A computer-supported oxystat system maintaining steady-state O_2 partial pressures and simultaneously monitoring O_2 uptake in biological systems

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A feedback-controlled oxystat system is described maintaining steady-state O_2 partial pressures (pO_2) between 0.01 mmHg (14 nM- O_2) and 150 mmHg (210 μM - O_2) and simultaneously monitoring O_2 uptake at rates between 0.1 and 120 μM - $O_2 \cdot \text{min}^{-1}$ in suspensions of cells, in subcellular fractions and in solutions of enzymes. At pO_2 values between 0.2 and 150 mmHg (0.28 and 210 μM - O_2) a polarographic O_2 sensor was used, and below a pO_2 of 0.2 mmHg (0.28 μM - O_2) the O_2 -dependent luminescence of the photobacterium *Vibrio fischeri* was utilized to monitor the actual pO_2 . At a selected pO_2 , O_2 supply is maintained by injecting appropriate amounts of O_2 -saturated aqueous medium into the reaction chamber by using a motor-driven burette. The oxystat system is under control of a computer that reads the O_2 sensors, interacts with the motor-driven burette, calculates the O_2 uptake from the amounts of O_2 -saturated medium added, collects data from further measuring devices and provides the documentation of the results during incubation.

INTRODUCTION

There are several methods of maintaining steady-state O_2 partial pressures (pO_2) during the study of metabolic reactions in respiring biological systems, some of which also permit one to monitor O_2 uptake simultaneously (see Jones, 1985, for a recent review). The most widely used technique is to deliver O_2 via diffusion from a gas phase and to measure manometrically the amounts of O_2 consumed (Warburg & Kubowitz, 1929). However, at physiological pO_2 , for example, in incubations of isolated hepatocytes between 1 and 20 mmHg (1.4 and 28 μM - O_2) with rates of O_2 uptake between 12 and 25 nmol of $O_2 \cdot \text{min}^{-1} \cdot 10^6 \text{ cells}^{-1}$ and at normally used concentrations of $(2\text{--}4) \times 10^6 \text{ cells} \cdot \text{ml}^{-1}$, the manometric device is difficult to maintain near equilibrium, since the rate of O_2 transfer from the gas to the liquid phase becomes limiting (Roughton, 1941; Myers & Matsen, 1955). Extension of the manometric method to lower pO_2 values can only be achieved by extensive dilution of the O_2 -consuming particles. The manometric determination of O_2 uptake under those conditions, however, is technically difficult and a quite laborious process (Warburg & Kubowitz, 1929).

Rate limiting of O_2 transfer from the gas to the liquid phase can be circumvented by injecting O_2 dissolved in an aqueous medium (Garrison & Ford, 1970). We focused on this principle and developed an incubation technique, the oxystat system, particularly applicable to the maintenance of steady-state O_2 conditions at low pO_2 while permitting the determination of O_2 uptake simultaneously. The oxystat system is based on a feedback circuit employing a polarographic O_2 sensor or O_2 -dependent bacterial luminescence for continuous pO_2 monitoring, a motor-driven burette for the introduction of O_2 dissolved in an aqueous medium, and a computer for process control. The reliability of the technique is verified at steady-state pO_2 values between 0.01 and 150 mmHg (14 nM- and 210 μM - O_2) and rates of O_2

uptake between 0.1 and 120 $\mu M \cdot \text{min}^{-1}$ in incubations of enzymes and isolated hepatocytes.

MATERIALS AND METHODS

Glucose oxidase (EC 1.1.3.4) and horseradish peroxidase (EC 1.11.1.7) were from Boehringer, Mannheim, Germany. Bovine serum albumin was from Behring, Marburg, Germany. *Vibrio fischeri* was from the German Collection of Micro-organisms, Munich, Germany, and photobacterium broth was from Difco Laboratories, Detroit, MI, U.S.A. 4-Aminophenazone and 3,5-dichloro-2-hydroxybenzenesulphonic acid were from Aldrich-Chemie, Steinheim, Germany. Argon, O_2 and gas mixtures were from Linde, Düsseldorf, Germany. All the other chemicals were obtained from Merck, Darmstadt, Germany.

Hepatocytes were prepared as described by Berry & Friend (1969) as modified by Sies *et al.* (1977). *Vibrio fischeri* was harvested and utilized as described by de Groot & Haas (1980). H_2O_2 was assayed by using the peroxidase-catalysed reaction of H_2O_2 with 4-aminophenazone and 3,5-dichloro-2-hydroxybenzenesulphonic acid to yield a quinonimine, which was measured at 546 nm (Fossati *et al.*, 1980). The quinonimine concentration was calculated by using a molar absorption coefficient (ϵ_{546}) of $11 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

SYSTEM DESCRIPTION

Incubations were performed in a glass incubation chamber (11 ml) closed by a glass stopper that is drilled by three syringe needles (Fig. 1). One of them projects into the chamber and is used as the inlet for O_2 -saturated infusion medium. The other two end at the base of the

Fig. 1. Diagram of the oxystat system

stopper and served as a chamber outlet and as injection port for addition of reagents throughout incubation. In those incubations where the bioluminescence of *Vibrio fischeri* is used as O₂ sensor, the incubation chamber was placed in a light-tight box.

The components of the control circuit were: 1, a polarographic O₂ sensor (type MT-1-AC; Eschweiler, Kiel, Germany) used in conjunction with a pO₂ monitor unit (self-constructed); 2, an O₂ sensor based on bacterial luminescence (Lloyd *et al.*, 1981) [the latter consists of a glass-fibre optic (Schott, Mainz, Germany), one end of which was placed in front of the photomultiplier of a fluorimeter (Baird-Atomic, Braintree, Essex, U.K.); at the other end, 5–10 µl of a suspension of the photobacterium *Vibrio fischeri* (4 mg of bacterial protein · ml⁻¹, suspended in 0.5 M-NaCl/0.1 M-phosphate buffer, pH 7.0) is covered under a Teflon membrane which is fitted by means of a rubber O-ring]; 3, a motor-driven burette (ABU 80 Autoburette; Radiometer, Copenhagen, Denmark) with a reservoir for O₂-saturated infusion medium; 4, a DEC PDP 11/23 computer (Maynard, MA, U.S.A.) equipped with an eight-channel analog-to-digital converter for data input and a four-channel digital-to-analog converter for data output, and used in conjunction with a printer (DEC LA 180; Maynard) and a graphics plotter (7470A; Hewlett-Packard, San Diego, CA, U.S.A.).

At the input, two channels of the analog-to-digital converter were connected with the pO₂ monitor unit and the fluorimeter to read the actual pO₂. A third channel is used to store the motor-driven-burette signal, which is proportional to the amounts of O₂-saturated infusion medium added. The residual five channels are used to store data from further measuring devices, e.g. a photometer. At the output, one channel of the digital-to-analog converter is connected with the motor burette to activate the device. Programs providing process control and, simultaneously, processing of data

from the measuring devices, are developed under a real-time operation system RT-11 (DEC, Maynard) in FORTRAN IV.

At running time, the respective O₂ sensor continuously determines the pO₂ of the magnetically stirred incubation mixture. The computer reads the sensor signal and compares it with a preselected value. If the actual pO₂ is below the set point, the motor-driven burette is activated to deliver O₂-saturated infusion medium into the reaction chamber until the set point is approached. At the outlet, the displaced incubation medium is fractionated into cups and can be used in further assays. At preselected intervals the computer reads the outputs of the motor-driven burette and of further measuring devices, stores the data into memory, calculates the O₂ uptake from the amounts of O₂-saturated medium added, provides the processing of the data from the measuring devices and displays the actual incubation data on the screen. At the end of an incubation it lists the incubation data on printer and plotter.

CALCULATIONS AND THEORETICAL CONSIDERATIONS

The oxystat technique makes use of the principle that the O₂ supply in suspensions of respiring particles is maintained by injecting O₂-saturated aqueous medium. Therefore steady state conditions are attained when the rate at which O₂ is taken up ($\dot{N}O_2 \times V_i$) is equal to the rate at which O₂ is infused $[(O_2)_b - (O_2)_i] \times \dot{V}$:

$$\dot{N}O_2 \times V_i = [(O_2)_b - (O_2)_i] \times \dot{V} \quad (1)$$

where $\dot{N}O_2$ is the rate of O₂ uptake (nmol · min⁻¹ · ml⁻¹), V_i is the volume of the incubation chamber (ml), $\dot{V}$ is the flow speed of the motor-driven burette (ml · min⁻¹), $(O_2)_b$ is the O₂ concentration of the infusion medium (nmol · ml⁻¹), $(O_2)_i$ is the O₂ concentration in the incubation chamber

($\text{nmol} \cdot \text{ml}^{-1}$) and $[\text{O}_2]_b$ and $[\text{O}_2]_i$ are calculated by the terms given by Forstner & Gnaiger (1983).

Since V_i and $[\text{O}_2]_i$ are constant for an incubation at a preselected $p\text{O}_2$ value, it is apparent that, for a given $\dot{N}\text{O}_2$ of the respiring particles, various couples of the variables $[\text{O}_2]_b$ and $\dot{V}$ can be chosen to attain steady-state conditions. The appropriate couple depends on whether a minimal dilution of the respiring particles (high $[\text{O}_2]_b$ and low $\dot{V}$) or the determination of O_2 uptake with high precision (low $[\text{O}_2]_b$ and high $\dot{V}$) is aimed for; the latter is of particular importance at low O_2 -uptake rates. In the present study, where steady-state $p\text{O}_2$ values are maintained between 0.01 and 150 mmHg ($[\text{O}_2]_i$; 14 nM–210 $\mu\text{M-O}_2$), $[\text{O}_2]_b$ is chosen between 0.13 mM and 1.3 mM and $\dot{V}$ between 0.01 and 1 $\text{ml} \cdot \text{min}^{-1}$. $[\text{O}_2]_b$ is always kept at least six times higher than $[\text{O}_2]_i$. If these values are put into eqn. (1), they define the range of $\dot{N}\text{O}_2$ that is allowed to occur, at a selected $p\text{O}_2$, during an incubation (Fig. 4 below).

The O_2 uptake at a time t in relation to the concentration of the O_2 -consuming particles is calculated as follows:

$${}_s\text{NO}_2(t) = \frac{([\text{O}_2]_b - [\text{O}_2]_i) \times V(t)}{V_i \times C_{\text{res}}(t)} \quad (2)$$

where ${}_s\text{NO}_2(t)$ is the specific O_2 uptake [$\text{nmol} \cdot \text{mg}$ of protein $^{-1}$ (or $\text{nmol} \cdot 10^6$ cells $^{-1}$)], $V(t)$ is the amount of O_2 -saturated infusion medium added (ml) and $C_{\text{res}}(t)$ is the concentration of the O_2 -consuming biological particles [mg of protein $\cdot \text{ml}^{-1}$ (or 10^6 cells $\cdot \text{ml}^{-1}$)].

Provided that the concentration of the biological material at zero time of an incubation [$C_{\text{res}}(0)$] is known, the actual concentration [$C_{\text{res}}(t)$] can be calculated according to:

$$C_{\text{res}}(t) = C_{\text{res}}(0) \times e^{-[V(t)/V_i]} \quad (3)$$

From this equation it can be deduced that, at a volume of the incubation chamber of 11 ml, half-dilution of the respiring particles takes place after addition of 7.6 ml of O_2 -saturated infusion medium. For example, in an incubation of hepatocytes with 3×10^6 cells $\cdot \text{ml}^{-1}$ at the start and at an O_2 -uptake rate of 25 $\text{nmol} \cdot \text{min}^{-1} \cdot 10^6$ cells $^{-1}$, half-dilution occurs at about 23 min.

APPLICATIONS

Glucose oxidase-catalysed O_2 uptake and H_2O_2 formation was chosen as a first example to verify the validity of the oxystat system. As shown for an incubation at a steady-state $p\text{O}_2$ of 150 mmHg (210 $\mu\text{M-O}_2$) (Fig. 2), upon the addition of glucose oxidase to glucose-supplemented incubation mixture, O_2 is taken up and H_2O_2 is formed at constant rates. After 15 min the glucose oxidase concentration is doubled, which results in 2-fold increases in O_2 uptake and H_2O_2 formation. As would be expected, O_2 uptake and H_2O_2 formation proceed linearly with the respective rates throughout the incubation, and both parameters react stoichiometrically.

As a further example, the dependence of hepatocellular O_2 uptake on steady-state $p\text{O}_2$ between 0.01 and 150 mmHg (14 nM- and 210 $\mu\text{M-O}_2$) was studied. To yield a solution of the $p\text{O}_2$ desired, hepatocytes were mixed with appropriate amounts of incubation medium equilibrated with either O_2/CO_2 (19:1) or N_2/CO_2 (19:1). As shown for two representative incubations at $p\text{O}_2$ values of 0.2 and 20 mmHg (0.28 and 28 $\mu\text{M-O}_2$) (Fig. 3a)

Fig. 2. Glucose oxidase-catalysed O_2 uptake and H_2O_2 formation

Computer-generated plots of an incubation at 150 mmHg (210 $\mu\text{M-O}_2$) and 25 °C. The incubation mixture consists of air-saturated 1 M-glucose/0.1 M-phosphate buffer, pH 7.4, and peroxidase reagent comprising peroxidase (10³ units/litre), 3,5-dichloro-2-hydroxybenzenesulphonic acid (4.2 mM) and 4-aminophenazone (0.3 mM). The infusion medium was saturated with pure O_2 (1.3 mM- O_2) and was of the same composition as the incubation mixture. Glucose oxidase (GOD) (100 units/litre, measured at 100% O_2 , final activity) was added where indicated. The outlet of the incubation chamber is connected to a flow cuvette of a photometer to determine the quinonimine formed by the peroxidase-catalysed reaction of H_2O_2 with 3,5-dichloro-2-hydroxybenzenesulphonic acid and 4-aminophenazone.

respectively, O_2 uptake can be recorded immediately after the addition of the hepatocytes and it proceeds linearly for at least 30 min.

In the incubations described thus far, the O_2 concentration of the infusion medium was 1.3 mM. Thus, according to Fig. 4, at steady-state $p\text{O}_2$ between 0.2 and 150 mmHg (0.28 and 210 $\mu\text{M-O}_2$), $\dot{N}\text{O}_2$ is allowed to vary between 1.2 and 120 nmol of $\text{O}_2 \cdot \text{min}^{-1} \cdot \text{ml}^{-1}$. In agreement with these theoretical considerations, no significant deviation from the set-point $p\text{O}_2$ was observed at 20 mmHg (28 $\mu\text{M-O}_2$) (Fig. 3a). However, at 0.2 mmHg (0.28 $\mu\text{M-O}_2$) steady-state conditions are maintained with a signal-to-noise ratio of 5. The increase in the deviation at low $p\text{O}_2$ results from the electrical pick-up by the polarographic O_2 sensor that gains significance at the lower limit of its operation range. To enhance further the operation range of the oxystat system to $p\text{O}_2$ values below 0.2 mmHg (0.28 $\mu\text{M-O}_2$), the O_2 -dependent luminescence of the photobacterium *Vibrio fischeri* was chosen as O_2 sensor. Furthermore, the O_2 concentration of the infusion medium was decreased to 0.65 mM- O_2 . The biological O_2 sensor is calibrated by the addition of discrete amounts of O_2 -saturated infusion medium by means of either the burette or a gas-tight microlitre syringe (Fig. 3b). Afterwards the $p\text{O}_2$ was adjusted to 0.01 mmHg (14 nM- O_2) and the hepatocytes were added as a nearly anaerobic suspension and the oxystat system was started to monitor the O_2 uptake. After 10 and 20 min the set-point $p\text{O}_2$ was increased to 0.05 mmHg and 0.1 mmHg (70 and 140 nM- O_2) respectively. As shown for the preceding application, the oxystat system responds immediately, approaching the new set point without delay, and steady-state conditions are maintained with a signal-to-noise ratio above 8. Plotting the rates of O_2 uptake versus $p\text{O}_2$ reveals an apparent cellular respiratory $K_{0.5}$ ($p\text{O}_2$) of 0.5 mmHg (0.7 $\mu\text{M-O}_2$) (Fig. 3c), a value similar to that estimated by Jones & Mason (1978).

Fig. 3. pO_2 -dependence of hepatocellular O_2 uptake

(a) Computer-generated plot of incubations at a steady-state pO_2 of 0.2 and 20 mmHg (0.28 and 28 μM - O_2) using the polarographic O_2 sensor; (b) plot of an incubation at a steady-state pO_2 of 0.01, 0.05 and 0.1 mmHg (14, 70 and 140 nM- O_2) using the O_2 -dependent bacterial luminescence as O_2 sensor; (c) rate of hepatocellular O_2 uptake as a function of the pO_2 of three different preparations of hepatocytes. Hepatocytes $[(2.5-4) \times 10^6$ cells/ml] were suspended in Krebs-Henseleit saline supplemented with glucose (10 mM), lactate (2.1 mM) and pyruvate (0.3 mM). The infusion medium was saturated with O_2/CO_2 (19:1) (1.2 mM- O_2) or $O_2/N_2/CO_2$ (10:9:1) (0.65 mM- O_2) respectively and was of the same composition as the incubation medium. Incubations were performed at 30 °C.

DISCUSSION

The present experiments demonstrate that maintenance of O_2 supply by injecting O_2 dissolved in aqueous media is a useful technique for the study of O_2 metabolism in suspended biological material under steady-state O_2

Fig. 4. Operation range of the oxystat system

The allowed range of the O_2 -uptake rate ($\dot{N}O_2$) at a preselected pO_2 between 0.01 and 150 mmHg ($[O_2]_i$; 14 nM–210 μM) was generated under the following assumptions: the volume of the incubation chamber (V_i) is 11 ml, the O_2 concentration of the infusion medium ($[O_2]_b$) is between 0.13 and 1.3 mM- O_2 , and the flow speed of the motor-driven burette ($\dot{V}$) is between 0.01 and 1 ml · min⁻¹ (half-dilution of the respiring particles occurs between 25 h and 15 min respectively).

conditions. In comparison with the currently used incubation techniques providing O_2 supply via a gas phase, the feedback-controlled oxystat system permits the maintenance of steady-state pO_2 from 150 mmHg (210 μM - O_2), a level normally present in air-saturated aqueous solutions, down to the extremely low level of 0.01 mmHg (14 nM- O_2) with high precision, even if the rates of O_2 uptake vary over the range 0.1–120 μM - O_2 · min⁻¹. Therefore it is not necessary to use O_2 -consuming particles in highly diluted suspensions, conditions which may cause loss of viability. The use of normal concentrations of O_2 -consuming particles [e.g. hepatocytes at $(2-4) \times 10^6$ cells · ml⁻¹] also permits the simultaneous determination of O_2 uptake and detection of metabolites even if the latter are formed in minor quantities.

The oxystat system responds without delay to sudden changes in O_2 uptake without affecting equilibrium conditions. These control characteristics are due to the high velocity with which dissolved O_2 can be introduced independent of the selected steady-state pO_2 . Therefore the present technique of O_2 supply can be easily applied to monitor fast changes of O_2 uptake, as frequently induced by the addition of reagents, in order to study the metabolic flux in isolated cells and mitochondria.

A further advantage resulting from the principle of O_2 supply is that the amounts of O_2 taken up during incubation can be calculated from the amounts of O_2 -saturated medium added. In this regard, the computer is especially useful because it calculates the O_2 uptake by correcting for the dilution. Also, by processing the results from other measuring devices, it provides a comparison between O_2 consumption and other metabolic reactions in each single experiment.

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REFERENCES

- Berry, M. N. & Friend, D. S. (1969) *J. Cell Biol.* **43**, 506–520
- de Groot, H. & Haas, W. (1980) *FEBS Lett.* **115**, 253–256
- Forstner, H. & Gnaiger, E. (1983) in *Polarographic Oxygen Sensors* (Gnaiger, E. & Forstner, H., eds.), pp. 321–333, Springer-Verlag, Berlin
- Fossati, P., Prencipe, L. & Berti, G. (1980) *Clin. Chem.* **26**, 227–231
- Garrison, J. C. & Ford, G. D. (1970) *J. Appl. Physiol.* **28**, 685–688
- Jones, D. P. (1985) in *Oxidative Stress* (Sies, H., ed.), pp. 151–195, Academic Press, London
- Jones, D. P. & Mason, H. S. (1978) *J. Biol. Chem.* **253**, 4874–4880
- Lloyd, D., James, K., Williams, J. & Williams, N. (1981) *Anal. Biochem.* **116**, 17–21
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Myers, J. & Matsen, F. A. (1955) *Arch. Biochem. Biophys.* **55**, 373–388
- Roughton, F. J. W. (1941) *J. Biol. Chem.* **141**, 129–145
- Sies, H., Akerboom, T. P. M. & Tager, J. M. (1977) *Eur. J. Biochem.* **72**, 301–307
- Warburg, O. & Kubowitz, F. (1929) *Biochem. Z.* **214**, 5–18

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