Plant Physiol. (1986) 81, 854–858
0032-0889/86/81/0854/05/01.00/0

Assay of Photosynthetic Oxygen Evolution from Single Protoplasts[1,2]

Received for publication January 3, 1986 and in revised form February 5, 1986

RÜDIGER HAMPP*, WERNER MEHRLE, AND ULRICH ZIMMERMANN
Biologie I, Universität Tübingen, D-7400 Tübingen 1 (R.H.), *and Institut für Biotechnologie, Universität Würzburg, D-8700 Würzburg* (W.M, U.Z.), *Federal Republic of Germany*

ABSTRACT

A semiquantitative assay for light-dependent O_2 evolution by a single mesophyll protoplast is described. The assay indicator is the density of aerotactic bacteria (*Pseudomonas aeruginosa*, ATCC 10145; 'Engelmann experiment') attracted to the protoplast. Quantification is by dark field microphotometry. The sensitivity is about 50 femtomoles O_2 per protoplast per minute. The results demonstrate the biphasic nature of O_2 evolution of a single protoplast during photosynthetic induction. Computerized data acquisition yields traces which, until a steady state of photosynthetic O_2 evolution is reached, are identical to ordinary O_2 electrode traces.

Plant cell protoplasts are an important tool for investigating physiological, biochemical, or genetic problems. However, the isolation of protoplasts may result in physiologic perturbations that introduce artefacts. Thus, assessing the integrity of protoplasts should be a routine procedure for many types of investigations. The most commonly used indicators available are vital stains and vital fluorochromes (see Ref. 4). These are rapid procedures, but are less accurate than a test of the ability of protoplasts to regenerate a cell wall and divide. Unfortunately, the latter tests are not suitable for routine use, particularly when time is of the essence. In experiments on protoplast fusion, we used biochemical parameters such as the determination of the cellular energy state in addition to staining procedures (22). A system of higher complexity is photosynthesis. High rates of photosynthetic O_2 evolution are possible only if there is a high degree of cellular integrity. Our experiments with oat mesophyll protoplasts showed that damage not detected with staining (FDA[3]) is manifested by decreased rates of photosynthesis (W Mehrle, R Hampp, unpublished data).

Our need to assess the viability of hybrids obtained by electrofusion of mesophyll cell protoplasts (25) as well as that of protoplasts released from alginate matrices after exposure to ecotoxicological substances (18, 19), led to the development of a miniaturized assay for changes in O_2 concentration. Here, we present this semiquantitative technique for the assay of photosynthetic O_2 evolution on the single cell level. Its general applicability is demonstrated with green protoplasts isolated from several species.

[1] Dedicated to Prof. E. Bünning on the occasion of his 80th birthday.

[2] Supported by grants from the Deutsche Forschungsgemeinschaft to R. H. and the Bundesministerium für Forschung und Technologie to U. Z (no. 0373509).

[3] Abbreviation: FDA, fluorescein diacetate.

MATERIALS AND METHODS

Isolation of Protoplasts. Mesophyll protoplasts from *Avena sativa* L. (cv Arnold) were isolated from 7-d-old light-grown seedlings as reported (10), except, instead of cutting leaf segments, the lower epidermis was peeled off.

Nicotiana tabacum L. (cv Samsun) was grown at 20 to 22°C, 70% RH, and a 18-h-light period (about 8 W m^{-1}) for 4 weeks. Plants were kept in darkness before removing leaves. The abaxial epidermis was abraded (carborundum GC 120; Schleifmittelwerk, Düsseldorf) using a water color paintbrush (2). Then the leaves were rinsed with water and incubated for 1 h in 0.6 M mannitol (to plasmolyze the cells). Mesophyll protoplasts were isolated in 0.6 M mannitol, 1 mM $CaCl_2$, 5 mM Mes-KOH (pH 5.6), 10 mM ascorbic acid, 0.5% (w/v) BSA, 2% cellulase 'Onozuka R-10' (Serva), and 0.5% Macerozym R-10 (Serva) (2 h, 30°C, occasional shaking). Purification of protoplasts by floatation on a step gradient (100*g*, 90 s, swing out rotor) was carried out as described for oat (10).

Vicia faba L. (cv Hangdown) mesophyll protoplasts were isolated (17) from 2- to 3-week-old plants that were cultured like tobacco. Three-week-old *Phaseolus coccineus* plants (cv Preisgewinner) were grown in 9-h light/15-h dark cycle at 23°C to use for the isolation of pulvinus protoplasts (14). The primary pulvini of about 30 plants previously trimmed to 2 leaves each were excised. The extensor regions (abaxial part of the pulvinus; see Ref. 14) were dissected out, chopped into about 0.5-mm pieces, and rinsed in 0.6 M sorbitol, containing 0.1 mM $CaCl_2$, and 5 mM Mes (pH 5.6). The tissue slices were then transferred to 10 ml of digestion medium (0.5 M sorbitol, 0.1 mM $CaCl_2$, 5 mM Mes (pH 5.6), 0.5% (w/v) BSA, 0.5% (w/v) PVP (insoluble), 1 $\mu g\ ml^{-1}$ pepstatin A (Sigma), and enzymes (w/v): 2% cellulase (Sigma), 0.5% cellulase TC (Serva), 0.5% pectinase Rohament P5 (Serva), 0.05% pectolyase Y23 (Paesel)), and incubated for 3 h at 30°C, without shaking. The digested material was filtered through a 100 μm nylon mesh, and washed with 10 ml of 0.55 M raffinose. The resulting suspension was transferred to a centrifuge tube, overlayered with 400 μl 0.55 M sorbitol and centrifuged (2 min, 200*g*, swing out rotor). Protoplasts, that banded at the interphase were removed, resuspended in 10 ml 0.55 M raffinose and the purification step repeated as above, but using electrode medium (see below) as the upper layer instead of sorbitol only.

Protoplast numbers were counted on a Fuchs-Rosenthal hemocytometer. Chl was determined according to Arnon (1).

Culture of Bacteria. *Pseudomonas aeruginosa* (ATCC 10145; German Collection of Microorganisms, Göttingen) was cultured in nutrient agar (medium 1 [6]), fortified with 2 g/L NaCl and 0.1 g/L KCl. About 12 h before starting a protoplast experiment, bacteria were transferred from culture tubes to 10 ml medium 1 ([6] without agar) and kept at 37°C on rotatory shaker (60 oscillations min^{-1}). Aliquots (1 ml) from the bacterial suspension were taken during the logarithmic growth phase (O.D. at 578

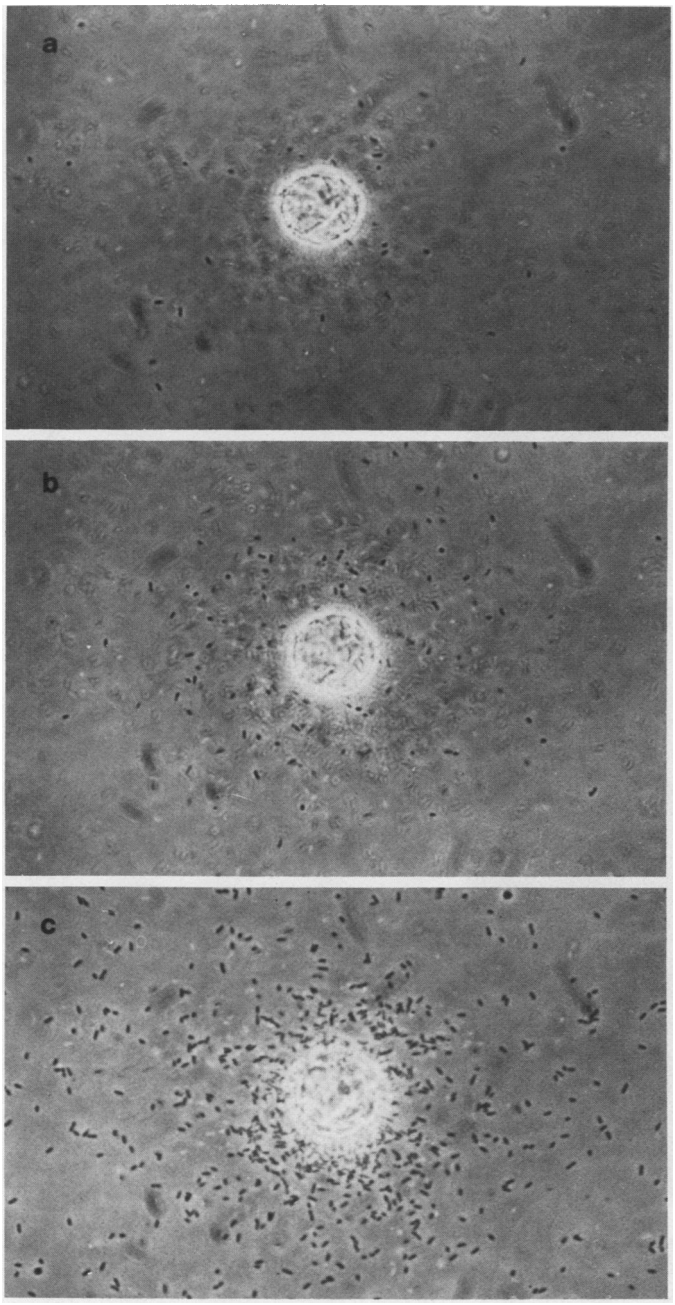

FIG. 1. Flash light photomicrographs of a *Phaseolus* protoplast isolated from primary pulvinus tissue and suspended together with *P. aeruginosa* bacteria. The bacteria respond positively by chemotactis towards O_2. a, Control (15 min dark incubation); b, same protoplast after 30 s of illumination (microscope lamp, 320 W m^{-2}); c, as (b), but after an illumination period of 3 min. Note the considerably increased density of the bacterial population around the photosynthesizing protoplast.

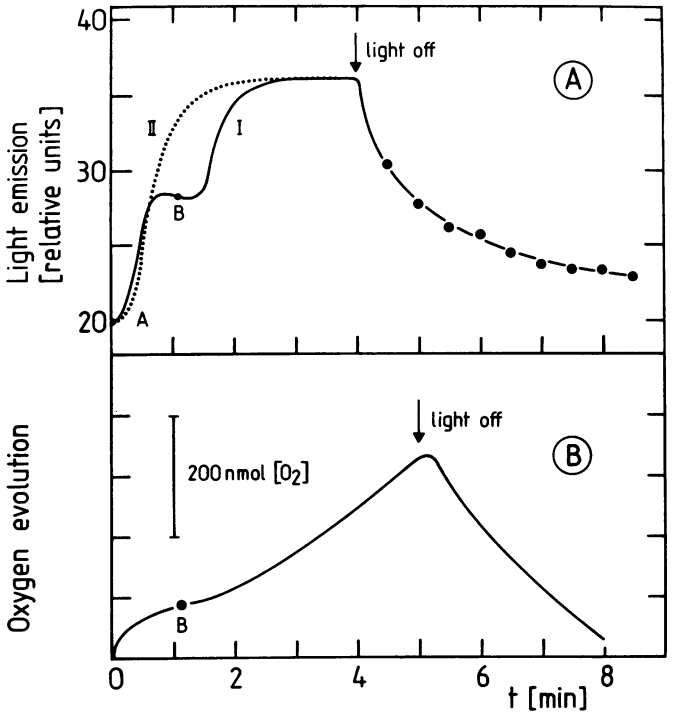

FIG. 2. Microphotometric recordings of light scattered by a mesophyll protoplast (tobacco) and *P. aeruginosa* under dark field-illumination (compare Fig. 3). Following a 15-min dark preincubation protoplasts were illuminated. The increase in light scattering during illumination is due to an accumulation of bacteria around the protoplast. Panel A, Trace I: light scattering during photosynthetic induction; background reading: level A, light scattered by the protoplast itself is measured (dark treatment, compare Fig. 3a). Intermediate halt in light-dependent O_2 evolution of the protoplast during photosynthetic induction: level B. Trace II, incubation after addition of 100 μM dichlorophenol indophenol, an electron acceptor. Panel B, O_2 electrode tracing of the protoplast suspension. B, same state as in trace I. The assay volume (1 ml) contained protoplasts equivalent to about 50 μg Chl.

nm: 1.5–1.6), and centrifuged for 1 min at 10,000*g*. The pellet was resuspended in 1 ml of O_2 electrode medium that was also used for the resuspension of protoplasts (7.5 mM $CaCl_2$, 25 mM Tricine (pH 7.6), 5 mM $NaHCO_3$, and sorbitol as required).

Assay of Photosynthetic O_2 Evolution. For the microscopic assay of photosynthesis, 6 μl of protoplast and *Pseudomonas* suspension (in 'electrode medium' see above; about 10^4 protoplasts ml^{-1}) were mixed on a microscope slide and covered with a coverslip, the edges of which were coated with Vaseline to reduce the rate of O_2 exchange between assay medium and ambient air. Before starting an experiment, the suspension was kept in darkness for at least 15 min. This dark incubation increased sensitivity (we speculate that respiration lowered the O_2 content of the system). Photosynthesis was induced by illumination (halogen lamp, 320 W m^{-2}; Leitz Diavert). Semiquantitative determinations of O_2 evolution were obtained with a Dialux 20 EB microscope (dark field illumination; Leitz), equipped with a photomultiplier (MPV compact, Leitz) and connected to a Hewlett-Packard 87 microcomputer (15). Readings were taken in 30 s intervals. In parallel to the *Pseudomonas*-based assay light-dependent O_2 evolution was monitored in a Hansa-Tech O_2 electrode (Bachofer, Reutlingen; see Ref 9).

Photomicrographs of bacteria were taken with a Leitz microflash (100 W s^{-1}).

RESULTS AND DISCUSSION

Assay of Light-Dependent O_2 Evolution of Single Protoplasts. *Microphotographic Studies.* After a 15-min dark preincubation of protoplasts (from *Phaseolus* pulvinus cells) and bacteria, the latter were randomly distributed (Fig. 1a). Illumination for as few as 30 s was sufficient to cause an increase in the number of bacteria surrounding a photosynthesizing protoplast (Fig. 1b). The maximum effect was observed after about 3 min of illumination (Fig. 1c). Due to the motion of the bacteria protoplasts sometimes started to rotate at this stage. These observations show that the bacteria respond to an O_2 gradient (compare Engelmann [8]). Thus the bacteria accumulate where the gradient is most

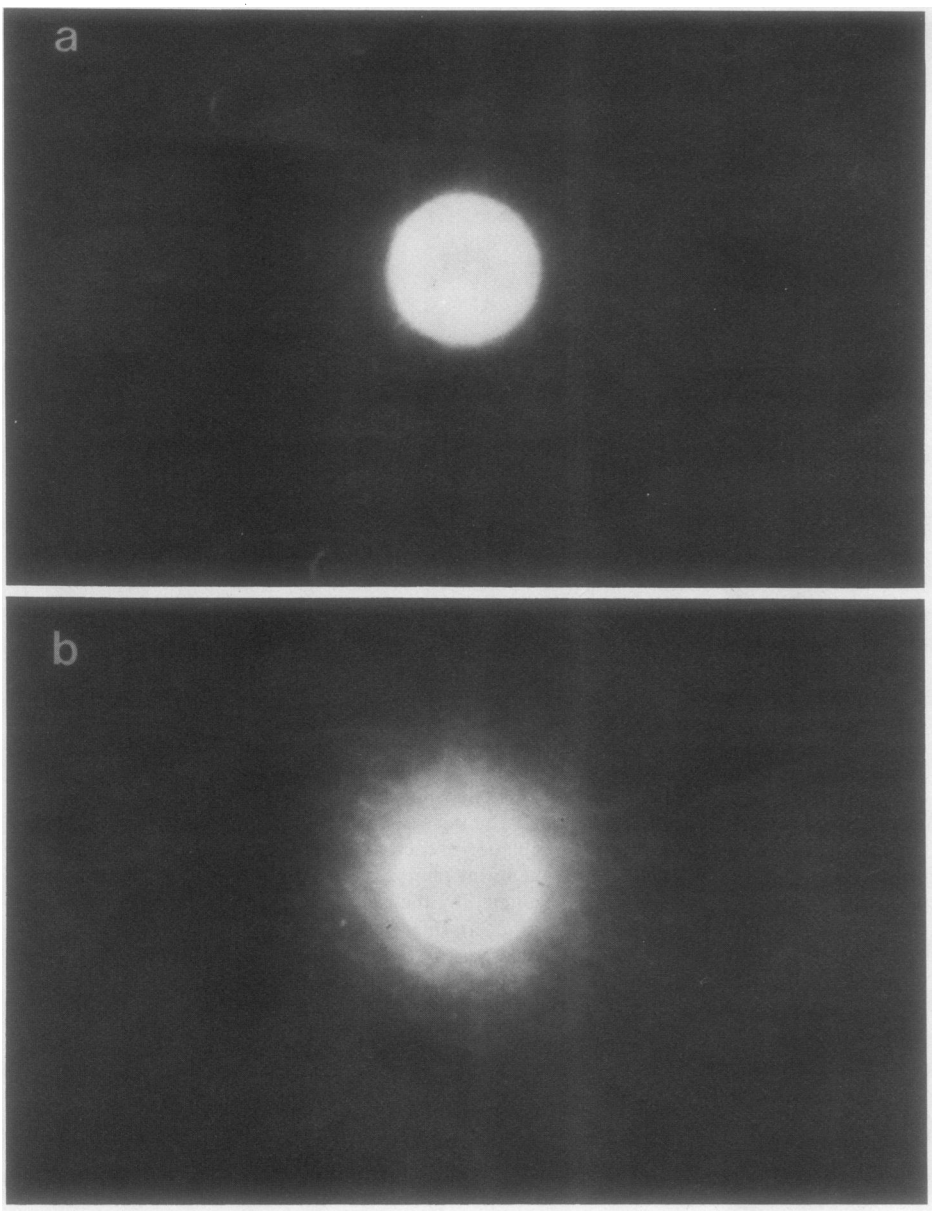

FIG. 3. Photomicrographs of a tobacco mesophyll protoplast under dark field illumination and in the presence of *P. aeruginosa.* a, Dark treated protoplast; b, same protoplast after 5 min of illumination (320 W M^{-2}). The halo results from light scattered by the aerotactic bacteria.

steep (*i.e.*, near the surface of a photosynthesizing protoplast). After prolonged illumination (no significant increase in temperature was detected), bacteria are not preferentially localized near protoplasts. We interpret this observation to mean that diffusion of O_2 had occurred in the incubation medium. The initial attraction is reversible. Returning the mixture to darkness resulted in a completely random distribution of bacteria within 10 to 15 min, and at this stage the experimental procedure could be started from the beginning. Even after keeping the sealed mixture (cover slip with vaseline) for about 24 h in the dark (20°C) the system was still functional. It should be noted that in fresh protoplast preparations the aerotactic bacteria also tend to be attracted by broken protoplasts. This effect, however, is not light-dependent and is obviously caused by leaking solutes. Due to diffusion, effective gradients of possible attractants disappear within about 10 min as well as the enhanced aggregation of bacteria around the respective protoplasts. There is still the possibility that ions, *e.g.* K^+, leaking from intact protoplasts could act as an attractant in the light rather then O_2. However, even after addition of 5 mM KCl the bacteria responded to illumination as described above.

Chloroplasts, which were present due to the rupture of protoplasts, were prevented from evolving O_2 by the addition of 7.5 mM $CaCl_2$ (13, 24). They were therefore not surrounded by bacteria in the light.

Addition of 15 μM dibromothymoquinone, a plastoquinone antagonist (7, 21), which was shown to inhibit light-driven electron flow in intact mesophyll protoplasts (9), rendered previously bacteria-attracting protoplasts unattractive. The motility of the bacterial suspension was not affected.

Kinetic Studies. A semiquantitative assay of light-dependent O_2 evolution by a single tobacco mesophyll protoplast is shown in Figure 2a (trace I). The determination is based on dark field illumination. With this technique only light scattered by particles is recorded, whereas the background is dark. Thus, with a protoplast surrounded by few bacteria (darkness), light scattering is at a low level (Fig. 2a [I, A]). From the onset of illumination (and O_2 evolution), bacteria respond aerotactically and accumulate around the cell (*cf.* Figs. 1 and 3). This accumulation is measured as an increase in light scattering. Interestingly, the time course of changes in light scattering is biphasic (Fig. 2a [I]); there are two periods exhibiting a fast increase in the population density (0–1 and 1.5–2.5 min of illumination) which are separated by a short period without major changes (about 30 s).

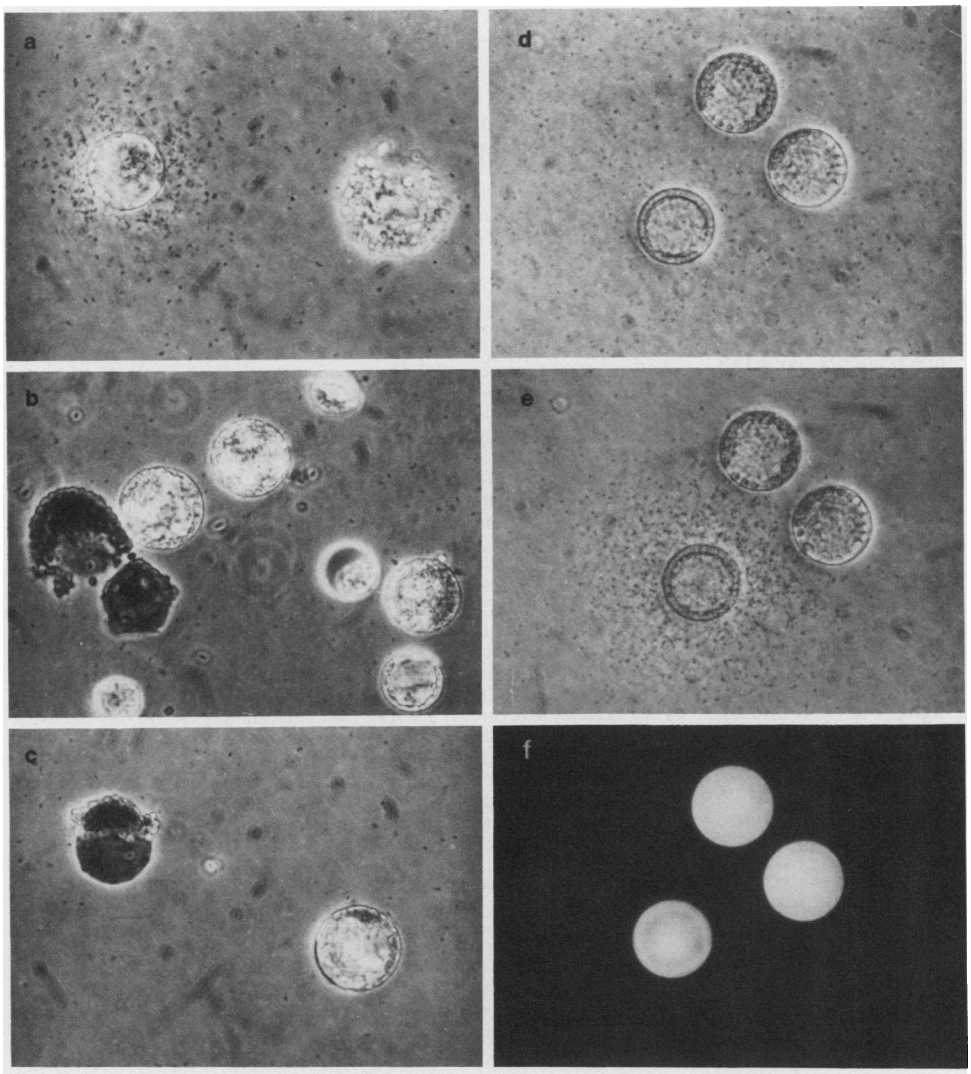

FIG. 4. Photomicrographs of *V. faba* mesophyll protoplasts. Comparison of different viability tests. a, *Pseudomonas* assay: only the intact protoplast attracts bacteria; (b) treatment with Evans blue: damaged protoplasts are dark colored; c, phenosafranin, as (b); d, e, and f, same group of protoplasts, but incubated either with bacteria in the dark (d) or light (e), or with FDA (f); note, while all protoplasts are FDA-positive (*i.e.* 'intact') only one of them is able to evolve O_2.

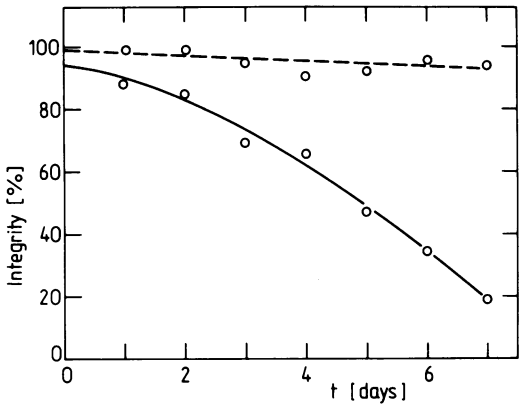

FIG. 5. Viability of a population of oat mesophyll protoplasts over 7 d at 4°C. Integrity is expressed as percentage of those protoplasts that still appeared intact under phase contrast microscopy. (- - - -) fluorescein diacetate staining; (——) *Pseudomonas* assay.

Thus, these kinetic studies, obtained by single cell techniques, are in substantial agreement with studies on the phenomenon of photosynthetic induction. "Induction" is used to describe the delayed onset of photosynthesis when illumination follows a period of darkness (23). This phenomenon is related to the coupling between carbon assimilation and the activity of the photochemical apparatus. Thus, NADPH and ATP accumulate during the initial 60 s of illumination (for mesophyll protoplasts, see Ref. 11), where enzymes involved in CO_2 fixation are still inactive (5). High stromal ratios of ATP/ADP and NADPH/NADP, however, will decrease photosynthetic electron transport and thus lower the rate of O_2 evolution.

We would like to emphasize that our method results in a different measurement than that of the O_2 electrode. While the latter measures overall O_2 concentrations, our method is sensitive to a gradient (*cf.* above). Thus, traces from the two methods should not be identical: when O_2 evolution ceases, the electrode trace should remain virtually horizontally. Under the same condition, the trace obtained with the bacterial method would decline because of diffusion and O_2 depletion in the vicinity of a protoplast (respiration; Fig. 2a [I, B]).

With the start of CO_2 fixation NADPH and ATP are consumed and electron flow is no longer restricted. This leads to a second steep increase in bacterial numbers around the protoplast. After about 3 min of illumination a new steady state is reached between O_2 evolution and diffusion that results in a constant O_2 gradient and therefore in a constant number of bacteria. When larger numbers of individual traces are accumulated and averaged, about the same curve develops as is obtained with a conventional O_2 electrode assay during the first minutes of illumination (Fig. 2b). When the light is switched off, bacteria respond immediately with a decrease in population density (Fig. 2a; determinations in

30 s intervals with 2.5 s of illumination each time) and the whole experimental sequence can be repeated several times without any signs of decay.

The induction-dependent intermediate break in O_2 evolution (Fig. 2a [I, B]) disappears when a membrane permeable electron acceptor like dichlorophenol indophenol (DCPIP; 100 μM) is added to the assay (Fig. 2a; trace II). In this case electrons, originating from the water splitting site are directly transferred to the oxidized dye and thus no limitation of O_2 evolution is observed. An interference of DCPIP with bacterial mobility was not detected.

Protoplast Viability as Assayed with *Pseudomonas versus* Conventional Techniques. Commonly used substances for assessing viability of intact protoplasts are fluorescence reagents like FDA (12), and for damage and death, exclusion dyes such as Evans blue, bromphenol blue, or phenosafranine (3). Other techniques like protoplasmic streaming and plasmolysis have also been employed to estimate the relative viability of plant cells. However, the most convenient and reliable method appeared to be that of vital staining.

To compare the degree of viability as assessed with *Pseudomonas* with that obtained by other commonly used techniques, 4-d-old *Vicia* mesophyll protoplasts (kept in darkness at 4°C) were either studied by phase contrast microscopy or incubated in the presence of Evans blue, phenosafranine, or FDA (Fig. 4). Out of three protoplasts appearing intact by FDA staining (Fig. 4f), only one was able to evolve O_2 in the light (Fig. 4, d and e). This example is typical for the different results on vitality obtained with staining methods compared to the physiological test. Based on protoplasts that appeared intact under phase contrast (100%), Evans blue (96%) and phenosafranine (98%), which are deflected by an intact plasma membrane, were excluded. By contrast, only 60 to 70% of the protoplasts were judged to be intact by the FDA method. This method is based on FDA permeability and its subsequent hydrolysis by cytosolic esterases to the fluorescent compound, fluorescein. Thus, fluorescein, which is impermeable, accumulates in intact cells only. The *Pseudomonas* assay yielded an even lower figure: only about 50% of the protoplasts judged to be intact by phase contrast microscopy evolved O_2 at detectable rates (the sensitivity of the bacterial assay is suggested to be as low as 1 pg O_2 (about 60 fmol) (16) (for comparison: 10^6 protoplasts evolve about 15 μmol O_2 h^{-1} or about 225 fmol [protoplast × min]$^{-1}$).

The reliability of staining procedures has been questioned previously. Bornman *et al.* (3) pointed out that even though conditions for vital staining may be rigorously standardized, caution should be exercised in the interpretation of the fluorescent image. These authors showed that not only were concentrations and freshness of both dye and protoplasts, and time of staining critical, but also the photographic exposure itself could result in progressive fading and damage of the protoplast due to UV irradiation. In addition it was frequently observed that fluorescence was limited to certain areas of "intact protoplasts" which was interpreted as indicating leakage. Similarly, Smith *et al.* (20) reported that staining properties of cell cultures did not correlate well with their ability to be subcultured. This obvious inaccuracy of viability tests that rely on vital staining is further corroborated by the experiment on protoplast senescence shown in Figure 5. In this example oat mesophyll protoplasts were kept at 4°C for up to 7 d. In parallel to the *Pseudomonas*-based assay, light-dependent O_2 evolution was measured in an O_2 electrode. As shown in Figure 5, the bacterial assay yielded by far the lowest number of intact protoplasts (based on the evaluation of about 200 phase contrast-positive protoplasts for each datum). Interestingly, integrity as measured with FDA is nearly unchanged with incubation time, while the capacity for O_2 evolution (*Pseudomonas* assay) indicates a considerable decrease. This difference shows that deterioration of physiological and plasma membrane integrity proceeds at a different speed.

In summary, the protocol reported here is a simple assay system that allows for a screening of the photosynthetic competence of individual protoplasts. This method is a more reliable indicator than commonly used staining procedures.

Acknowledgments—The authors are indebted to U. Heber for helpful suggestions and to W. H. Outlaw, Jr., for help in establishing a system of computerized data acquisition (microphotometry) and for a critical reading of the manuscript.

LITERATURE CITED

1. ARNON DI 1949 Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24: 1–15
2. BEIER H, G BRUENING 1975 The use of an abrasive in the isolation of cowpea leaf protoplasts which support the multiplication of cowpea mosaic virus. Virology 64: 272–276
3. BORNMAN JF, CH BORNMAN, LO BJÖRN 1982 Effects of ultraviolet radiation on viability of isolated *Beta vulgaris* and *Hordeum vulgare* protoplasts. Z Pflanzenphysiol 105: 297–306
4. BORNMAN JF, CH BORNMAN 1983 Protoplast viability—a relative concept. *In* I Potrykus, CT Harms, A Hinnen *et al.*, eds, Protoplasts 1983. Birkhäuser Verlag, Basel, pp 208–209
5. BUCHANAN 1980 Role of light in the regulation of chloroplast enzymes. Annu Rev Plant Physiol 31: 341–374
6. CLAUS D, P LACK, B NEU 1983 German collection of microorganisms. *In* Catalogue of Strains 1983. Göttingen, F.R.G., p 265
7. CROWTHER D, G HIND 1980 Partial characterisation of cyclic electron transport in intact chloroplasts. Arch Biochem Biophys 204: 568–577
8. ENGELMANN, TW 1881 Botanische Zeitung, p 446. Cited in Pfeffer (16)
9. GOLLER M, R HAMPP, H ZIEGLER 1982 Regulation of the cytosolic adenylate ratio as determined by rapid fractionation of mesophyll protoplasts of oat. Planta 156: 255–263
10. HAMPP R, H ZIEGLER 1980 On the use of *Avena* protoplasts to study chloroplast development. Planta 147: 485–494
11. HAMPP R, M GOLLER, H FÜLLGRAF, I EBERLE 1985 Pyridine and adenine nucleotide status, and pool sizes of a range of metabolites in chloroplasts, mitochondria and the cytosol/vacuole of *Avena* mesophyll protoplasts during dark/light transition: effect of pyridoxal phosphate. Plant Cell Physiol 26: 99–108
12. LARKIN PJ 1976 Purification and viability determinations of plant protoplasts. Planta 128: 213–216
13. LEEGOOD RC, DA WALKER 1983 Chloroplasts. *In* JL Hall, AL Moore, eds, Isolation of Membranes and Organelles from Plant Cells. Academic Press, New York, pp 185–210
14. MAYER WE, D FLACH, MVS RAJU, N STARRACH, E WIECH 1985 Mechanics of circadian pulvini movements in *Phaseolus coccineus* L. Shape and arrangement of motor cells, micellation of motor cells, and bulk moduli of extensibility (ε_a). Planta 163: 381–390
15. OUTLAW WH, JR, SA SPRINGER, MC TARCZYNSKI 1985 Histochemical technique. A general method for quantitative enzyme assays of single cell 'extracts' with a time resolution of seconds and a reading precision of femtomoles. Plant Physiol 77: 659–666
16. PFEFFER W 1897 Pflanzenphysiologie, Vol I. Verlag W. Engelmann, Leipzig, p 292
17. SCHNABL H, CH BORNMAN, H ZIEGLER 1978 Studies on isolated starch-containing (*Vicia faba*) and starch-deficient (*Allium cepa*) guard cell protoplasts. Planta 143: 33–39
18. SCHNABL H, P SCHEURICH, U ZIMMERMANN 1980 Mechanical stabilization of guard cell protoplasts of *Vicia faba*. Planta 149: 280–282
19. SCHNABL H, RJ YOUNGMAN, U ZIMMERMANN 1983 Maintenance of plant cell membrane integrity and function by the immobilisation of protoplasts in alginate matrices. Planta 158: 392–397
20. SMITH BA, ML REIDER, JS FLETCHER 1982 Relationship between vital staining and subculture growth during senescence of plant tissue cultures. Plant Physiol 70: 1228–1230
21. TREBST A, E HARTH, W DRABER 1970 On a new inhibitor of photosynthetic electron transport in isolated chloroplasts. Z Naturforsch 25b: 1157–1159
22. VERHOEK-KÖHLER B, R HAMPP, H ZIEGLER, U ZIMMERMANN 1983 Electrofusion of mesophyll protoplasts of *Avena sativa*. Determination of the cellular adenylate levels of hybrids and its influence on the fusion process. Planta 158: 199–204
23. WALKER DA 1976 Photosynthetic induction. *In* G Akoyunoglou, ed, Photosynthesis. IV. Regulation of Carbon Metabolism, Balaban International Science Services, Philadelphia, pp 189–202
24. WIRTZ W, M STITT, HW HELDT 1980 Enzymic determination of metabolites in the subcellular compartments of spinach protoplasts. Plant Physiol 66: 187–193
25. ZIMMERMANN U, J VIENKEN, J HALFMANN, CC EMEIS 1985 Electrofusion: a novel hybridization technique. *In* A Mizrahi, AL van Wezel, eds, Advances in Biotechnological Processes. Alan R Liss, New York, pp 79–150