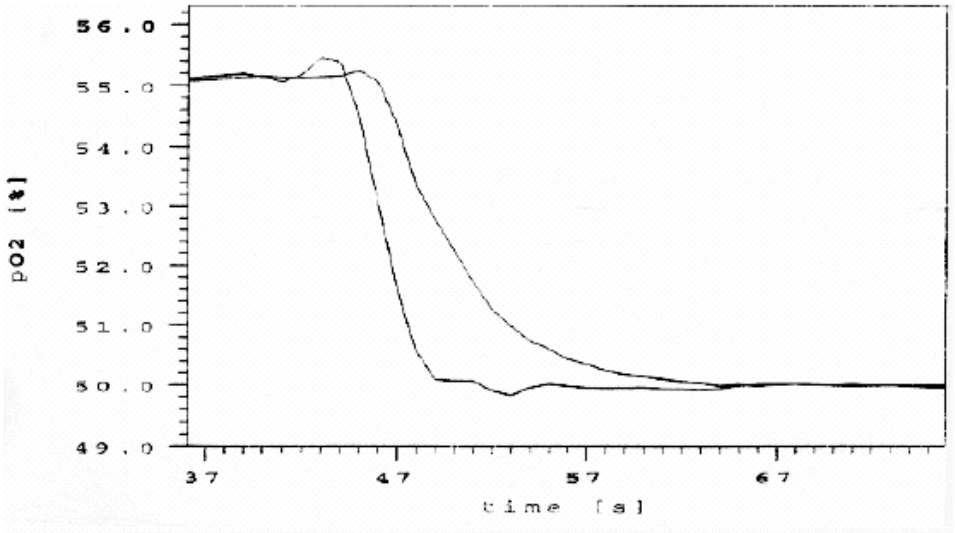


Fig. 3A Determination of the time constant, τ, by repetitive pulse-titration of anoxic water into previously air-saturated water, at 5-min intervals. At the end of the experiment, dithionite was added for the zero oxygen calibration. 25 °C; stirring speed 500 rpm; chamber volume 2 cm^3; titration volume 200-250 mm^3.

Fig. 3B Single titration of anoxic water into air-saturated water. The right trace represents the recorded signal (raw). The left trace is the time-corrected signal. 25 °C; stirring speed 300 rpm; chamber volume 2 cm^3; titration volume 200 mm^3; τ = 3.9 s.

3 Effect of experimental conditions on τ

3.1 Effect of stirring speed on the time constant

Stirring speed influences τ theoretically only when *(1)* mixing is slow of the injected (anoxic) solution with the (oxygenated) Oxygraph medium (i.e., if the time constant of the mixing process is in the same range or

higher than the time constant of the oxygen sensor), or when *(2)* unstirred layers (Fig. 1) play a significant role in oxygen diffusion limitation to the cathode.

τ was virtually constant at stirrer speeds between 100 and 700 rpm (Fig. 4). Although the slope of the linear regression was not significantly different from zero, there may be an approx. 5 % increase of τ with the decline from 700 to 100 rpm. Such a 5 % increase is fully consistent with the data in Figure 5, showing a 5 % decrease in the recorded Oxygraph signal for air-saturated water between 700 and 100 rpm. The coincidence of both findings points to more pronounced unstirred layer effects at lower stirring speeds and, at the same time, excludes a significant contribution of the mixing process to τ.

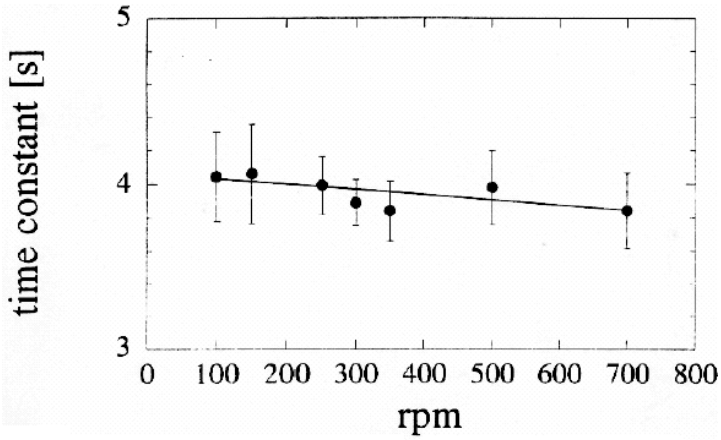


Fig. 4 Effect of stirring speed on the time constant τ, determined with the titration method (Figure 3). 25 °C; chamber volume 2 cm^3; titration volume 200-250 mm^3.

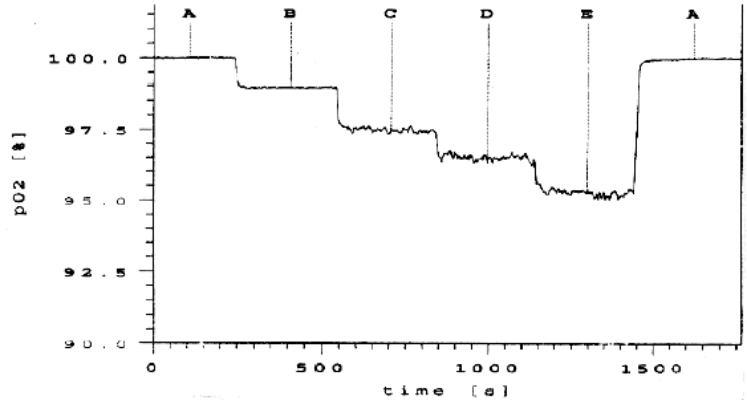


Fig. 5 Effect of stirring speed on the oxygraph signal at 100 % air saturation. Air-saturated water in contact with a gas phase was present in the oxygraph chamber throughout the experiment. The effect of changes in stirring speed on the recorded oxygraph signal were analyzed at (rpm): A, 700; B, 500; C, 300; D, 200; E, 100; 25 °C; chamber volume 2 cm^3. The oxygraph signal declined by c. 4 % in air-saturated water between 700 rpm and 100 rpm. Note the difference in signal stability between 700 and 500 rpm on one hand and 300, 200 and 100 rpm on the other hand. Noise at lower stirring speeds is due to instabilities of the PEEK stirring rod. High stirring speeds increase signal stability, whereas the optimum stirring speed depends on the application (750 rpm is the standard setting in the OROBOROS Oxygraph-2k, but may be varied).

3.2 Effect of temperature on the time constant

As expected for a diffusion-controlled process, the time constant τ strongly depends on the experimental temperature. A logarithmic plot of time constant τ *vs.* temperature results in a straight line (Fig. 6), indicating

a 31 % decrease in τ for a 10 °C increase in temperature.

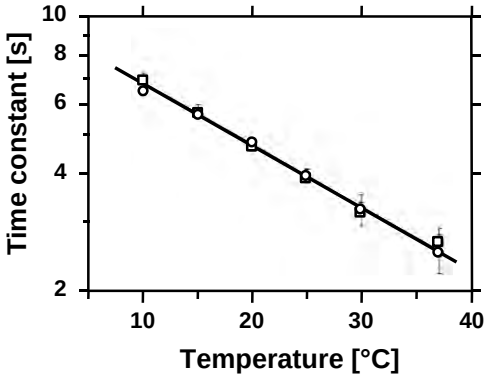


Fig 6 Effect of temperature on the time constant τ. The temperature was varied between 10 and 37 °C, and the time constants of two sensors (in chambers A and B) in the same oxygraph were determined by the titration method (Fig. 3). Stirring speed 300 rpm; chamber volume 2 cm^3; titration volume 200-250 mm^3. Each value represents the mean ± SD of 5-6 measurements.

3.3 Effect of dextrans on the time constant

The oncotic or colloid-osmotic pressure of the medium critically influences a variety of mitochondrial parameters, and dextrans can be used to change the colloid-osmotic pressure of the medium (Gellerich et al., 1994). The effect of dextrans on the time constant was evaluated. The increase in viscosity associated with the addition of 10% dextran 15,000-20,000 or 10% dextran 70,000 to the experimental medium did not significantly affect τ (Fig. 7).

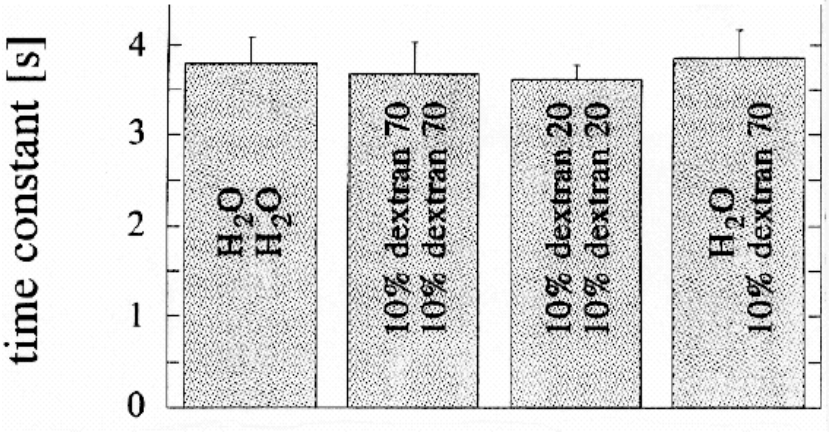


Fig. 7 Effect of dextrans on the time constant determined by the titration method (Figure 3). Titrations into previously air-saturated medium: Anoxic H_2O into H_2O; 10% dextran 70,000 into the same; 10% dextran 15,000-20,000 into the same; anoxic H_2O into 10% dextran 70,000. Means ± SD; *N*=6.

4 DatLab Analysis: DATLAB MACRO TIMECONS

For analysis in DatLab 2, open a data file (*.DLR) containing a step change of the oxygen signal, achieved either in a stirrer test (**970101A1.DLR**) or an oxygen titration test. The file to be analyzed must be the active data set, and the time unit must be seconds [s]. Data must not be smoothed. They do not have to be calibrated. If a background analysis has been completed, dynamic calibration can be performed on these data without opening the file again and without

closing other data sets.

For analysis of a stirrer test performed with the Oxygraph-2k and DatLab 4 (use the F11 and F12 keys to switch both stirrers off and on), it is a convenient option to save in DatLab 4 specifically a marked section of the experiment (the section immediately before switching off the stirrer until the end of the steady-state period after switching the stirrer back on). Export the recorded data (O2L or O2R) to DatLab 2 for analysis (File\Export). DatLab 2 can only open (exported) files in the *.DLR format (or ASCII files), but not the original data files (*.DLD) saved in DatLab 4.

TIMECONS.{M} Press [**F5**], select macro **TIMECONS.{M}** and confirm by [**Enter**].

☞ Other data sets are deleted when proceeding with the macro. Store data before if necessary. All marks on the active data set are deleted, otherwise the new marks may not be interpreted correctly. Follow the step-by-step instructions for analysis of the exponential time constant.

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1. Set an initial MARK before step change; 3 MARKS at steady state.

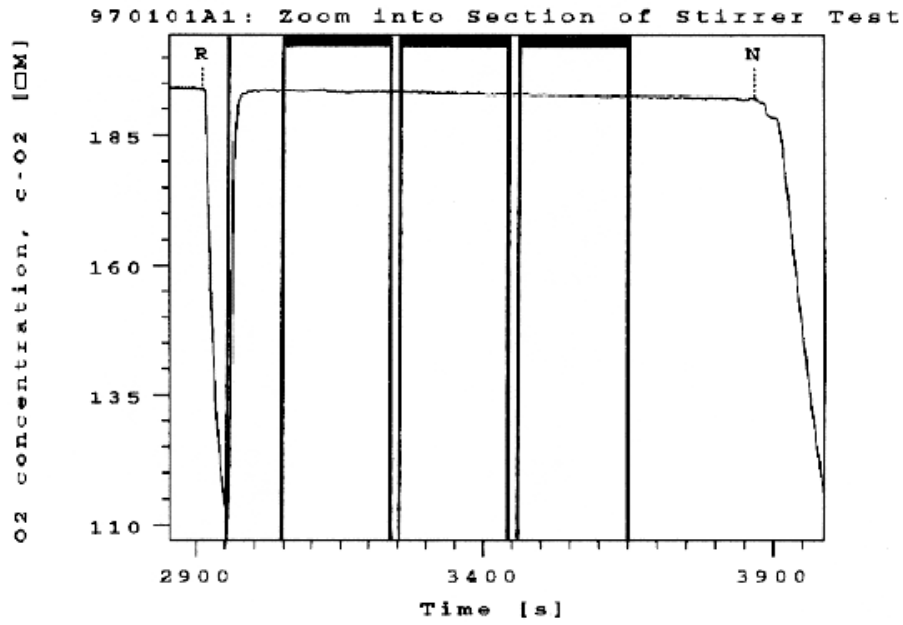

Fig. 8 Zoom into stirrer test, showing the raw signal after switching the Oxygraph stirrer off <R>, and on. The stirrer is switched off for a short period only, but the full response is recorded after switching the stirrer on.

Mark the initial and final oxygen signal level. The **first mark** may be very short, just defining the initial signal level before the step change caused by switching the stirrer on (do not mark a section before switching the

stirrer off). The **second mark** is set at steady state of the signal. This mark is long and **partitioned into several sections**, to obtain a series of marks interrupted by very short periods of unmarked data, for linear extrapolation of any slope of the signal after full response is reached.

A calibration of the time constant of the oxygen sensor may be performed in the open or closed chamber, by following the signal after a step change until the full response is obtained. In a closed chamber, the full response does not result in a constant signal but is followed by a period of steady oxygen consumption. Identical time constants are observed in an open chamber in association with air calibration.

Any steady change of the signal after reaching full response is taken into account by a baseline correction. The change may occur from any level of initial to final signal. Therefore, the data are aligned to constant reference values of 0 % and 100 % (arbitrary units).

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2. MARK the exponential increase of the signal.

See Fig. 9 for setting the mark in the exponential range, left of the marks defining the maximum level. The first mark, at minimum oxygen, has been deleted automatically (Figure 8). Start the mark at a signal between 25-75% responses and set the mark over c. 30 s into the region of full response.

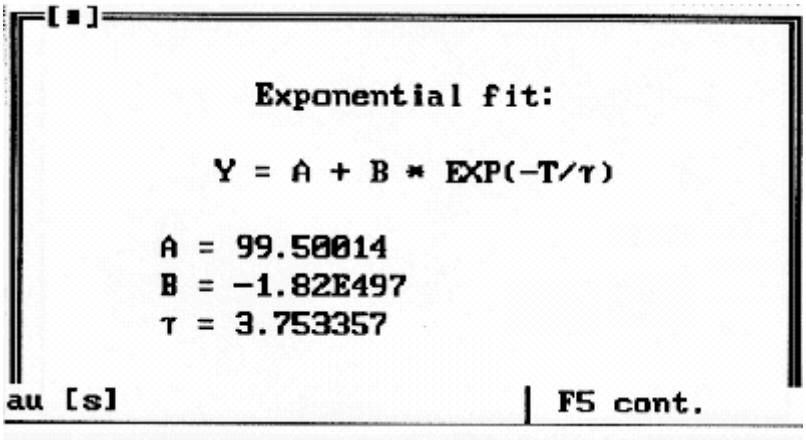
a) Stirrer test: The stirrer accelerates its rotation after being switched on, reaching maximum speed after a few seconds. Therefore, the initial part of the increasing signal is not a true step change. Start the mark between 50-75% responses.

b) Oxygen titration: The titration into the chamber may cause a disturbance of the signal, superimposed on the step change in oxygen concentration. Therefore, the initial part of the increasing signal should not be marked. Start the mark at c. 25% response.

The time constant, τ [s], is calculated as a parameter in an exponential fit, according to the equation,

Eq. 3. $$Y_t = A + b \cdot \exp(-t/\tau)$$

where by comparison with Eq.(1), $Y_t = p_{O_2}(t)$, and $b = B\text{-}A$.



3. TIME CONSTANT, tau [s]

Note the value calculated for the time constant in the last line. The exponential time constant, τ [s], is the time required until the signal has reached 63 % of the full response.

On the basis of the calibrated time constant, the data are time-corrected and displayed after pressing [**F5**].

4. Normalized and time corrected signal.

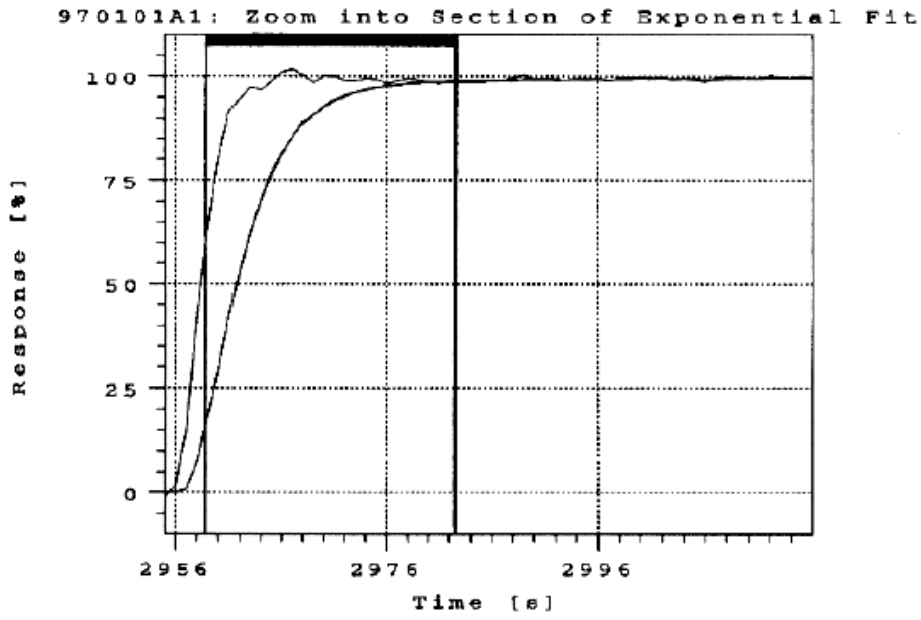


Fig. 9 Baseline corrected signal, normalized to a change from 0 % to 100 % response. The marked section over the uncorrected oxygraph signal (right curve) is the basis for calculating the time constant. The corrected signal (left curve) aids in evaluating the quality of the time constant as applied to the raw data.

Time corrected data are more noisy, but the initial 10 s of delayed response and high noise level are partly due to the slow-start function of the magnetic stirrer.

You may **edit the mark** and compare the time constant displayed at the end of macro execution.

Good sensors show an exponential increase. Then the calculated time constant is independent of the duration of the mark. Sensors with an increased time response show frequently second-order time responses. Then the positioning and duration of the mark critically influences the result, and an initial overshoot of the corrected signal may be observed. A mark over 30 s may yield an appropriate estimation of the first-order exponential time constant under these conditions.

The following steps allow for an iterative optimization of the time constant and evaluation on the basis of the corrected curve.

5. EDIT time constant, tau [s] **| F1 Help**

Choose a numerical value of the time constant, approximating the calculated value of τ displayed above. Compare the time corrected curve to Fig. 9.

6. Normalized and time corrected response (RED).

An overshoot of the time-corrected response is due to overestimation of the time constant.

7. Varation of time constant, tau [s] **| F1 Help**

Choose a numerical value of the time constant, different from the first value according to the result in the previous figure. Compare the time corrected curve to the previous figure.

8. Normalized and time corrected signal (RED).

The calculated time constant is displayed according to the mark edited in the last figure.

9. NOTE tau. **EDIT in macro TIMEC_2.**

Which time constant should be used? An optimum decision can be made after critical examination of the effects of varying the time constant, avoiding overshoots associated with high time constants, and keeping undershoots in check associated with low time constants.

Insert the proper time constant (**3.7** s in the example) into the **Calibration Table** [MiPNet02.03, Appendix].

TIMECONS.{M} Play macro **TIMEC_2.{M}** for further optimizing the time constant.

Correction for the time response by using an accurate time constant is essential for high-resolution analysis of kinetic studies, such as ADP pulse titrations and oxygen kinetics involving rapid transitions to anoxia (Gnaiger, 2001).

☞ The OROBOROS® DATLAB MACROS have been tested under a variety of conditions, but no guarantee can be given for their proper functioning outside the scope of our test conditions. Deviations from the initial conditions described for each macro may lead to failures of program execution and loss of unsaved data.

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5 References

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OROBOROS INSTRUMENTS Software - DatLab 4 (Windows) programmed by Lukas Gradl, Innsbruck, Austria; export to DatLab 2 for analysis.

OROBOROS Oxygraph-2k Produced by WGT Elektronik, Philipp Gradl, Austria