modifies the final result: oxidation, staining with CH, differentiation, phloxinic staining, and the differentiation of this latter.

Thus, for the same tissue, by varying time between 1 and 5 min, we may obtain clearly different results (table). If the duration of oxidation is short (1 min), the affinity of type A secretion for CH is slight, and if the actual staining step is not prolonged certain cells remain unstained ($A_2$NSC). If oxidation is excessive (5 min), the affinity for phloxin of type B substances will decrease and disappear. An overly pronounced phloxin coloration, after medium or weak oxidation will, on the other hand, yield a uniform color for a large number of cells, some of which are not neurosecretory. Depending upon the mode of application of technique, there is variation in the number of B NSC, and demonstration of 1 or 2 different categories in this population.

For azan, the situation is similar. Results depend upon azocarmin differentiation, the intensity of staining by the orange G-blue aniline mixture, constituting Heidenhain blue, and the differentiation of this stain (see table). The different shades of coloration of $A_1$ and $A_2$ NSC, as well as the 2 categories of B NSC, depend upon the exact application of the method.

While a time standard may appear desirable, it will not resolve these problems since, depending upon species, results vary slightly, due either to the permeability of the neurilemma, or the pH of the medium.

*Concluding remarks.* The complexity of the neurosecretory system of insects, the differences among various species and the variations in applying staining techniques lead, in some cases, to conflicting results. Our experiments on *Clitumnus* and *Roscius* suggest adoption of the following methods for the determination of the various types of neurosecretory cells: use of several techniques including azan, HC and FP in double or triple staining, and application of each technique not according to a single method, but using a range of variants.

1 Dedicated to Professor Dr M. Gersch's on the occasion of his 70th birthday.
2 M. Raabe, A.A. Panov, E.D. Davydova and D. Chervin, Experientia *35*, 404 (1979).
3 M. Gersch, Forschn Fortschr. *31*, 9 (1957).
4 M. Gersch, R. Bräuer and H. Birkenbeil, Experientia *29*, 425 (1973).
5 M. Gersch, Coll. Intern. C.N.R.S. n° 251, 281 (1976).
6 M. Gabe, C. r. Soc. Biol. *149*, 462 (1955).

## Soybean photosynthesis: simultaneous $^{14}CO_2$ and $O_2$ estimates

P.D. Kemp and L.C. Campbell[1]

*Department of Agronomy and Horticultural Science, University of Sydney, Sydney, N.S.W. 2006 (Australia), 27 November 1979*

*Summary.* A method of estimating the photosynthetic rate of soybean leaves using an oxygen electrode is presented. The procedure is rapid, requires small samples and compares favourably with estimates by other techniques. Light saturation occurs at 1200 $\mu E \cdot m^{-2} \cdot sec^{-1}$. The apparent $K_m$ for $HCO_3^-$ is 3.2 mM at pH 7.6.

This communication demonstrates that net photosynthesis of soybean leaf slices measured in an oxygen electrode is the same as that reported for intact leaves using an infrared gas analyzer. Furthermore, the photosynthetic responses to light intensity and carbon dioxide concentration are consistent with those reported in the literature. The rate of $O_2$ evolution by leaf slices is compared directly with the rate of carbon fixation, measured with $^{14}CO_2$.

Soybeans (*Glycine max* (L.) Merrill) cv. Wayne were grown in solution culture (pH 5.5) under glasshouse conditions (24–19 °C). All experiments were performed on the 2nd expanded trifoliate leaf. Leaf slices (1 mm wide, 10 mm long) were hand cut, rinsed and kept in 0.5 mM $CaSO_4$ (pH 7.6) in the dark, then illuminated for 1 h at 200 $\mu E \cdot m^{-2} \cdot sec^{-1}$ before use. Oxygen exchange was measured at 25 °C with a Clark-type oxygen electrode. A 20–30 mg subsample of leaf slices from a single leaflet was added to 4 ml 20 mM HEPES (N-2-hydroxyethylpiperarzine-N[1]-2-ethanesulphonic acid), pH 7.6, in the incubation chamber. The solution was partly de-oxygenated with $N_2$ to 60% of its saturated soluble $O_2$ content and the $CO_2$ concentration obtained by adding $KHCO_3$. Dark respiration was measured for 8 min followed by net photosynthesis for 5 min. For $^{14}CO_2$ fixation, 2 $\mu$Ci $NaH^{14}CO_3$ was injected into the incubation solution (sp. act., 21.8 mCi/mole $HCO_3^-$). After illumination for 35 sec, the slices were rinsed in fresh incubation solution, blotted dry, killed, decolorized with benzoyl peroxide and counted for radioactivity in a liquid scintillation counter. Controls were performed to account for adsorption or dark fixation.

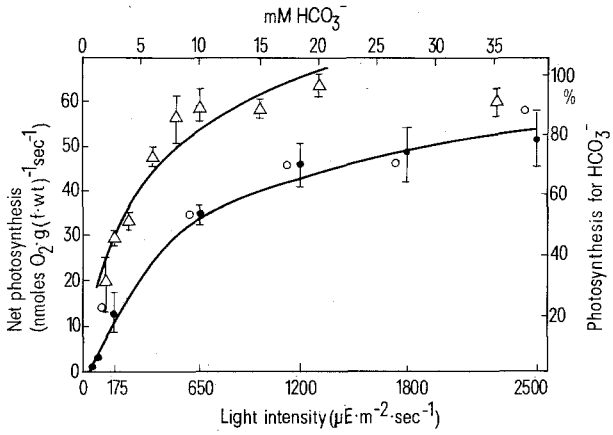
$CO_2$ response: Under the prevailing conditions of pH 7.6 and 2000 $\mu E \cdot m^{-2} \cdot sec^{-1}$ light intensity, there was no significant increase in the rate of oxygen evolution by leaf slices from 8 mM to 35 mM $HCO_3^-$. Thus photosynthesis was saturated by 8 mM $HCO_3^-$ (figure). The concentration which gave half-maximal rates (apparent $K_m$) was calculated from the regression equation as 3.2 mM. The $CO_2$ concentration in solution was not significantly depleted as the rate of $O_2$ evolution by the slices during exposure to light was always linear. The calculated free $CO_2$ concentration[2] at saturation was 450 $\mu$M and the apparent $K_m$ was 180 $\mu$M; it is the free $CO_2$ rather than the $HCO_3^-$ which is the chemical species taken up by leaf slices[3,4].

Light response: Light saturation occurred at 1200 $\mu E \cdot m^{-2} \cdot sec^{-1}$, at 20 mM $HCO_3^-$, when the photosynthetic rate was expressed in terms of chlorophyll content or fresh weight (figure). Photosynthesis, determined by infrared gas analysis on individual soybean leaves at 25 °C, is light

Net photosynthetic and respiration rates for soybean leaf slices (mean ± SE)

| Units | Photosynthesis (n = 9) | Respiration (n = 11) |
|---|---|---|
| nmoles $CO_2 \cdot g\ (f.wt)^{-1} sec^{-1}$ | 77 ± 9* | |
| nmoles $O_2 \cdot g\ (f.wt)^{-1} sec^{-1}$ | 67 ± 3** | 6.5 ± 0.4** |
| ng $CO_2 \cdot cm^{-2} sec^{-1}$ | 49 ± 6* | |

* Determined as $^{14}CO_2$ fixation. ** Determined as $O_2$ evolution (consumption). f.wt, fresh weight.




The response of net photosynthesis (△) to 1. varying $HCO_3^-$ concentrations at pH 7.6 and a light intensity of 2000 $\mu E \cdot m^{-2} \cdot sec^{-1}$ or 2. varying light intensity at 20 mM $HCO_3^-$. Rates are expressed on a fresh weight (●) or chlorophyll (○) basis. Vertical bars represent SE. Regression equations ($p < 0.001$) are: $y = -63.5 + 15.0 \ln x$ ($n = 18$) and $y = -64.5 + 15.4 \ln x$ for fresh weight and chlorophyll bases respectively. Chlorophyll values are slightly offset to the left. The $CO_2$ regression equation is: $y = 18 + 27 \ln x$ ($p < 0.001$) omitting the highest concentration.

saturated in the range 750–1400 $\mu E \cdot m^{-2} \cdot sec^{-1}$[5-7]. From the fitted curves, the light compensation point was calculated as about 65 $\mu E \cdot m^{-2} \cdot sec^{-1}$, which is the same as that based on other methods[6].

$^{14}CO_2$ fixation versus $O_2$ evolution: There was no difference in the photosynthetic rate measured by $^{14}CO_2$ fixation compared with that measured as $O_2$ evolution (table). The $^{14}CO_2$ fixation values were adjusted by subtracting the rate of $^{14}CO_2$ fixed by leaf slices kept in the dark to account for dark $CO_2$ fixation and possible adsorption of $H^{14}CO_3^-$ (or $^{14}CO_2$) in such places as cell walls and intercellular air spaces of the slices. No attempt was made to partition the dark fixation rate of 6.4 ng $CO_2 \cdot cm^{-2} \cdot sec^{-1}$ into these components.

The net photosynthetic rate of 49 ng $CO_2 \cdot cm^{-2} \cdot sec^{-1}$ (table) is the same as that for Wayne soybean seedlings estimated by infrared gas analysis[5]. The cut surface area of the slices should not be considered when calculating photosynthetic rate, as it is not the main area of $CO_2$ diffusion[4]. Thus, in these leaf slices, gaseous exchange is through the stomata.

Using leaf slices in an oxygen electrode we find that rates of $O_2$ evolution and $CO_2$ fixation expressed on a fresh weight, chlorophyll or leaf area basis are the same as those for whole leaves using other methods.

1 To whom correspondence should be addressed.
2 G.H. Aylward and T.J.V. Findlay, in: SI Chemical Data, 2nd edn, 136 pp. Wiley, Sydney 1974.
3 H.G. Jones and C.B. Osmond, Aust. J. biol. Sci. *26*, 15 (1973).
4 C.I. Ullrich-Eberius, U. Luttge and L. Neher, Z. PflPhysiol. *79*, 336 (1976).
5 P.E. Curtis, W.L. Ogren and R.H. Hageman, Crop Sci. *9*, 323 (1969).
6 W.A. Brun and R.L. Cooper, Crop Sci. *7*, 451 (1967).
7 K. Ohki, Crop Sci. *18*, 79 (1978).

## Anchorage-dependent culture of line 10 guinea-pig hepatoma cells

S.J. Kleinschuster, H.J. Rapp[1], A.V. Johnston, K.R. Van Kampen, C.C. Muscoplat[2] and J. Bier[3]

*Department of Animal, Dairy & Veterinary Sciences, Utah State University, Logan (Utah 84322, USA), 22 November 1979*

*Summary.* A simple method for the anchorage-dependent culture of line 10 guinea-pig hepatoma cells is described.

The transplantable cell line commonly known as 'line-10 guinea-pig hepatoma' is an hepatocellular carcinoma converted to ascites form from primary hepatomas in strain-2 guinea-pigs originally induced by feeding diethylnitrosamine[3]. Intradermal (i.d.) inoculation of $10^6$ ascites line-10 tumor cells leads to progressive local growth and metastases to the regional lymph nodes[4,5]. The animals die about 60 days following i.d. inoculation[6]. This neoplasm has been extensively investigated and multidirectional information concerning the antigenic and biologic properties of the transplanted tumors has led to its recognition as one of the most valuable animal tumor models used in cancer research today[7]. Immunotherapy of this tumor system has been very successful and predictions from this research have been extrapolated for use in the immunotherapy of naturally-occurring tumors in allogeneic species[8,9]. New approaches to studies of the mechanism of these maneuvers would be possible if continuous anchorage-dependent cultures derived from this tumor system were available; hitherto only suspension cultures have been attainable. Therefore, we now report a simple method for the propagation of guinea-pig line-10 hepatoma cells in an anchorage-dependent fashion.

*Methods and materials.* Plastic culture flasks (25 $cm^2$) were pretreated with human fibronectin (Collaborative Research Inc., Waltham, Mass., USA) at a concentration of 2.5 $\mu g/cm^2$ of growth area before addition of line-10 ascites cells. Fibronectin is a plasma protein isolated and purified from human fibrinogen which enhances and facilitates the culture of anchorage-dependent cells, and in this case, appears to induce and promote anchorage-dependency within many cells of the seeded population. Following pretreatment with fibronectin, the culture flasks were seeded with $4 \times 10^6$ line-10 hepatoma cells. The cells were obtained in ascites form, washed 2 times with MEM (Microbiological Associates, Bethesda, MD, USA) and numbers adjusted with MEM to seeding density. The culture medium consisted of MEM, 20% fetal bovine serum (Sterile Systems Inc., Logan, Utah USA), 100 units/ml penicillin and 100 $\mu g$/ml streptomycin. The cultures were allowed to incubate undisturbed for 4 days at 37 °C. Phase microscopy revealed the presence of many settled and anchorage-dependent islands of cells at that time (figure 1). Unattached cells were decanted and the cultures washed gently with MEM. Medium (MEM/FBS) was then added and the cultures allowed to propagate until maximum density was attained (figure 2). The viability of the cultured cells was tested by trypan blue exclusion and the induction of malignant metastatic lesions following the i.d. inoculation of the cultured cells (figure 3).

*Discussion.* The line-10 guinea-pig hepatoma transplant-