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An improved microrespirometer and extension of its application over plants with big leaves

by

W. STARZECKI

INTRODUCTION

This paper presents the description of some improvements in the construction of the microrespirometer devised by J. Zurzycki (1955 a and b) and destined for researches on photosynthesis and respiration. Due to its great sensitivity permitting the evaluation of 10^{-3} microliters of gas, it could be applied for measurements of assimilation rates of small objects not exceeding several square milimeters area. Its construction enabled also a microscopic observation (magnification 100—150) of the assimilating object, during the whole time the measurements were performed. Changes of illumination intensity were achieved by shifting the lightening equipment on the optical bank.

With the aid of the microrespirometer of Zurzycki the following problems made the subject of a detailed study:

1. The influence on photosynthesis of the arrangement of chloroplasts in a cell (Zurzycki 1955a and b),
2. The influence of polarised light on photosynthesis (Zurzycki 1955c),
3. The action of inhibitors on the intensity of respiration and photosynthesis (Zurzycki 1955 d),
4. The harmful action of light of high intensity on leaves (Zurzycki 1957),
5. A research on the photosynthesis and respiratory activity (including the determination of the points of compensation and light saturation) of various forms of the fern *Asplenium trichomanes* grown in intensive and poor natural illumination (Starzecki 1958),
6. Dependence of photosynthesis on light intensity and thickness of the leaf of *Asplenium trichomanes* (Starzecki 1959),
7. Quantum yield of photosynthesis of leaves of *Asplenium trichomanes* (Starzecki, in preparation),

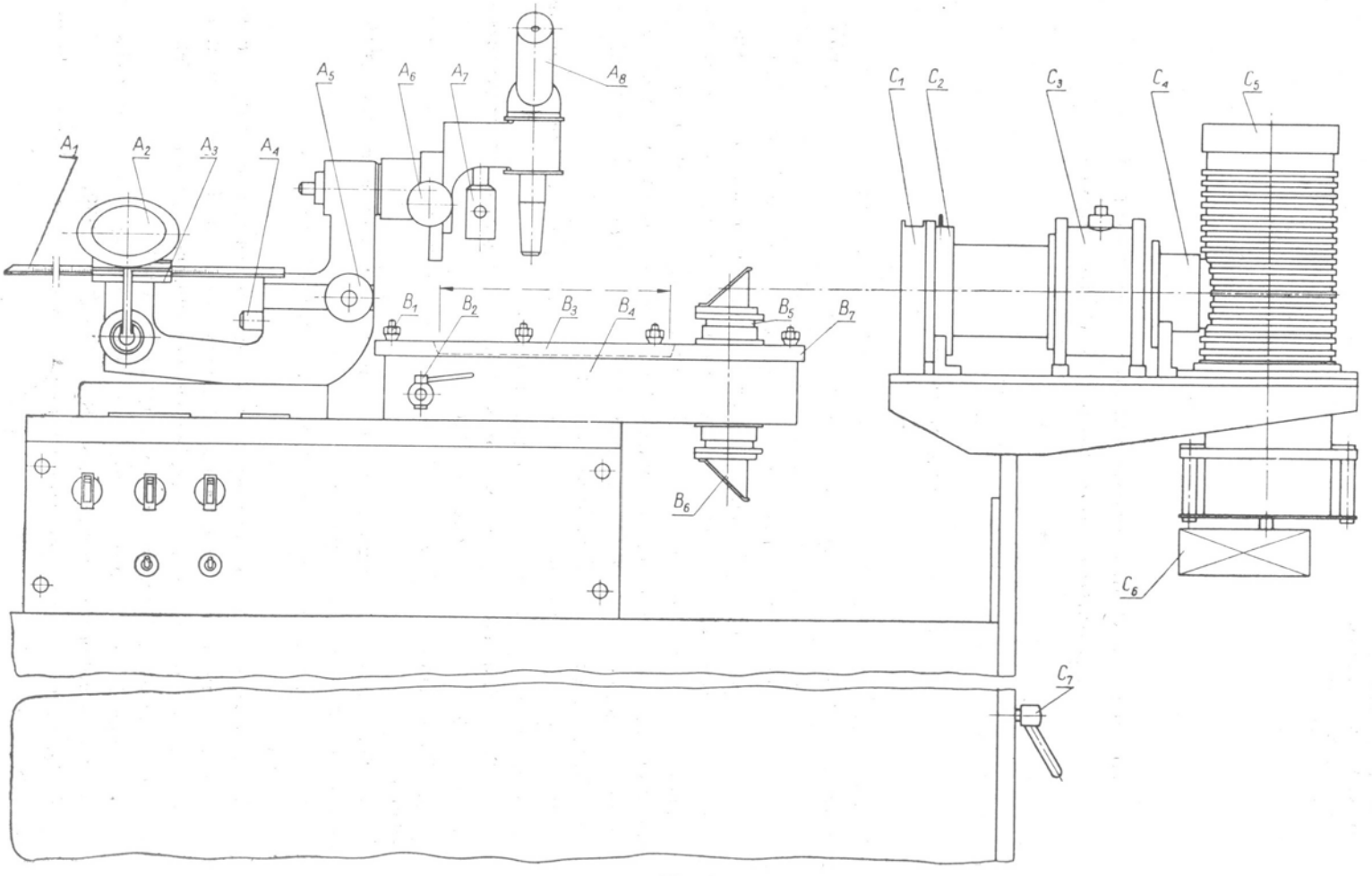

Fig. 1

8. Finally a detailled study on the influence of rhodamine B on photosynthesis was performed with the help of the modified microrespirometer (Zurzycki and Starzecki 1961).

Assimilating organs of following plant species provided the material used in the above mentioned studies: *Mougeotia* (fragments of filament), *Spirogyra* (fragments of filament), *Lemna trisulca* (individual fronds), *Asplenium trichomanes* (individual leaflets).

This paper consists of two parts: the first is a description of an improved microrespirometer in the line of attaining a greater number of repetitions in the same period of time, an easier handling, a better utilization of the light energy emited by the light source and a possibly wide range of regulating the light intensity and temperature. In the second part the possibilities of applying the improved microrespirometer for the determination of photosynthesis of large leaves are examined.

CHANGES IN THE CONSTRUCTION OF THE MICRORESPIROMETER

a. No changes in the working principle of the apparatus of Zurzycki (1955a and b) have been introduced into the new design of construction (fig. 1). Its principle is based on measurement of the change of volume caused by uptake or emission of oxygen. The application of bicarbonate buffer of Warburg assures a constant concentration of CO_2 in the atmosphere of the chamber.

Changes of volume are read from the position of a karosene drop in the capillary.

The respirometer constructed by Zurzycki in 1955 was equiped with two micro-chambers: the reaction chamber and thermobarometric chamber were of the same size and shape. The new modification contains

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Fig. 1. Microrespirometer (whole set)
Indications: A — refer to the microscope with a horizontal movement; B — to the main chamber; C — to the lightening equipment

A_1 — a rod with a millimeter scale; A_2 — magnifying glass for reading the scale; A_3 — handle with the nonius; A_4 — screw blocking the sliding movement; A_5 — screw for the horizontal mouvement; A_6 — screw for focusing adjustment; A_7 — lamp for illumination the scale; A_8 — microscope; B_1 — screws to fasten the cover; B_2 — stopcock to equalize the pressure; B_3 — window through which the kerosene meniscus is read; B_4 — base of the chamber; B_5 — prism with holder for illuminating the object from the upper side; B_6 — prism with holder for illuminating the object from the lower side; B_7 — cover of the chamber; C_1 — frame (handle) for filters; C_2 — holder of the lens and the iris diaphragm; C_3 — holder for liquid filter; C_4 — holder of the lens; C_5 — holder of the lamps; C_6 — motor of the exhaustor; C_7 — screw blocking the horizontal movement of the lightening equipment.

4 micro-chambers bored in a brass plate 45 × 32 × 3 mm in size (fig. 2). The circular chambers Ø 7 mm are closed on their lower side with a glass plate ±0,7 mm thick which is stuck tightly with araldite. On the upper side of the plate, elliptical hollowings 0,6 mm deep enclose the reaction chambers connected with the ends of respective capillaries. For experiments the micro-chambers are prepared in the following manner: kerosene columns 10 mm long are introduced into the capillaries by means of a pipette. They are deplaced through the whole length of the capillaries

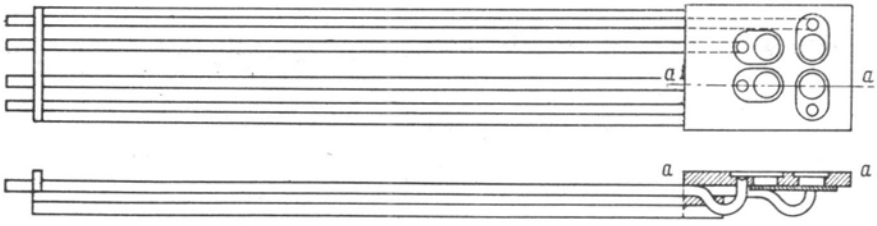

Fig. 2. Plate with four micro-chambers: at the top — view from above, at the bottom — view from one side and in the section through the axis a—a

in order to moisten the inner walls. After this had been accomplished the kerosene columns are placed near the middle of the capillary length. Then 35 µl Warburg's carbonate buffer No. 10 are pipetted into every chamber. The chambers are closed on their upper side with a cover glass fitted to the elliptical shape of the brim round the chambers. The experimental material is placed in hanging drops on three elliptical cover glasses. The fourth chamber with no material is also covered with a glass plate and acts as a thermobarometer. The fissures between the glass and the edges of the elliptical brim of the plate are filled with melted vaseline, in order to make them tight, but a small part of these fissures

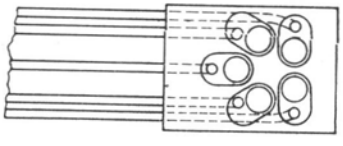

Fig. 3. Plate with five micro-chambers

remains unfilled. The micro-chambers are finally closed when their temperature is equalized with that of the macro-chambers. Micro-chambers with five capillaries (4 reaction chambers and one thermobarometer chamber) may also be used in the apparatus. Fig. 3 shows the distribution of the 5 micro-chambers on the brass plate.

b. Macro-chamber. The macro-chamber (fig. 1 B) serves to place the micro-chambers in, to maintain a constant temperature during the whole

experiment and acts also as compensation chamber. In the previous construction this chamber constituted formally a separate unit.

Micro-chambers are placed in the elongated cavity of the water bath with water circulation, supplied by a Hoepler's ultrathermostat. There is a small window under the brass plate with the micro-chambers through

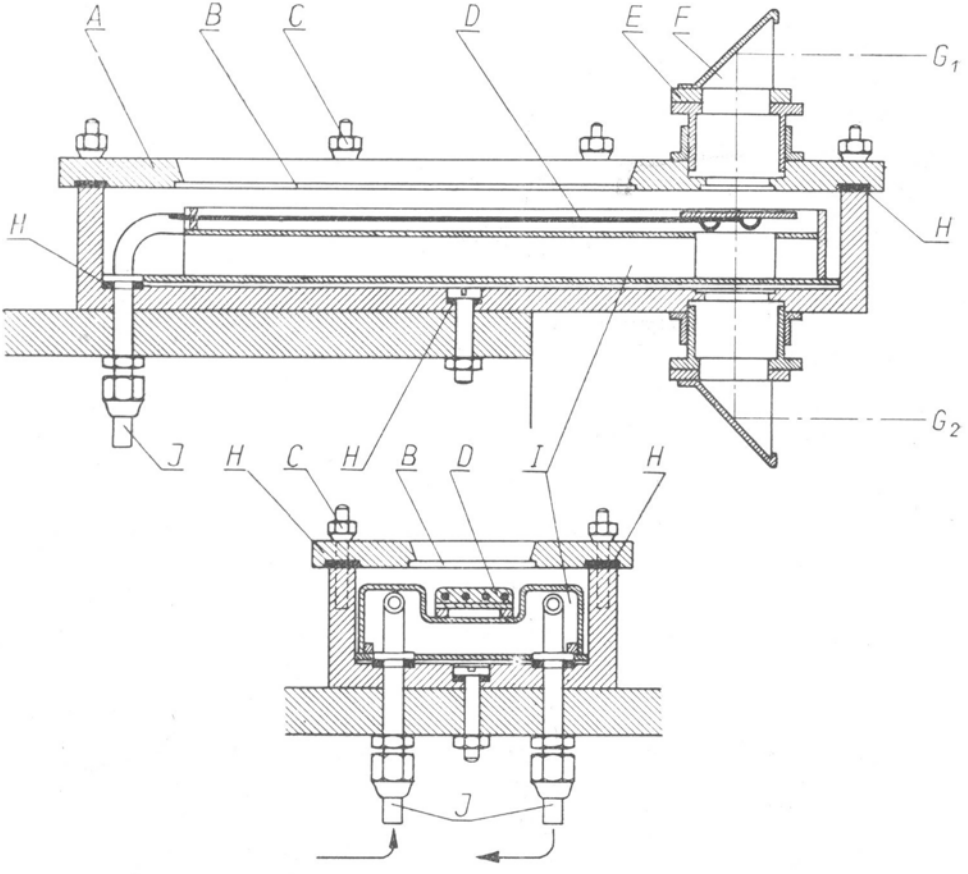

Fig. 4. Main chamber (sections):

A — cover of the chamber; *B* — window through which the kerosene meniscus is read; *C* — screws to fasten the cover; *D* — plate with micro-chambers; *E* — prism holder; *F* — prism; *G* — boundle-light; *H* — liners; *I* — water bath; *J* — connector for the ultrathermostate

which light can penetrate. There is a similar window in the cover too. Both these windows are hermetically closed with glass plates. Glass prisms attached to a piece movable in vertical direction are placed on the outer side of the windows. These prisms change the horizontal direction of the

light beams to a vertical and perpendicular to the surface of the plate of the micro-chambers (fig. 4). Having removed the upper prism with the movable cylindrical piece it is possible to set up a microscope deprived of its table and provided with a COOK's A.E.L. × 20 or × 40 objective.

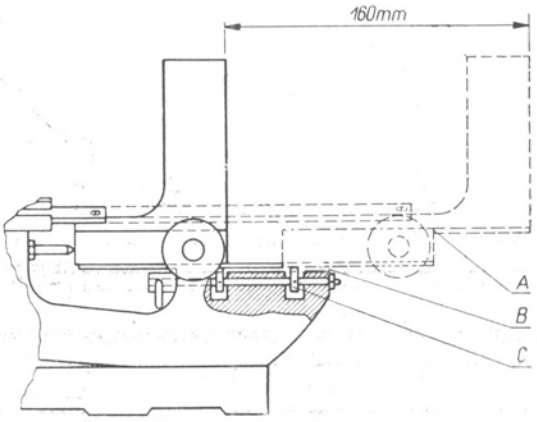

Fig. 5. Scheme presenting the principle of the horizontal movement of the microscope: *A* — microscopic mouvement macro; *B* — sliding mouvement; *C* — arrangement blocking the sliding movement

In this way a direct observation of the object during the experiment is achieved. The cover is screwed hermetically on a ruber liner with 8 screws. The ruber liner is fixed in the brim of the cover. The equalization of inner pressure and outer atmospheric pressure is achieved by means

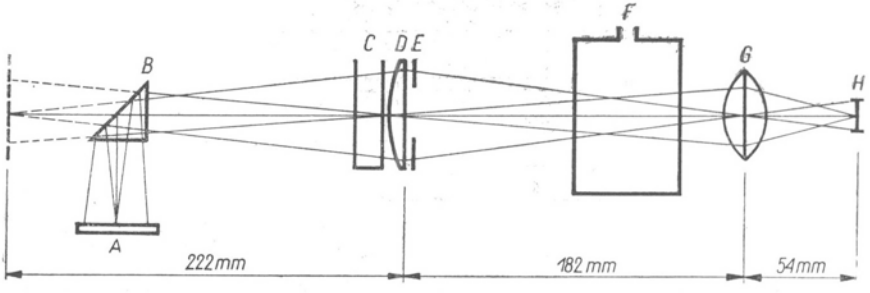

Fig. 6. Scheme of the lightening equipment: *A* — plate with micro-chambers; *B* — prism; *C* — holder of the glass and interference filters; *D* — lens; *E* — iris diaphragm; *F* — glass cell for liquid filter for absorption of heat radiations; *G* — lenses; *H* — filament of the bulb

of a stop-cock fastened to the sidewall of the macro-chamber. There is still in the cover a small window 165 × 35 mm. It is hermetically closed with a glass plate 3 mm thick. This window enables the reading of the position of kerosene meniscus in the capillaries.

c. Microscope with a horizontal movement. This microscope is used for readings of the position of the meniscus of kerosene drops in the capillaries (fig. 1A). A range of movement restricted to 80 mm was a great inconvenience of the formerly used microscope, as the length of the capillaries was 160 mm. An increase of the range of reading was achieved by moving the microscope along the capillar axis. This structural inconvenience has been improved by introducing an additional sliding arrangement which extended the range of readings to the whole length of

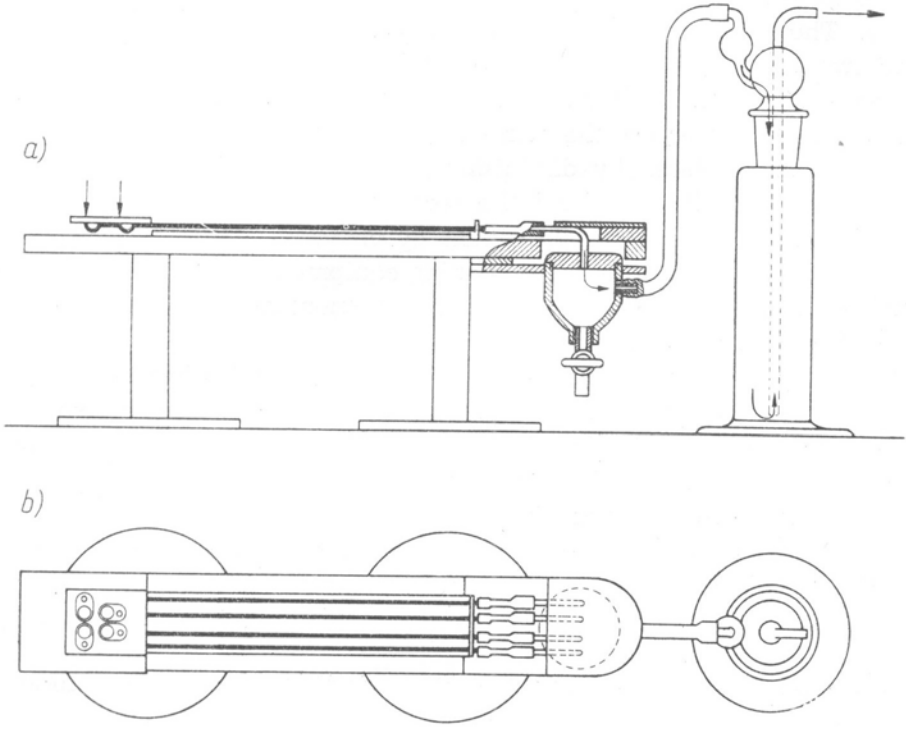

Fig. 7. Arrangement for washing the capillaries. At the top — view from one side, arrows show the air circulation (*a*), at the bottom — view from above (*b*)

the capillaries. The working scheme of this arrangement is shown in fig. 5.

The procedure adopted for reading the position of the index in the capillary has been also modified. In the former type the microscopic ocular was provided with a scale consisting of 50 divisions. The optical equipment of the microscope was chosen in such a way that the 50 divisions in the ocular corresponded exactly to 5 mm. an interval corresponding to 5 mm of the scale of the capillaries. To perform the readings, the scale of the ocular had to be adjusted for the place where the meni-

scus of kerosene was. It was possible then to read the position of the kerosene index, with accuracy to 0,1 mm.

In the new modification the ocular scale has been replaced by a line perpendicular to the scale of the capillaries. A rod with a milimeter scale is fastened to the microscope and moves in the handle fixed to the microscopic table when the microscope moves horizontally. A nonius is attached to the handle by means of which any change of the position of the microscope can be read with accuracy to 0,1 mm. A 3 times magnifying glass is placed above the nonius in order to make the readings easier.

d. The lightening equipment consists of a projection lamp 250 W 220 V and two lenses (25 and 12 dioptres) which are placed as shown in the scheme (fig. 6). This equipment gives an uniformly illuminated light spot of 34 mm diameter on the windows of the micro-chambers. The light intensity is regulated by diminishing or enlarging the diameter of the iris diaphragm placed behind the second lens. The light intensity may also be modified to a certain extent by changing the voltage by means of an autotransformator. The lightening equipment is fixed to rails which enable the vertical movement of the equipment and the illumination of the object from its upper and lower side.

e. A special equipment for washing the capillaries has been established (fig. 7). Cleaning fluid, distilled water and ethanol are introduced in turn into the capillaries by means of a water pump. The washed capillaries are then dried with a current of air.

For other details concerning the preparation of the apparatus for experiments and their execution the reader is referred to Zurzycki (1955a and b).

EMPLOYING OF THE MICRORESPIROMETER FOR PLANTS WITH LARGE LEAVES

As it was already said the microrespirometer was destined for examinations performed on small objects not exceeding 5 mm in length. To employ this microrespirometer for greater organs, for instance leaves, fragments of these organs must be used. This part of work aimes to examine whether a disk cut out of a leaf blade can provide convenient experimental material. In this connection the question arose whether the rates of respiration and photosynthesis undergo any changes in the course of experiment and whether there is a period of time with no fluctuations of greater importance.

It should be stressed however, that the rates of assimilation and respiration obtained on a leaf fragment do not represent the analogous

rates of the function of a whole leaf organ not separated from the mother plant. If, however, a leaf fragment proved to keep in constant conditions, for a time on the same level, without showing any visible changes, it could be used as experimental material for certain investigations.

a. Material and methods

Discs 4 mm in diameter cut out of leaves of *Pelargonium* and *Tropaeolum* provided the material used in this study. Only leaves of fully grown plants were used. Measurements started 1 hour after the discs were cut. The period of performing measurements was gradually extended from 2,5 hours to 72 hours. In the two first experiments the discs remained in the microrespirometer during the whole experiment. In other experiments the discs were kept in a weighing bottle between two successive measurements.

Cotton wool saturated with tap water was placed at the bottom of the bottle and covered with filter paper. Discs were cut out with cork-borer and placed on this paper (fig. 8). In this way the discs were completely protected from drying. The investigated material (experiment 3) remained

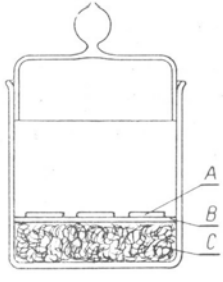

Fig. 8. The way of keeping the discs of leaf blades in a vessel: *A* — discs of a leaf blade; *B* — filtration paper; *C* — cotton wool

in a dark or light thermostat in about 21°C. The light thermostat was illuminated with fluorescent tubes which gave 1.100—1.200 lux light intensity on the surface of the bottle. In the last experiment (Nr. 4) the weighing bottles were kept in a room from which immediate sun radiation was excluded. Figures given in tables are the result of interpolation of 6 measurements performed in 5 minutes long intervals.

b. The experimental results

E x p e r i m e n t 1. The first experiment was performed in order to examine the respiration intensity of a disc cut out of a leaf blade immediately after cutting and 2,5 hours later. It is impossible to perform

Table 1

Respiration intensivity of discs cut from *Tropaeolum* leaves

Disc No.	*A*	*B*	$100\% - \left(\frac{B}{A} \cdot 100\right)\%$
1	102	47	54
2	51	35	32
3	55	43	22
4	63	35	45
5	63	39	38
6	39	31	21
7	39	39	0
8	43	43	0
9	55	35	36
10	66	39	41

(x^{10-2} µl/5 min/disc), *A* — 1 hour, *B* — 2,5 hours after cutting the discs

experiments immediately after cutting because it takes 40—60 minutes to prepare the microrespirometer for measurements. So the first measurement was made 1 hour after the disc was cut. Subsequently 6 measurements were performed within 30 minutes in 5 minutes intervals. The measurement was repeated 2,5 hours after cutting. Results are shown in table 1 and 2. In almost all cases the respiration intensity was higher when measured after 1 hour than that measured after 2,5 hours. No differences were observed only exceptionally. Respiration intensity A was never observed to be lower than result B obtained after 2,5 hours. On the average, for *Pelargonium* respiration A is 16,9% and for *Tropaeolum* 28,9% more intensive.

Table 2

Respiration intensivity of discs cut from *Pelargonium* leaves

Disc No.	*A*	*B*	$100\% - \left(\frac{B}{A} \cdot 100\right)\%$
1	59	59	0
2	86	70	13
3	71	51	28
4	63	51	19
5	67	55	18
6	63	55	13
7	78	55	3
8	55	55	0
9	84	62	26
10	55	43	2

(x^{10-2} µl/5 min/disc), *A* — 1 hour, *B* — 2,5 hours after cutting the discs

Experiment 2. It has been observed in the previous experiment that the respiration rate decreases gradually with the course of time. The purpose of this experiment was to analyse these changes in details. Observations were made on *Tropaeolum*. The first measurement was performed 1 hour after cutting the disc. The oxygen uptake (fig. 9) was measured in 5 minutes long intervals.

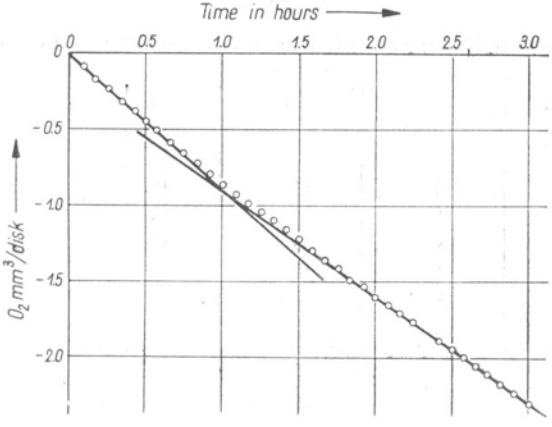

Fig. 9. Changes in the respiration of the disc cut from a leaf blade, first measurement performed 1 hour following cutting

Respiration intensity attains the highest value for the first half an hour following the start of measurement and then begins to decrease gradually. After 1,5 hour it becomes stabile at a lower level. Considering the period of 1 hour preceding the first measurement the respiration intensity of the disc in this case became stable after 2,5 hours. In other cases the period of time necessary for the stabilization of respiration intensity fluctuated between 1,5 to 2,5 hours. Altough in no case the period of stabilization exceeded 2,5 hours, it is safer however, to assume at least a 3 hours long period of time as necessary for respiration intensity of the disc to become stable.

Experiment 3. In this experiment the measurements were performed for 10 hours following cutting.

The material remained in a weighing bottle between two succesive measurements as it was described in the method. Since however, an isolated disc has no possibility of translocation of assimilates, there arose the question of examining whether the storage of assimilates in light conditions or decrease of the sugar level in darkness influences the course of metabolic processes occuring in the disc.

This experiment was performed in two different ways and was carried on for 10 hours and 30 minutes. Four measurements of respiration inten-

sities A, B, C, D were performed at the following hours after cutting: A — 1 — 1,30, B — 4 — 4,30, C — 7 — 7,30, D — 10 — 10,30. In the first case the discs remained all the time in darkness except for 5 — 10 minutes necessary for placing them in the microrespirometer. In the second case the discs remained during the time of measurements in darkness for 40 minutes. Data shown in the tables (3 and 4) corroborate in both cases

Table 3

Respiration intensivity of discs cut from *Tropaeolum* leaves

Disc No.	*A*	*B*	*C*	*D*
1	82	47	47	47
2	51	36	35	—
3	68	39	39	35
4	63	37	35	36
5	55	43	51	47

(x^{10-2} µl/5 min/disc), *A* — 1 hour, *B* — 4 hours, *C* — 7 hours, *D* — 10 hours after cutting the discs

Table 4

Respiration intensivity of discs cut from *Pelargonium* leaves

Disc No.	*A*	*B*	*C*	*D*
1	47	47	47	47
2	55	35	43	39
3	66	43	47	47
4	63	35	39	35
5	51	43	43	47

(x^{10-2} µl/5 min/disc), *A* — 1 hour, *B* — 4 hours, *C* — 7 hours, *D* — 10 hours after cutting the discs

the results obtained in previous experiments. The highest rate of respiration appeared in measurement A (1 hour after cutting). No essencial differences were observed in the successive measurements (B, C, D). It results out of these experiments that the fact of keeping the material in darkness or in faint light has no influence on the intensity of respiration. These results permit to assume that experiments on discs cut from leaves may be performed within 7 hours following the metabolical stabilization of the material.

Experiment 4. The purpose of this experiment was to observe whether there is any possibility of a still further prolongation of the

experiment. The material was observed for three days (72 hours) following cutting. In this experiment the rates of respiration and photosynthesis were measured once a day (24 hours). For measurements of photosynthesis light intensity of 3000 lux was applied. *Pelargonium* leaves provided the experimental material. This experiment was performed according to the following scheme: discs were placed immediately after cutting in weighing bottles kept not in the thermostat but in a room. After 24, 48, 72 hours the intensities of respiration and photosynthesis were measured. After measurements had been performed the discs were placed back into the weighing bottles.

Table 5

Intensivity of respiration and real photosynthesis of discs cut from *Pelargonium* leaves

Dise No.	*A*		*B*		*C*	
	respiration	photo-synthesis	respiration	photo-synthesis	respiration	photo-synthesis
1	47	124	47	124	54	132
2	30	109	30	109	30	113
3	38	149	34	133	38	145
4	46	132	74	165	55	150

(x^{10-2} µl/5 min/disc), *A*—24 hours, *B*—48 hours, *C*—72 hours after cutting the discs

Data obtained in this experiment are shown in tab. 5. It results out of this table that during this time there are no essential differences either in the results of respiration or in photosynthesis.

DISCUSSION

The experimental part of this work consists in utilization of discs of leaf blades for observations in the microrespirometer. It is well known that a mechanical injury of a leaf tissue causes disturbances in the plant. Niklewski (1933) established that respiration intensity of an injured tissue can increase by 50%. In more recent work Said and Shishiny (1944) examined the respiration on fragments of roots of *Raphanus sativus Aegyptiacus*. The fragments were 0,3, 1,0, 2,0 4,0 mm thick. The respiration intensity decreased with the increase of thickness and in distilled water the corresponding values were: 0,40, 0,32, 0,27, 0,18 g of CO_2 for 100 g fresh weight. For three days the respiration intensity only slightly decreased. The authors did not examine the influence of a comparatively more serious injury of the tissue in thinner fragments but they maintain

that a more intensive respiration depends on a better access of oxygen to the tissue.

Similar investigations were made by Allen and Price (1950) on a myxomycete *Physarum polycepharum*. They used fragments of plasmodium of various weights. The highest respiration intensity was observed with fragments of 100 mg weight. A less intensive respiration was observed with fragments which weight was above or below this level. The authors explain that the cause of the observed decrease of respiration intensity is different in both these cases. Respiration in plasmodia below 100 mg weight is less intensive because the injury is relatively great in comparison to the whole fresh weight of the plasmodium, whereas, the decrease of the intensity of CO_2 evolution is caused, according to the authors, by a more difficult access of oxygen, similarly as it was already suggested by Said and Shishina in their work.

Dr. Dwuraźna enabled me to make use of the results of unpublished observations. She experimented on 1,9 4,3 6,4 mm in diameter fragments of leaves of *Nicotina Tabacum* variety White Burley. She employed the Warburg apparatus for measurements of respiration, and established that 50—60 minutes following cutting the discs the respiration intensity remained on a constant level, but that the respiration of discs 1,9 mm in diameter was most intensive (6,85 µl CO_2 for 1 mg dry weight) and in other discs, 4,3 and 6,4 mm in diameter, respiration was lower and lower and attained successively 5,99 and 4,88 µl CO_2 for 1 mg dry weight. She observed, moreover, that after 60 minutes the respiration intensity gradually decreased. Basing the opinion on these data it is difficult to establish whether a more intensive respiration of smaller discs was caused by a greater injury or by a better access of oxygen. A decrease of respiration intensity after 60 minutes corroborates the observed changes in experiments performed in this work.

E. C. Wassink (1946) performed measurements of photosynthesis by means of the Warburg apparatus on discs 5 mm in diameter, cut out of a series of leaves of land plants. Measurement were performed in Warburg's carbonate buffer Nr. 9 or in water enriched by CO_2. Wassink thinks that his method of preparing the samples enables a considerable reduction of the dispersion and is suitable for establishing true photosynthesis of leaves of land plants. The author is not interested, however, either in the metabolical injuring of the leaf or in the influence of the wound on the metabolic processes.

In this work the respiration intensity was the criterion of possible disturbances. In the three first experiments the measurement of photosynthesis was practically impossible because it took place in time when greatest changes in respiration occured (1,5 hour after cutting the discs

from the leaf blade). In the second experiment measurements of respiration intensity could be performed every 50 minutes in turn with measurements of photosynthesis. In this case, however, it was not possible to follow the changes so detailful as it was with measurements only of respiration intensity. It was possible to observe changes of apparent photosynthesis but concomitant changes in respiration could lead to completely false conclusions. In the third experiment photosynthesis was not measured to avoid an additional illumination of the disc. It was only in experiment 4 when measurements were repeated every 24 hours that respiration and photosynthesis could be measured on one and the same disc without running any risk.

In these experiments it was examined whether the increase of respiration intensity caused by injuring is durable or if not — what its behavior is. The obtained results indicate that the respiration intensity stabilizes after 3 hours following cutting the discs and remains on this level for 72 hours. To examine respiration and photosynthesis on fragments of leaves we dispose of a material of stabilized physiological properties for a long period of time. The here applied method may also be employed for investigations on the influence of various factors on photosynthesis and respiration (light, temperature, stimulants, inhibitors and other substances). It may also be used for local measurements of photosynthesis on individual parts of a leaf. It may also be very helpful when comparing necrotic and healthy parts of a leaf. It also seems possible to apply it in order to compare the activity of photosynthesis and respiration in various varieties in genetical, selectional and culturing works especially when relative data are sufficient for this purpose. As there are no comfortable field methods the microrespirometer was also used to determine respiration intensity, compensation point and saturation point of photosynthesis of plants with small leaves (S t a r z e c k i 1958) collected on various habitats. It is, however, still a problem to settle to what an extent discs of leaves may be used for this kind of experiment. The results of this work have not determined to what an extent the results obtained from leaf discs may represent values of respiration and photosynthesis of the whole organ. It may be supposed that differences in the leaf blade itself (different thickness of the leaf blade, concentration and thickness of veins, differences in the content of chlorophyll, differences in structure etc.) will influence the results obtained with individual discs. On the other hand as the individual respiration intensity of the whole organ is not known, one can not say whether the stabilized respiration intensity after 3 hours following cutting of the discs drops to its initial level or if it is higher or lower in

comparison with it. We do not possess, however, a method which would permit to perform measurements of respiration intensity and photosynthesis of leaves of average size. This would make possible a direct comparison of results obtained from a whole organ and from discs cut from it (this remains a subject for a separate work).

The above given examples and the various papers mentioned in the introduction give evidence of a large range of possibilities of using the above described microrespirometer for investigations on photosynthesis and respiration.

SUMMARY

1. This paper presents a detailed description of an improved microrespirometer destined for researches on photosynthesis and respiration.

2. The modifications were introduced to enlarge the number of replications in the same period of time (from one to three or four). Besides the newly introduced modifications aimed at making the reading and the handling of the microrespirometer easier and at a better utilization of the intensity of the light source and regulation of temperature.

3. Keeping the discs in weighing bottles on a moistened paper in faint light or in darkness permits the conservation of material for three days in an unchanged and fresh state.

4. Discs cut out of a leaf blade may be used for observations after three hours following cutting the discs.

5. The apparatus can be applied when studying the following problems of respiration and photosynthesis:

a) the influence of external factors (light, temperature, inhibitors, stimulants and other substances),

b) local measurements within the leaf blade,

c) comparative measurements in genetical, selectional and breeding investigations,

d) in ecological investigations for measurement of respiration, compensation point and saturation value of photosynthesis.

I am indebted to the chief of the Institute prof. dr. F. Górski for his valuable advice and critics. I am also indebted to doc. dr. J. Zurzycki for his personal assistance in elaboration of the new technical solution of the microrespirometer.

Laboratory of Plant Physiology
Polish Academy of Sciences
Cracow

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