Some improvements of the microrespirometric technique

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INTRODUCTION

The microrespirometer, which allows to measure the gas exchange of small objects and permits illumination and microscopical examination of the objects during measurements, was elaborated and described in 1955 (Zurzycki 1955 a and b). Some construction improvements and detailed technical description of the microrespirometer was given by Starzecki (1961). Since then the microrespirometric technique has been used for many research works on leaves and leaf segments, and also on fungi and protozoans. Within the last 10 years some new modifications and improvements of this technique have been done. The aim of this paper is to describe some of them i.e. the new optical system for illumination, technique of light measurements, modification which enables to mix two drops of liquid during measurements.

OPTICAL SYSTEM FOR ILLUMINATION

The scheme of the old and new optical systems is shown in figure 1. In the old system the plane of the condensor lens was projected by means of an collector lens on the plane of microchambers and formed there a homogenous illuminated disc of about 20 mm diameter. A part only of this area was utilized for illumination of the objects and the numerical aperture of the system was very low. The new optical system consists besides a condensor lens of a double collector lenses, correction lens and a raster of 4 small lenses situated below the microchambers and concentrating the light in these chambers. In the plane where biological objects are present four light discs (each in every chamber) of about 3 mm diameter are formed. The raster of lenses together with the correction lens are built in the lower part of the compensation chamber. The new system has two important advantages. For the first much higher illumination intensities can be obtained due to the concentration of light. For example using 750 W, 110 V incandescent lamp supplied by 90 V and interference filter 454 nm in the old system we got the intensity 2500 erg/cm²sec., whereas in the new one 29200 and for 676 nm 7620 and 73000 erg/cm²sec.

Fig. 1. Scheme of the optical paths in the old and new system for illumination of objects

respectively. The other advantage is the increase of the numerical aperture of the illumination. This is important for microscopical observations. The quality of microscopic picture is much better by the new illumination system than by the old ones.

MICROSCOPE FOR OBSERVATIONS OF OBJECTS

The microscope (PZO — M1 2) was reconstructed and adapted for control of objects during gas exchange measurements. The mechanical stage of the microscope movable in two directions in the horizontal plane was mounted out. This stage serves as a base for the microscope (Fig. 3). The optical axis of the microscope can be shifted in two directions in the horizontal plane and put above each reaction chamber. The reading scales on the stage make it easy to set up the optical axis of the microscope in a proper position. As objectives Cook's long distance working objectives 20 x and 40 x were used.

MEASUREMENT OF THE INTENSITY OF ILLUMINATION

By the old illuminating system it was possible to measure the light intensity with Kipp and Zonnen thermopile. The thermopile had to be put in such a way that the plane of the thermostrips should be situated at the

Fig. 2. Raster lens.

level of the objects. For measuring light intensity in small light discs in the new optical system another light detector must be used. For this purpose a photomultiplier was adopted. A RCA 21 P photomultiplier is closed in a light tight container. The only whole of this box is supplied with a conical tube ending with a window of 2 mm diameter. An opal plate is mounted in that window (Fig. 4) During light measurements the box with the photomultiplier stands on a special plate covering the lower part of the compensation chamber (Fig. 5); the position of the window may be set up exactly at the level of the biological objects by means of four screws.

The photomultiplier is supplied by a stabilizing voltage 750 to 1500 V and the current is read with a Zeiss mirror galvanometer. A high sensitivity of the photomultiplier permits to measure with this arrangement very low light intensities of the order of 1 erg/cm²sec. The described system allows to measure the light intensity not only in the place of microchambers but also in microchambers containing buffer. It permits also to check equality of illumination in each chamber and (using a smaller window) the homogeneity of illumination across the light spot.

Fig. 3. Microscope adapted to observations of objects in microchambers in the course of gas exchange measurements.

Fig. 4. Cross section through the box with a photomultiplier.

a — photocell, *b* — box, *c* — conical tube, *d* — opal glass window, *e* — neutral wire screen
filters put in the light beam by knob — *f*

Fig. 5. Position of the photomultiplier during light measurement.

MIXING OF FLUIDS IN THE MICROCHAMBERS

The biological objects used for measurements are usually situated in a hanging drop on the glass covering the microchambers (Fig. 6). The drop contains 1—5 μ l of liquid. Sometimes it is necessary to use a bigger amount of liquid containing biological material. A small plastic ring cemented to the cover glass permits to use the amount up to 25 μ l of liquid and prevents from overflowing (Fig. 6 b) (Pado 1967).

Fig. 6. Covers of the reaction chambers with a — object in a small drop of medium, b — 25 μ l of cell suspension, c — two drops of liquid designed for mixing.

For mixing two liquid drops another cover of the microchambers was introduced. In a brass plate of the dimension of the cover glass and 1 mm thick an elongated whole about 5 x 3 mm was made. The whole is closed from one side by a glass cemented to the brass surface. In this way we get a small chamber. The brass surface is covered with a layer of chemically inactive resin. To each side of the chamber 1—2 μ l of different liquids were put. In one drop a "flea" made of a small piece of iron embedded in resin (Glick, 1949). The flea may be shifted from one side of the chamber to the other one by means of a magnet moved above the upper window of the compensation chamber without opening it. In this way it is possible to mix two drops of liquids in the course of gas exchange measurements.

APPLICATION OF CARBONATE BUFFERS

In order to maintain a constant CO_2 concentration in the reaction chambers usually 0,1 M/l carbonate buffers was used. For instance Warburg buffer No 9 which keeps the CO_2 concentration on the level 0,3% (at 25°C) (Warburg 1919). In the modern manometric techniques application of a higher concentration of buffers is recommended (Kleinzeller *et al.* 1965). The use of various buffers has been studied in the

Table 1

Concentration	Composition	
0.1 M/l	Na ₂ CO ₃	15 parts
	NaHCO ₃	85 ..
0.2 M/l	Na ₂ CO ₃	24 ..
	NaHCO ₃	76 ..
3 M/l	K ₂ CO ₃	60 ..
	KHCO ₃	40 ..

following experiments. Table 1 presents the composition of the tested buffers, at which carbon dioxide concentration is kept at the level 0,3% (25°C) in the gas phase.

In some experiments a crude enzyme preparation of carboanhydrase prepared according to Negelein and Noll (1963) 4 mg/ml was added into of the buffer solution.

The first experiment was performed without any living objects. One drop (2 µl) of 0.02 M/l KHCO₃, and separately one drop (2 µl) of 0.1 nHCl was added into the described arrangement where liquids can be mixed.

Table 2

Intensity of gas exchange µl O ₂ /10 min	Limiting volume of 0,2 M buffer	
	1 hour	3 hours
0,2	12	24
0,37	24	36
0,55	36	—

Different amounts of buffer solutions were pipeted onto the bottom of the microchambers. About 1/2 hour after closing the microrespirometer (necessary to equalize temperature) the two drops were mixed and CO₂ released. Changes of gas volume were measured in the meantime. The results are present in figure 7. After mixing the carbonate solution with HCl CO₂ is quickly released and in 2 minutes a new volume of the gas phase becomes constant. Absorption of the released CO₂ by buffers depends on the volume of buffers. Even 6 µl of 0,2 M buffer cause a rather good absorption (retension is negligible) but the time necessary for reaching equilibrium is rather long (40—50 min). No retension at all can be found when greater volumes of buffers were used the more buffer is in the microchamber the shorter is the time of CO₂ absorption. This time is 50, 30, 15 and 10 min. for 6, 12, 24 and 36 µl of buffer respectively. Application of enzyme carboanhydrase shortened the time of equilibrium by one half. One can generally state that under steady release of

Fig. 7. Volume changes of the gas phase after mixing carbonate ($2 \mu\text{l} - 0,02 \text{ M/l}$) with HCl ($2 \mu\text{l} - 0,1 \text{ n}$) in microchambers containing no buffer (0), and different amounts (figures by the curves — volume in μl) of $0,2 \text{ M/l}$ carbonate buffer. E — buffer contains carboanhydrase preparation.

Fig. 8. Rate of photosynthesis as measured by various amounts (figures by the curves — volume in μl) of $0,2$ — (solid line), and $0,1$ (dash line) of buffers and for various amounts of *Chlorella* packed volume (figures above).

Fig. 9. Short time registration of gas exchange by *Chlorella* in the transition period after changing light conditions. a — 0,2 M/l buffer without enzyme preparation, b — with carboanhydrase preparation.

CO_2 24 μl of buffer should suffice in the used conditions. To registrate rapid changes in CO_2 concentration application of enzyme is recommended.

The second series of experiments was made with alga *Chlorella pyrenoidosa*. The suspension of alga was centrifugated and various amounts of packed alga were pipeted onto the cover glass of the reaction chamber with the same amount of water. The dry mass of 1 μl of packed volume of alga was 0,164 mg. Photosynthesis was measured succesively during 3 hours.

Application of 3 M/l carbonate buffer induces a very great dessication of the biological material. Within $1/2$ —1 hour algae become completely dry. Therefore concentrated buffers were no more used. In 0,2 or 0,1 M buffers the experiments may be continued up to 5—6 hours, the material being not desicated. The measured photosynthetic activity is presented in fig. 8. It is to be seen that if the registered gas exchange should reflect the constant in time process the amount of buffer must exceed a certain limit. This limit depends on the intensity of gas exchange and on the time of measurements (Tab. 2). The values for 0,1 M buffer are bigger. Addition of enzyme preparation does not change the intensity of registered gas exchange. On the other hand the registration of gas exchange in short time intervals following sudden change of the CO_2 production or consumption showed (Fig. 9) that when using only buffers there is a cer-

tain lag period (about 5 min) necessary to obtain the readings constant in time; when applying an enzyme preparation this lag period nearly disappears and production or consumption of CO_2 is constant just in the first minutes.

SUMMARY

Some improvements and modifications of microrespirometric technique were described. They consist of

- 1) a new optical system which permits to obtain 10 x higher light intensities with the same light source and improves the numerical aperture of illumination.
- 2) adaptation of the microscope for observations of objects
- 3) technique of light measurements on small areas
- 4) modification allowing to use cell suspension up to 25 μl
- 5) modification allowing to mix two drops of liquids in the reaction chamber during measurements.

Application of carbon buffers was studied critically. The amount of buffer necessary to obtain real and constant results of gas exchange depends on the intensity of gas exchange and the time of measurements. Limiting values for some conditions have been given. Addition of carboanhydrase preparation to buffer is recommended for registration of rapid changes of gas exchange in short time intervals.

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Pewne udoskonalenia techniki mikrorespirometrycznej

Streszczenie

Opisano szereg modyfikacji i udoskonaień metody mikrorespirometrycznej, a mianowicie:

1. Nowy system optyczny, który pozwala uzyskać 10-krotnie wyższe natężenie oświetlenia przy zastosowaniu tego samego źródła światła i znacznie polepsza aparaturę numeryczną.

2. Adaptację mikroskopu do obserwacji obiektów.

3. Technikę pomiarów intensywności światła na małych powierzchniach.

4. Modyfikację pozwalającą stosować zawiesinę komórek objętości do 25 μl .

5. Modyfikację pozwalającą na mieszanie 2 kropeł płynów w komorach reakcyjnych w trakcie pomiarów.

Przeprowadzono krytyczne badania nad stosowalnością buforów węglanowych. Objętość buforu konieczna dla uzyskania rzeczywistych i stałych w czasie pomiarów przemiany gazowej zależy od intensywności tej przemiany i czasu pomiarów. Wartości ograniczające zostały podane dla kilku warunków. Dodatek preparatu karboanhydrazy do buforu jest szczególnie godne polecenia przy rejestracji nagłych zmian natężenia przemiany gazowej.