Fractionation of the Proteins of Plant Microbodies

By RICHARD H. BROWN, J. MICHAEL LORD and MICHAEL J. MERRETT

Postgraduate School of Biological Sciences, University of Bradford, Bradford, Yorks. BD7 1DP, U.K.

(Received 28 May 1974)

1. Glyoxysomes and peroxisomes have been isolated from dark- and light-grown seedlings of pumpkin (*Cucurbita pepo*) by sucrose-density-gradient centrifugation. 2. Pumpkin microbodies and castor-bean (*Ricinus communis*) glyoxysomes may be fractionated, by a combination of osmotic shock and treatment with KCl, into three distinct groups of proteins: readily soluble (matrix enzymes), solubilized in the presence of KCl (membrane-bound enzymes) and relatively insoluble (membrane 'ghost' proteins). 3. Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis of 'ghost' fractions indicated that the membrane proteins were generally of low molecular weight; one gel band (mol.wt. 27000–28000) was common to all three microbodies. 4. Although there were major differences in the soluble protein components of pumpkin glyoxysomes and peroxisomes, electrophoresis of the pumpkin microbody 'ghosts' indicated that the membrane proteins were similar, four main components being common to each class of microbody (monomer molecular weights 42000, 34000, 27000 and 17000).

The widespread occurrence of microbodies in plant tissues is now well established (Tolbert, 1971). Two main categories of plant microbody are known: glyoxysomes, present in germinating seeds actively converting fats into carbohydrate and which house enzymes of the gluconeogenic pathway (Beevers, 1969), and peroxisomes, present in green leaves and containing enzymes of the photo-respiratory sequence (Tolbert & Yamazaki, 1969).

Electron micrographs of microbodies *in situ* have frequently shown these cytoplasmic organelles adjacent to sections of the endoplasmic reticulum, and direct continuity between the endoplasmic reticulum and microbody membrane has been reported (Hruban & Rechcigl, 1969; Vigil, 1970; Gruber *et al.*, 1970). Such observations have given rise to speculation that microbodies might originate from the endoplasmic reticulum by a process of vesiculation.

Recent studies using castor bean-endosperm tissues have established that several phospholipids, including lecithin, are exclusively synthesized on the endoplasmic reticulum (Lord *et al.*, 1972, 1973; Moore *et al.*, 1973). This work provided a biochemical basis for the previous observation (Kagawa *et al.*, 1973a) that [¹⁴C]choline supplied to intact endosperm tissue was initially incorporated into lecithin in the endoplasmic-reticulum membrane fraction and subsequently appeared in the glyoxysomal membrane, suggesting a possible precursor-product relationship between these membrane components.

At present no information is available regarding the protein components of the microbody membrane; data concerning the nature and origin of such proteins

might be relevant to the question of microbody biogenesis.

The present paper reports the protein profiles obtained after sodium dodecyl sulphate-polyacrylamide-gel electrophoresis of the membrane of castor-bean glyoxysomes and the glyoxysome and peroxisome membrane from developing pumpkin cotyledons.

The results obtained with the pumpkin system are considered in terms of the developmental relationship between glyoxysomes and peroxisomes during the transition from fat breakdown to photosynthetic metabolism during germination.

Materials and Methods

Germination. Castor-bean (*Ricinus communis*) and pumpkin (*Cucurbita pepo*) seeds were soaked overnight in running tap water and germinated in moist vermiculite in darkness at temperatures of 30°C and 25°C respectively. After 8 days' growth in darkness, the pumpkin seedlings were exposed to 5000 lx white light provided by fluorescent tubes to allow the development of photosynthetic capacity and peroxisomal function (Kagawa *et al.*, 1973b).

Homogenization of tissue and microbody isolation.

(a) **Glyoxysomes.** Castor-bean endosperm halves removed from 4–5-day-old seedlings and pumpkin cotyledons removed from 8-day-old dark-grown seedlings were homogenized by chopping for 10 min with a single razor blade in grinding medium contained in a Petri dish on ice. The grinding medium contained 150 mM-Tricine buffer, pH 7.5,

10mM-KCl, 1mM-MgCl₂, 1mM-EDTA, pH7.5, and 18% (w/w) sucrose. In general, 1ml of grinding medium was used per two castor-bean endosperm halves and 1ml per four pumpkin cotyledons. The homogenate was filtered through four layers of cheesecloth and cell debris was removed by centrifuging at 300g and 0°C for 10min. A portion (5ml) of homogenate was layered on to a 30ml sucrose gradient increasing linearly in concentration from 32% to 60% (w/w) sucrose over a 2ml cushion of 60% sucrose contained in a 38.5ml polyallomer tube. The gradient was centrifuged at 53000g and 4°C for 4h in the SW 27 rotor on a Beckman L2-65B ultracentrifuge and, after centrifugation, 1.0ml fractions were collected over the gradient by using a Beckman gradient fractionator.

(b) Peroxisomes. Green pumpkin cotyledons (25g) removed from 8-day dark-grown followed by 2-day light-grown seedlings were chopped with scissors and homogenized in 50ml of grinding medium (as above) in an MSE top-drive homogenizer operating at top speed for 30s. The homogenate was filtered through four layers of cheesecloth and cell debris was removed by centrifuging at 300g and 0°C for 10min. The supernatant was then centrifuged at 20000g and 0°C for 10min to obtain a crude organelle pellet. This pellet was carefully resuspended in 5ml of grinding medium and the peroxisomal fraction was separated by centrifugation on a discontinuous sucrose gradient as described by Tolbert *et al.* (1969). The peroxisome fraction thus obtained was then applied to a second sucrose gradient, 30ml in volume, and increasing linearly in concentration from 50% to 60% (w/w) sucrose. The gradient was centrifuged at 53000g and 4°C for 2h, and then fractionated as described above.

All sucrose solutions used to prepare gradients contained 1mM-EDTA, pH7.5.

Isolation and treatment of microbody membranes. Gradient fractions containing microbodies were established by the presence of isocitrate lyase (glyoxysomes) and glycollate oxidase (peroxisomes). Microbody membranes were obtained from the pooled microbody peaks collected from four sucrose gradients by the procedure of Huang & Beevers (1973). The microbody fraction [6–10ml in 54% (w/w) sucrose] was diluted 1:3 with 50mM-Tricine, pH7.5, to osmotically disrupt the organelles. This suspension was incubated at 0°C for 30min, and the crude membrane fraction was obtained by centrifuging at 100 000g for 30min at 0°C. The membranes were washed with 50mM-Tricine, pH7.5, containing 18% (w/w) sucrose alone or with either 0.15M- or 1.0M-KCl and re-pelleted.

Gel electrophoresis. Membrane pellets were incubated for 2h at 37°C in 100μl of 2% (w/v) sodium dodecyl sulphate and 2% (v/v) β-mercaptoethanol in 20mM-potassium phosphate, pH7.2. Membrane

proteins were separated by electrophoresis in buffer containing sodium dodecyl sulphate as described by Weber & Osborn (1969), except that 3% (w/v) urea was included in the gel mixture.

The gels were stained in Coomassie Blue, destained electrophoretically and scanned in a Gilford gel scanner at 600nm. The gels were calibrated with standard proteins; the relationship between mobility and log(mol.wt.) was linear between 0.3 and 0.9 relative mobility.

Labelling of glyoxysomal membrane lecithin. Castor-bean endosperm halves removed from 4–5-day-old seedlings were treated with [methyl-¹⁴C]choline chloride (2.5μl; 0.25μCi per endosperm half) and incubated at 30°C for 6h before homogenization and isolation of microbodies. The details of this procedure, including the counting of radioactivity of [¹⁴C]-lecithin-labelled membrane fractions, were exactly as described by Kagawa *et al.* (1973a).

Enzyme assays. All enzymes were assayed spectrophotometrically, with a Unicam SP. 500 or Gilford 222 spectrophotometer linked to a Servoscribe 541 recorder. All assays were conducted at 20°C, with the exception of glyoxylate reductase which was measured at 25°C. One unit of enzyme catalysed the disappearance of 1μmol of substrate or the appearance of 1μmol of product/min. Isocitrate lyase (EC 4.1.3.1) was assayed by the method of Dixon & Kornberg (1959), malate synthase (EC 4.1.3.2) by the method of Cook (1970), malate dehydrogenase (EC 1.1.1.37) by the method of Ochoa (1955), glyoxylate reductase (NADP-linked; EC 1.1.1.79) by the method of Tolbert *et al.* (1970), catalase (EC 1.11.1.6) by the method of Lück (1963), glycollate oxidase (EC 1.1.3.1) by the method of Feierabend & Beevers (1972) and cytochrome oxidase (EC 1.9.3.1) by the method of Smith (1955).

Protein determination. Protein was determined by the method of Lowry *et al.* (1951), bovine serum albumin being used as the standard.

Materials. Castor-bean (*Ricinis communis*) and pumpkin (*Cucurbita pepo* var. Hundredweight) seeds were obtained from D. T. Brown and Co., Poulton-le-Fylde, Blackpool FY6 7HX, U.K. [methyl-¹⁴C]-Choline chloride (specific radioactivity 59μCi/μmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Other chemicals used were of the highest purity commercially available.

Results and Discussion

Development of microbody enzymes during germination

The glyoxylate cycle (Kornberg & Krebs, 1957) plays a necessary role in the conversion of fats into carbohydrate, which characterizes the early post-germinative growth of lipid-storing seeds (Canvin & Beevers, 1961). In the endosperm of castor-bean seedlings, the activity of the key enzymes of this

Fig. 1. Development of glyoxysomes in pumpkin seedlings

Microbodies were isolated from 20 cotyledons, removed at various stages from dark-grown seedlings, by sucrose-density-gradient centrifugation (Fig. 3) and assayed for isocitrate lyase (Δ) and protein ($\square$). $\circ$, Total wet weight of 20 cotyledons.

cycle, and the number of glyoxysomes which house them, increases until the fifth day of germination. Subsequently, enzyme activity and the rate of fat utilization declines (results not shown here; see Beevers, 1969).

When pumpkin seeds were grown at 25°C in the dark, maximal activity of the glyoxysomal enzyme isocitrate lyase and, accordingly, total glyoxysomal protein present in the cotyledons, was found on the eighth day of growth (Fig. 1). There was no apparent change in the position of the recovered glyoxysomes on the sucrose gradients with extracts from tissue of different ages. The reported change in enzyme activity could, however, be influenced by the ability of the microbodies to withstand the extraction procedure (Feierabend & Beevers, 1972). Nevertheless, 5 days and 8 days were taken as the ages at which maximal glyoxysome recovery could be obtained from castor-bean endosperm and pumpkin cotyledons respectively.

When dark-grown pumpkin seedlings were continuously illuminated from day 8 onwards, the cotyledons rapidly became green and photosynthetically competent. There was a rapid decrease in glyoxysomal function, exceeding that in seedlings which remained in darkness (Fig. 1), and a striking increase in peroxisomal function. This transition was essentially complete after 2 days of illumination (results not shown); the residual isocitrate lyase activity was less than 5% of that in 8-day-old tissue and the glycolate oxidase more than 95% of its maximal activity (per fresh weight of cotyledon tissue). The effect of illumination on the activities of glyoxysome and peroxisome marker enzymes reported here is

in agreement with data obtained with watermelon seedlings by Kagawa *et al.* (1973b).

Isolation of microbodies

Isopycnic sucrose-gradient centrifugation of castor-bean endosperm homogenates separated glyoxysomes (equilibrium density 1.24 g/cm³) from mitochondria (density 1.19 g/cm³) and other particulate components, as described previously (Breidenbach & Beevers, 1967). To avoid significant contamination of the glyoxysomal fraction with proplastids (density 1.22–1.23 g/cm³), only the more dense portion of the glyoxysomal peak was collected and used in membrane preparations.

The effectiveness of such gradients in isolating glyoxysomes present in pumpkin homogenates is shown in Fig. 2. As can be seen from the distribution of isocitrate lyase, glyoxysomes (density 1.27 g/cm³) were clearly separated from mitochondria (density 1.21 g/cm³ for cytochrome oxidase) and partially separated from proplastids, which were located by the presence of NADPH-linked glyoxylate reductase (Tolbert *et al.*, 1970) at a density of 1.25 g/cm³. After fractionation of the gradient, further studies on isolated glyoxysomes were performed on the more dense glyoxysomal peak fractions, thus avoiding contamination by proplastids.

Peroxisomes were isolated from homogenates prepared from pumpkin cotyledons exposed to light by differential centrifugation to obtain a crude organelle pellet enriched in peroxisomes, mitochondria and plastids. This pellet was resuspended in grinding medium and centrifuged on a non-linear

Fig. 2. Sucrose-density-gradient centrifugation of a cell-free extract of dark-grown pumpkin seedlings

An extract of 20 cotyledons from 8-day seedlings was applied to a linear sucrose density gradient (---) and centrifuged for 4 h at 53000g and 4°C. Cell organelles were located on the gradient by assaying for isocitrate lyase (Δ , units $\times 10^2$), NADP-linked glyoxylate reductase ($\bullet$, units $\times 5 \times 10^3$), cytochrome oxidase ($\blacktriangle$, units $\times 10^2$) and protein ($\square$).

Fig. 3. Sucrose-density-gradient centrifugation of a crude peroxisomal fraction of light-grown pumpkin seedlings

A crude peroxisomal fraction derived from a preliminary discontinuous gradient (Fig. 2) was applied to a linear sucrose gradient (---) ranging from 50% to 60% (w/w) sucrose. After centrifugation for 2 h at 53000g and 4°C, peroxisomes and plastids were located on the gradient by assaying for glyoxylate oxidase ($\circ$), NADP-linked glyoxylate reductase ($\bullet$) and protein ($\square$).

sucrose gradient (Tolbert *et al.* 1969), which separated the peroxisomes [located by glyoxylate oxidase activity and trapped at the 60–50% (w/v) sucrose interface] from mitochondria (mitochondrial malate dehydrogenase at the 50–43% sucrose

interface) and chloroplast fragments (heavy, chlorophyll-containing band at the 43–38% sucrose interface). The small amount of glyoxysomal isocitrate lyase activity remaining in the tissue at this stage was found in the peroxisomal fraction.

The peroxisome peak was collected and re-centrifuged on a linear sucrose gradient (Fig. 3). This second gradient effectively separated the peroxisomes (glycollate oxidase activity giving a peak at a density of 1.27g/cm³) from plastids (NADPH-linked glyoxylate reductase activity giving a peak at a density of 1.25g/cm³).

Preparation and properties of microbody membranes

Pumpkin. Isolated microbodies were fractionated into membrane 'ghosts' and soluble protein components by osmotic shock (Huang & Beevers, 1973; Bieglmayer *et al.*, 1973) and KCl treatment (Huang & Beevers, 1973).

The pattern of enzyme release from pumpkin glyoxysomes treated with increasing concentrations of KCl after osmotic shock (Table 1) is essentially the same as reported for castor-bean glyoxysomes by Huang & Beevers (1973). Some enzymes, for example catalase and isocitrate lyase, were readily solubilized on osmotic shock in the absence of KCl and are presumably freely soluble in the microbody matrix. Other enzymes, for example malate synthase and malate dehydrogenase, were largely recovered in the membrane pellet upon centrifuging osmotically shocked microbody suspensions, but were more readily solubilized when the membrane 'ghosts' were washed with increasing concentrations of KCl. Such enzymes appear to be loosely bound to the microbody membrane. In all cases the total recovered enzyme activity was within $\pm 10\%$ of the initial activity measured before fractionation.

The residual, hydrophobic, 'structural' protein of the membrane 'ghosts' is not amenable to study by

Table 1. Residual enzyme activity or [¹⁴C]lecithin present in washed membranes obtained from pumpkin or castor-bean glyoxysomes

Glyoxysomes were broken by osmotic shock, washed with increasing concentrations of KCl and the membranes recovered by centrifugation. Values are given as % of the total recovered enzyme activity present in the membrane pellet. The recovery of microbody membrane under these conditions is given by the % recovery of radioactivity for castor-bean glyoxysomes in which the lecithin had been labelled by prior treatment of the tissue with [¹⁴C]choline.

	Enzyme or ¹⁴ C in membrane pellet (%)		
	Tricine only	0.15M-KCl	1.0M-KCl
Pumpkin glyoxysomes			
Catalase	1.0	0.2	0.1
Isocitrate lyase	10.0	4.0	2.0
Malate dehydrogenase	25.0	9.0	2.0
Malate synthase	44.0	14.0	10.0
Castor-bean glyoxysomes [¹⁴ C]Lecithin	93.0	86.0	64.0

Fig. 4. Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis of fractions derived from pumpkin glyoxysomes

Isolated pumpkin glyoxysomes were broken by osmotic shock, the membranes washed in various solutions and the washed membranes and the washings were examined electrophoretically. Traces (a), (b) and (c) were obtained from membranes which had been washed in Tricine, 0.15M-KCl and 1.0M-KCl respectively.

enzymic analysis. Further characterization of the membrane fraction was therefore attempted by sodium dodecyl sulphate-gel electrophoresis. Intact pumpkin glyoxysomes gave a total of 17 different bands after electrophoresis. Of these, 13 were recovered from the microbody membrane which had been washed in Tricine buffer (Fig. 4a). Washing the membranes in 0.15M-KCl resulted in the removal of two of these bands (bands 2 and 9)

(Fig. 4b); washing in 1.0M-KCl largely removed band 7 (Fig. 4c).

Most of the peaks seen on the sodium dodecyl sulphate-gel traces therefore fall readily into one of three categories: easily solubilized, solubilized in the presence of KCl or relatively insoluble. It is reasonable then to conclude that gel peaks correspond to the monomers of the enzymes/proteins in the three similar categories established from the enzyme-release studies.

Tentative assignments may be made for the four main soluble proteins (peaks 3, 5, 6 and 8), on the basis of their appearance in the various washed membrane fractions (Fig. 4) and by comparison with the enzyme-release studies. These fractions were released with decreasing ease in the order 3, 8, 6, 5 (with respect to the intact glyoxysome gels) and would appear to correspond to monomers of four major enzymic proteins: catalase, isocitrate lyase, malate dehydrogenase and malate synthase respectively. The identification of peak 5 was confirmed by purifying malate synthase by sucrose-density-gradient centrifugation (Breidenbach, 1969). Approximate monomeric molecular weights of the peaks are: 3, 100000; 5, 90000; 6, 75000; 8, 63000.

Gels were also run of fractions derived from pumpkin peroxisomes. Gel scans for the intact peroxisomes again showed 17 bands, although they differed somewhat in position and intensity from the glyoxysomal bands. In particular, the relative heights of peaks 5 and 8 were decreased and that of peak 9 increased in the peroxisome.

This was not unexpected, since these peaks probably represent soluble matrix enzymes, which differ, with one or two exceptions, in the two types of organelle.

A comparison of 0.15M-KCl-washed membranes from the two types of organelle, however, shows a marked similarity, especially in the lower part of the gel, i.e. peaks 12, 13, 14 and 16 of the glyoxysome gel (Fig. 5). These peaks probably represent membrane proteins. If so, then the two types of organelle contain similar membrane proteins, in addition to having similar morphological and physical properties and certain enzyme activities in common. It would seem likely that the mechanism and site of synthesis within the cell are common to both classes of organelle membrane. However, our data do not allow us to differentiate between two hypotheses, either that pre-existing glyoxysomes are capable of differentiating into peroxisomes (Trelease *et al.*, 1971) or that peroxisomes are a separate population and are not derived from pre-existing glyoxysomes (Kagawa *et al.*, 1973b). Approximate molecular weights of the peaks (representing monomers of the proteins) are: 12, 42000; 13, 34000; 14, 27000; 16, 16,000.

Castor bean. Sodium dodecyl sulphate-gel electro-

Fig. 5. Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis of pumpkin microbody membranes

Glyoxysomes (—) or peroxisomes (·····), isolated from pumpkin cotyledons, were broken by osmotic shock, washed in 0.15M-KCl and examined electrophoretically.

phoresis of the original glyoxysome supernatant fraction after osmotic shock (results not shown) indicated the presence of four major and several minor components. The major components had approximate molecular weights of 100000, 86000, 80000 and 61000, and probably represent monomers of the readily solubilized matrix enzymes such as catalase and isocitrate lyase (Huang & Beevers, 1973). A comparison of the gel trace obtained for Tricine-washed membranes (Fig. 6a) with the 0.15M-KCl-treated membranes (Fig. 6b) showed that several peaks were removed and the relative content of others was considerably decreased by 0.15M-KCl. This response suggested that this group of proteins probably included malate synthase, malate dehydrogenase and β -oxidation enzymes (Huang & Beevers, 1973). Of particular interest is peak n, corresponding to a monomer molecular weight of 28000, which was virtually unaffected by the KCl treatments (Fig. 6) and seems to be a major protein component of the microbody membrane. The pumpkin glyoxysome membrane also contains a component of similar molecular weight (Fig. 4, peak 14, molecular weight 27000).

Protein removal by washing, particularly in KCl, probably represents selective removal of individual protein components rather than a general disintegration of the membrane. A measure of the residual membrane phospholipid in the washed pellets was obtained from the [14 C]lecithin content in experiments performed on glyoxysomes isolated from castor-bean endosperm which had been preincubated with [14 C]choline (Kagawa *et al.*, 1973a).

The bulk of the membrane-bound lecithin (over

Fig. 6. Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis of fractions derived from castor-bean glyoxysomes

Isolated castor-bean glyoxysomes were broken by osmotic shock, the membranes washed in various solutions, and the washed membranes and the washings were examined electrophoretically. Traces (a), (b) and (c) were obtained from membranes which had been washed in Tricine, 0.15M-KCl and 1.0M-KCl respectively.

60%) was still recovered even after washing in 1.0M-KCl (Table 1).

Washing the membranes in Tricine containing 0.01% (v/v) Triton X-100 had little effect on the recovery of [14 C]lecithin-labelled membranes, and a gel trace of such a preparation was quantitatively and qualitatively the same as the Tricine-washed membranes (Fig. 6a) (data not shown). The presence of 0.1% (v/v) Triton X-100, however, resulted in greater than 90% solubilization of the membrane [14 C]lecithin.

In spite of the fact that glyoxysomes from both castor-bean endosperm and pumpkin cotyledons were expected to have very similar physical and enzymic properties, the gel traces showed qualitative and quantitative differences for corresponding preparations from the two plant species (compare Fig. 4 and Fig. 6). However, this comparison reveals several bands apparently common to both plant preparations on the basis of mobility. For example, band 14 (Fig. 4) and band n (Fig. 6) both appear to be components of the microbody membrane with an approximate molecular weight of 27000–28000. The significance of the differences between the two sets of gels (Fig. 4 and Fig. 6) in terms of individual protein components of the microbodies cannot be assessed at present. In general, however, protein components of castor-bean glyoxysomes, as in pumpkin, seem to fall into three categories: those readily lost upon osmotic shock, those selectively removed by KCl treatment and residual proteins that appear to be tightly bound to the membrane.

References

- Beevers, H. (1969) *Ann. N.Y. Acad. Sci.* **168**, 313–324
- Bieglmayer, C., Graf, J. & Ruis, H. (1973) *Eur. J. Biochem.* **37**, 553–562
- Breidenbach, R. W. (1969) *Ann. N.Y. Acad. Sci.* **168**, 342–347
- Breidenbach, R. W. & Beevers, H. (1967) *Biochem. Biophys. Res. Commun.* **27**, 462–469
- Canvin, D. T. & Beevers, H. (1961) *J. Biol. Chem.* **236**, 988–993
- Cook, J. R. (1970) *J. Protozool.* **17**, 232–235
- Dixon, G. H. & Kornberg, H. L. (1959) *Biochem. J.* **72**, 3 p
- Feierabend, J. & Beevers, H. (1972) *Plant Physiol.* **49**, 28–32
- Gruber, P. J., Trelease, R. N., Becker, W. M. & Newcomb, E. H. (1970) *Planta* **93**, 269–288
- Hruban, Z. & Rechcigl, M. (1969) *Microbodies and Related Particles, Int. Rev. Cytol. (Suppl. 1)*, Academic Press, New York
- Huang, A. H. C. & Beevers, H. (1973) *J. Cell Biol.* **58**, 379–389
- Kagawa, T., Lord, J. M. & Beevers, H. (1973a) *Plant Physiol.* **51**, 61–65
- Kagawa, T., McGregor, D. I. & Beevers, H. (1973b) *Plant Physiol.* **51**, 66–71
- Kornberg, H. L. & Krebs, H. A. (1957) *Nature (London)* **179**, 988–991
- Lord, J. M., Kagawa, T. & Beevers, H. (1972) *Proc. Nat. Acad. Sci. U.S.A.* **69**, 2429–2432
- Lord, J. M., Kagawa, T., Moore, T. S. & Beevers, H. (1973) *J. Cell Biol.* **57**, 659–667
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Lück, H. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H., ed.), pp. 885–894, Academic Press, New York
- Moore, T. S., Lord, J. M., Kagawa, T. & Beevers, H. (1973) *Plant Physiol.* **52**, 50–53

- Ochoa, S. (1955) *Methods Enzymol.* **1**, 735-739
Smith, L. (1955) *Methods Biochem. Anal.* **2**, 427-434
Tolbert, N. E. (1971) *Annu. Rev. Plant Physiol.* **22**, 45-74
Tolbert, N. E. & Yamazaki, R. K. (1969) *Ann. N.Y. Acad. Sci.* **168**, 325-341
Tolbert, N. E., Oeser, A., Yamazaki, R. K., Hageman, R. H. & Kisaki, T. (1969) *Plant Physiol.* **44**, 135-147
Tolbert, N. E., Yamazaki, R. K. & Oeser, A. (1970) *J. Biol. Chem.* **246**, 5129-5136
Trelease, R. N., Becker, W. M., Gruber, P. J. & Newcomb, E. H. (1971) *Plant Physiol.* **48**, 461-475
Vigil, E. L. (1970) *J. Cell Biol.* **46**, 435-454
Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406-4412