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Oxygraph-2k: Oxygen Calibration and O2k-Background Tutorial

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It is not uncommon, that we receive the question '*What is wrong?*', despite of highly satisfactory calibration traces and proper instrumental background function. Two examples from our correspondence within the Mitochondrial Physiology Network may provide guidelines complementary to information in the relevant chapters of the O2k-Manual.

The first example presents traces obtained during air calibration, *(1)* explaining the advantage of opening the third graph with display of temperature and Peltier power and using the Excel template for listing numerical calibration parameters, *(2)* commenting on the sampling interval in relation to evaluation of noise in the plot of the time derivative (flux), *(3)* using the mark statistics function [F2] for extended analysis of signal stability during long-term calibrations, and *(4)* introducing the function of the 'gas spacer' during air calibrations.

The second example discusses instrumental background flux.

1. Air calibration of the polarographic oxygen sensors

'We are having a lot of problems trying to get a steady signal when doing calibrations, and thus the signals during our experiments are also in question. I have let the Oxygraph run during an air calibration (using solution B) for two hours with little improvement in signal stability.'

The traces (Fig. 1) indicate accomplishment of a perfect sensor service: The oxygen signal is stable, yet the apparently 'noisy' line (red) may be disturbing at first sight.

Interpretation of such traces, obtained during oxygen calibration in a first functional evaluation of high-resolution respirometry, requires a short explanation.

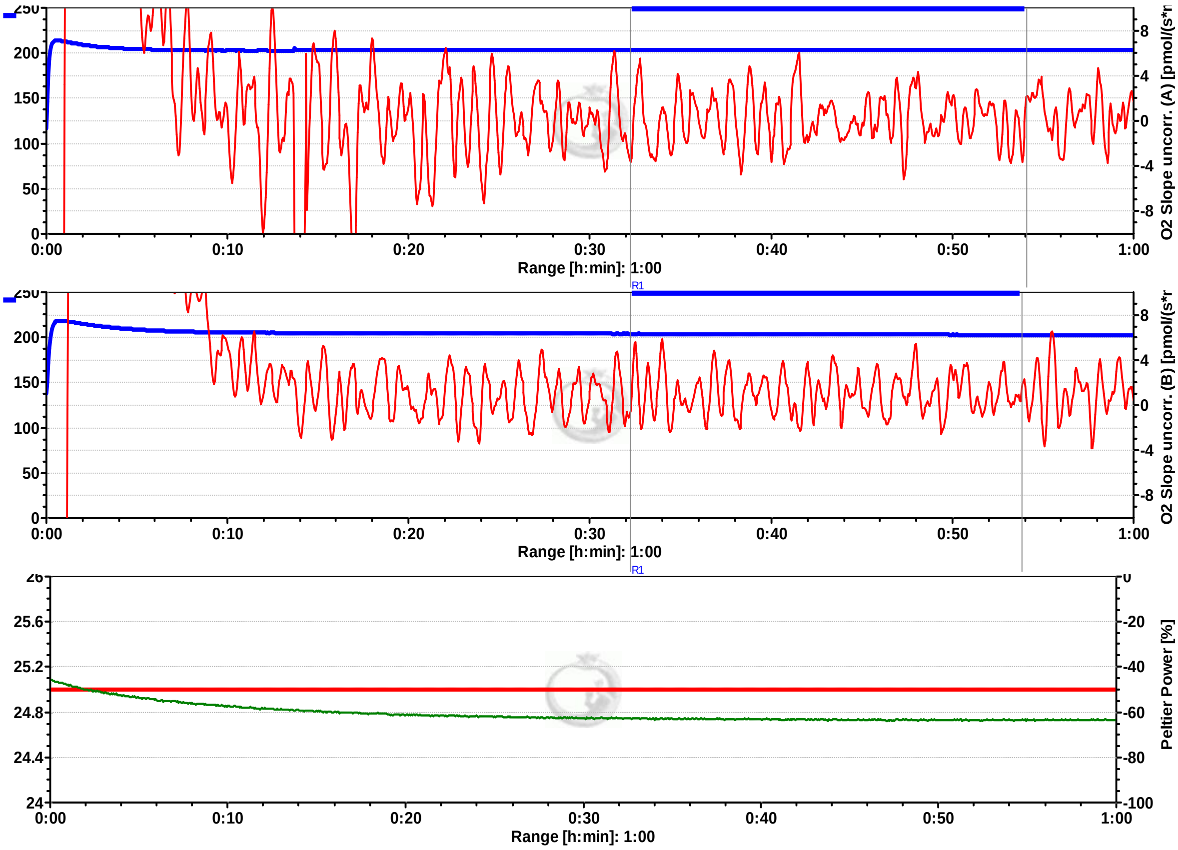
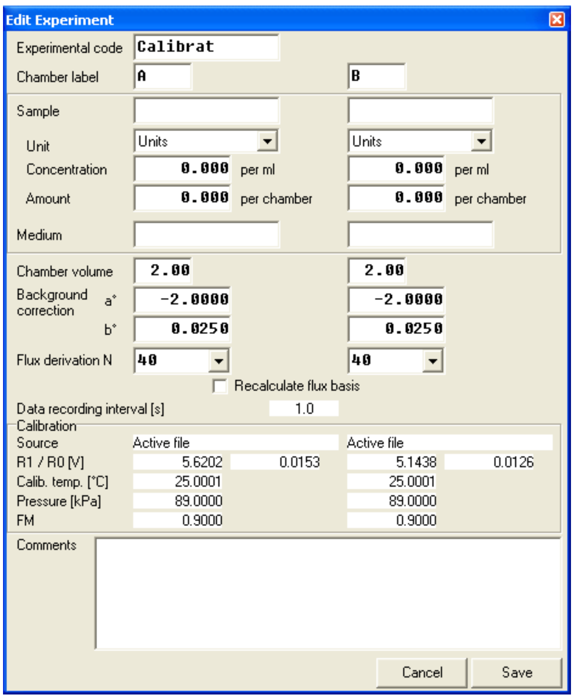


Figure 1. Calibration trace in Graph layout '1 Calibration Exp. Gr3-Temp', displaying oxygen concentration (blue) and uncorrected slope (red) in the upper two graphs, and block temperature (red) and Peltier power (green) in the lower graph.



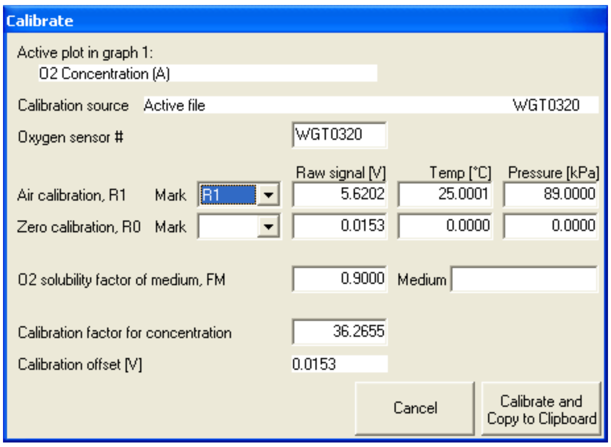


Figure 2. Experimental conditions during calibration are shown in the 'Edit Experiment' window (left), after air calibration (right) based on the marks 'R1' in Fig. 1.

(1) Using the graph layout '1 Calibration Exp. Gr3-Temp' (Fig. 1), it is apparent that the traces for oxygen concentration (blue) stabilize within a time frame of 30 min, closely related to the time course of stabilization of Peltier power (Fig. 1, bottom graph; c. 60 % of cooling power is maintained at steady state). Thermal equilibrium is a key for signal stability and equilibration of oxygen between the gas and aqueous phases. After reaching equilibrium, a mark is set (Fig. 1; 'R1'), oxygen calibration [F5] is performed (Fig. 2, right), and data are copied from the clipboard and saved in the excel template (Table 1).

Table 1: After calibration [F5] data are automatically copied to clipboard. The Excel template 'O2k-Calibration_Clipboard' is opened, and data for each pasted into a new line, consecutively for each chamber. The Excel table is copied here in two parts, continuing with F_M (the solubility factor for the medium) in a new line.

Date @ connect	Time @ connect	Filename	Chamber Label	POS #	Calibration source	Gain	Temp.$_{R1}$ °C	Medium
			A					
2007-09-24	11:11:30	2007-09-24 AB-02	A	WGT0320	Active file	4.0	25.0	
			B					
2007-09-24	11:11:30	2007-09-24 AB-02	B	WGT0321	Active file	4.0	25.0	

F_M	R_1 Mark	R_1 V	R_0 Mark	R_0 V	F_c µM/V	$p_{b,R1}$ kPa	c_{R1} µM	Instrumental background	$a°$ pmol.s^{-1}.ml^{-1}	$b°$
0.90	R1	5.620		0.0153	36.266	89.000	203.2645	Instrumental background	-2.00	0.025
0.90	R1	5.144		0.0126	39.614	89.000	203.2648	Instrumental background	-2.00	0.025

(2) Using the graph layout '2 Background Experiment', and setting the time axis [F6] at a range of 2:00 hours, starting at 0:20 min, we obtain an apparently very stable oxygen signal in each chamber (blue), at low resolution (250 µM O2 full scale; Fig. 3). At the same time, the time derivative (Fig. 3; 'O2 Slope uncorr.', right *Y*-axis) is shown with very high amplification, giving an apparently high (disturbing?) noise level. In fact, this noise level is perfectly acceptable, for meeting the demanding criteria of high-resolution respirometry. The slope is shown in this graph layout at high amplification (range is ±10 pmol·s^{-1}·ml^{-1}), to obtain a graphic representation of signal stability (about ±4 pmol·s^{-1}·ml^{-1} in Fig. 3). The data recording interval was set at 1 s (see Edit Experiment window), which is used only for high time resolution in advanced kinetic applications. Our recommended data recording interval, sufficient for all routine applications, is 2 s, which then results in only half the 'apparent' noise of the traces for flux (at an identical setting for the number of data points used for calculating flux, 'Flux derivation N' of 40 in the 'Edit Experiment' window; Fig. 3).

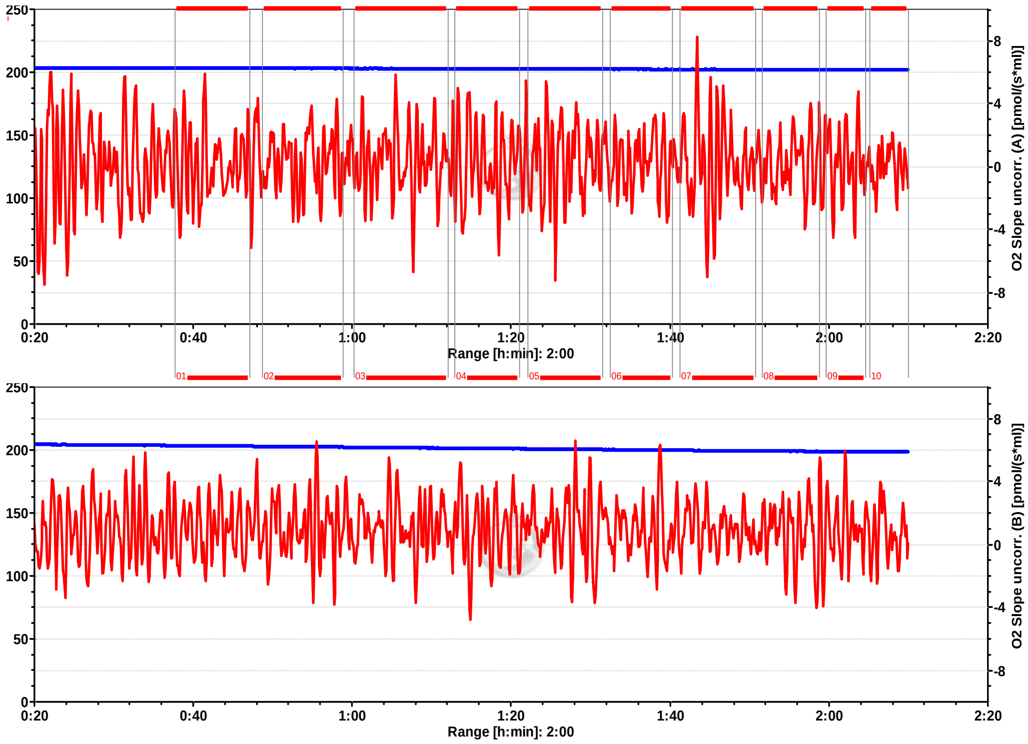


Figure 3. Oxygen signal stability over a two hour test, with a gas phase (air) maintaining a constant oxygen level, compensating for the minimum oxygen consumption of the polarographic oxygen sensor.

Typical experimental ranges of flux are 50 to 100 pmol·s^{-1}·ml^{-1}, such that the highly amplified apparent noise on the trace of flux becomes less disturbing. And as the oxygen concentration declines during experimental runs, noise is reduced an almost 'disappears' towards zero oxygen.

(3) Irrespective of the selected data recording interval, signal stability is evaluated by setting marks on the (red) plots for the slope, e.g. over consecutive intervals of c. 5, 10 or 20 min (marks in Fig. 3; only 6 of 10 marks are shown in Table 2). Signal stability (expressed as negative slope) was on average in chamber (A) 0.16 ±0.15 SD and chamber (B) 0.89 ±0.16 SD [units: pmol·s^{-1}·ml^{-1}] for 10 marks (474 ±143 s).

Oxygen signal stability in chamber (A) is just perfect at a slope close to zero (0.16 ±0.15 pmol·s^{-1}·ml^{-1}). For chamber (B) it is acceptable (close to specification: ±1 pmol·s^{-1}·ml^{-1}). In chamber (A) a practically stable signal was obtained, changing from 203.3 to 202.0 µM in two hours. Stability in chamber (B), however, was good but not optimal, with the apparent concentration dropping from 203.3 to 198.5 µM within two hours, despite the practically constant barometric pressure. A possible explanation is provided below.

Table 2. Mark statistics [F2] for evaluation of signal stability (Fig. 3).

2007-09-24 AB-02.DLD			Mark	Mark	Mark	Mark	Mark	Mark
	Averages	Unit	**1**	**2**	**3**	**4**	**5**	**6**
	Start	hh:mm:ss	00:37:39	00:48:39	01:01:58	01:12:51	01:22:05	01:32:25
	Stop	hh:mm:ss	00:47:01	00:58:51	01:12:02	01:21:00	01:31:28	01:40:18
	N Points		563	611	603	489	562	473
	O2 Concentration (A)	nmol/ml	203.3	203.1	202.9	202.7	202.5	202.4
X	O2 Slope uncorr. (A)	pmol/(s*ml)	0.2	0.2	0.4	0.2	0.1	0.2
	O2 Concentration (B)	nmol/ml	203.3	202.6	201.8	201.2	200.7	200.1
	O2 Slope uncorr. (B)	pmol/(s*ml)	0.9	0.9	1.0	1.0	1.0	0.9
	Block Temp.	°C	25.000	25.000	25.000	25.000	25.000	25.000
	Barom. Pressure	kPa	89.0	89.0	88.9	88.9	88.9	88.9
	Peltier Power	%	-63.3	-63.5	-63.5	-63.5	-63.4	-63.4

(4) The smaller the gas phase above the aqueous medium in the chamber, the smaller is the capacity of the system to compensate for the minimum oxygen consumption by the oxygen sensor. When a volume of 2.1 ml medium is added to the O2k chamber calibrated at 2.0 ml, then the volume of the gas phase depends on the precise position of the stopper, i.e. its displacement from the final 'closed' position. A new 'gas spacer' for the PVDF stoppers ensures that the stopper is optimally spaced during air calibration, and during any exchange re-oxygenation or oxygen depletion by purging the gas phase with a specific gas or gas mixture. After long-term air calibration with a small gas space, the gas phase may be slightly O_2 depleted due to the slow oxygen consumption by the POS. A simple control is possible to check for any oxygen depletion of the gas phase during such stability tests: The stopper is fully inserted (closed chamber), extruding the entire gas phase, and immediately return to the air calibration position (using the gas spacer). This re-generates the gas phase, and an increase of the signal after a short equilibration time would indicate a previous oxygen depletion of the gas phase, in contrast to sensor drift.

Air calibration should be performed, therefore, during the first hour of stabilization, followed by the background test requiring <2 h.

2. Instrumental background: the 'chamber calibration'

Long stability tests at air calibration were followed by an instrumental background test. Some results raised questions, if O_2 sensor (POS) service might be required. Importantly, POS function is evaluated during air calibration (and a quick stirrer test), performed before an instrumental background test. Sensor signal, stability (drift) and noise (and response time) are fully assessed during this calibration. The subsequent instrumental background test relates to the function of the O2k chamber. Zero calibration of the POS may be performed at irregular intervals independent of air calibration.

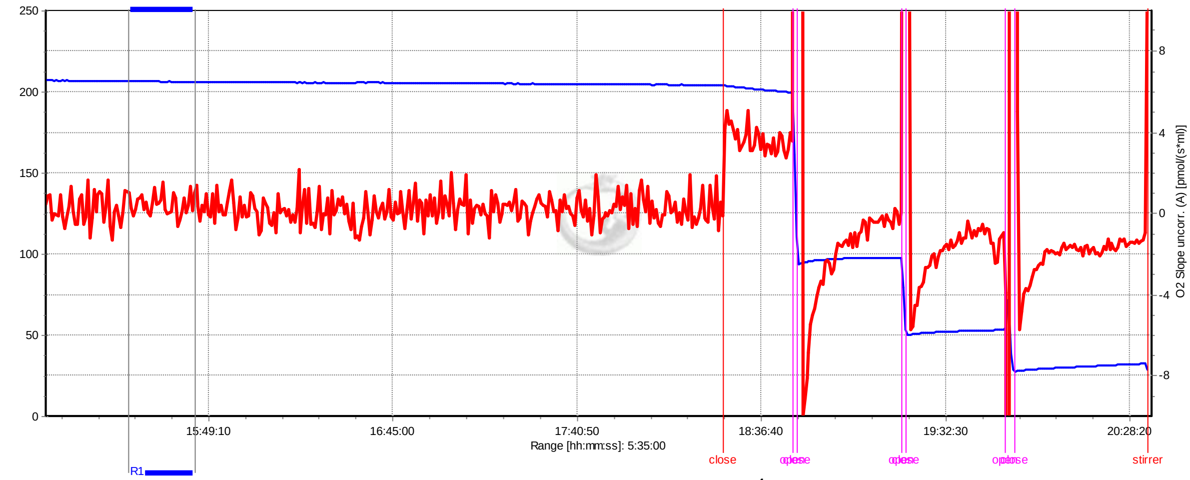
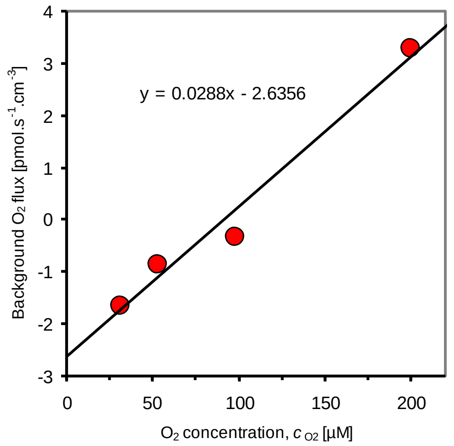


Figure 4. Air calibration followed by instrumental background test. After closing the chamber, the slope (red plot) indicates that, perhaps due to the long incubation for 18 h, $J°_{O2}$ is slightly elevated at air saturation, although 3.3 pmol·s^{-1}·ml^{-1} is in the specified range (top), as are the linear parameters (right: instrumental background O_2 flux as a function of O_2 concentration; experiment 2007-06-25 A-03).



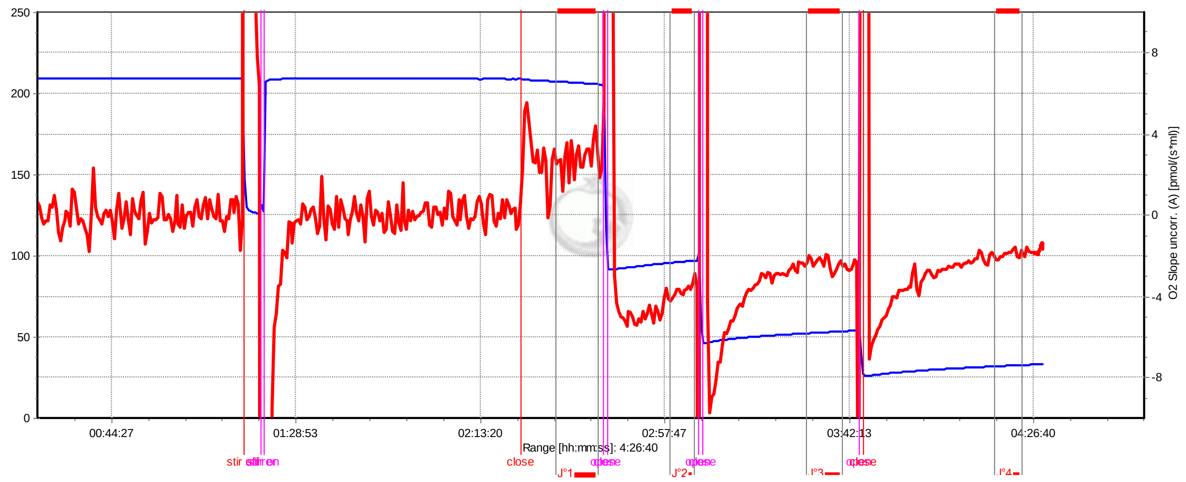


Figure 5. Air calibration, stirrer test and instrumental background. $J°_{O2}$ at air saturation is 2.8 pmol·s^{-1}·ml^{-1}. After closing the chamber at the O_2 level of c. 100 µM, a trapped gas bubble may cause a very negative background slope. Excluding this point (diamond, right), reproducible background parameters are obtained by comparison with Fig. 4 (experiment 2007-06-27 A-04).

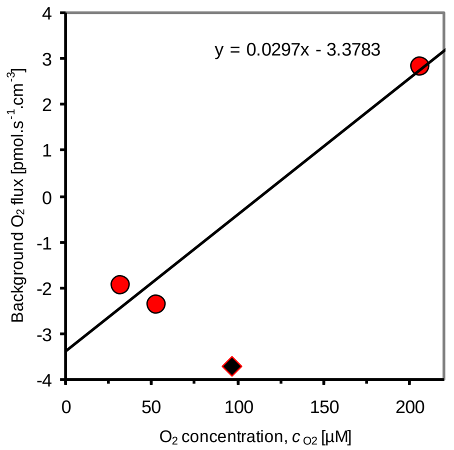
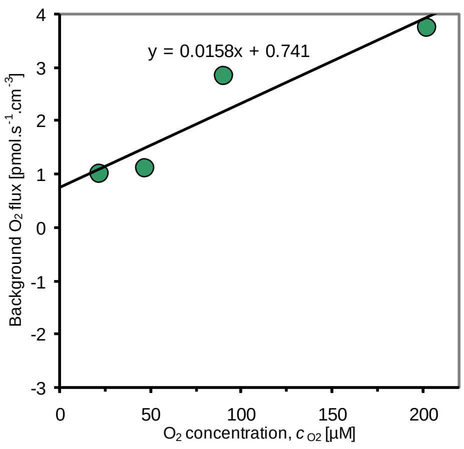


Figure 6.: In a parallel test in chamber B, there is an even further elevated $J°_{O2}$ at air saturation. Then - perhaps due to the repeated opening/closing of the chamber in the long-term test (>18 h) - oxygen consumption is elevated and over-compensates for the usual back-diffusion at low oxygen. Sensors etc. work perfectly fine, no reason for change of membrane or POS service. For standard cleaning of the chamber, see [MiPNet03.02 experiment 2007-06-25 B-03].



After 18 h at 37 °C in the O2k-chamber, there is a slight danger of microbial growth (Fig. 4). Evidence for such contamination comes from the results in Fig. 6, particularly since in a subsequent background run (comparable to Fig. 5), typical background parameters were obtained. Standard experiments never take that long, hence a routine background test including calibration should not take longer than 2-3 h.

Such a test was performed in the same chamber two days later (Fig. 5). The instrumental background oxygen consumption, $J°_{O2}$, at air saturation is now slightly lower and corresponds accurately to the expected value (2.8 pmol·s^{-1}·ml^{-1}). After the first reduction of oxygen concentration (to c. 100 µM), back-diffusion was untypically large, with a steep trend towards normal levels (perhaps due a small gas bubble?). The next steps, however, were quite normal, suggesting that there is no leak in the chamber (O-rings etc. are intact). These instrumental tests provide an excellent basis for starting an experiment.

'I thought that a lot of things were wrong and started to wonder if I would ever get the machine working. But it's already working properly, only I didn't see it.'