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AGEING OF ISOLATED CHLOROPLASTS IN A STABILIZING MEDIUM: INFLUENCE OF DIFFERENT TYPES OF PHOTOPHOSPHORYLATION*

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SUMMARY

Preparations of isolated chloroplasts show a reduction of photophosphorylation activity due to ageing even when stored in the dark at 0–4 °C in a stabilizing medium. The rate of activity loss depends strongly upon the type of photophosphorylation. With non-cyclic photophosphorylation (ferricyanide or NADP-ferredoxin) reduction of activity is rapid immediately after isolation of chloroplasts, whereas it decreases to much slower rates after 3–4 hours. In the presence of PMS, which under our reaction conditions serves as electron acceptor for PS-2 and electron donor for PS-1, photophosphorylation activity is hardly affected by the increasing age of chloroplasts. However, upon addition of low concentrations of DCMU, reduction of this phosphorylation activity due to ageing shows the same kinetics as non-cyclic photophosphorylation. Photophosphorylation of PS-1 (reduced DCPIP-MV-DCMU) is hardly affected by the ageing of chloroplasts.

Abbreviations: ATP: adenosine-5'-triphosphate, BSA: bovine serum albumin, DCMU: 3(3-4)dichlorophenyl-1,1-dimethyl urea, DCPIP: 2,6-dichlorophenolindophenol, MV: methylviologen, NADP: nicotinamide adenine dinucleotide phosphate, ^{32}P: the radioactive isotope phosphorus-32, PS-1 (2): photosystem 1 (2), PMS: phenazine methosulphate.

1. INTRODUCTION

Ageing of isolated chloroplasts suspended in salt solutions is known to lead to an increase in their volume, cf. SIEGENTHALER (1968, 1969, 1972) and HOSHINA et al. (1975). Swelling occurs slowly in the dark, but is accelerated in the light, cf. SIEGENTHALER (1968). Furthermore, the ageing of chloroplasts is accompanied by inactivation of photochemical activities such as electron transport, cf. HARNISCHFEGER (1972) and SIEGENTHALER (1969), ATP-ase, photophosphorylation and photoshrinkage, cf. SIEGENTHALER (1968, 1969, 1972). These phenomena suggest that during swelling and/or ageing there is a deterioration in the chloroplast membrane system. This hypothesis has gained support as a result of electron microscopic studies which have shown that light-activated and (slower) dark-induced swelling or ageing cause drastic morpholo-

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gical changes in the structure of the chloroplast cf. DEAMER et al. (1967) and HARNISCHFEGER (1970).

Biochemical studies have provided evidence that chloroplast ageing *in vitro* may be due to differen causes, *e.g.*

(a) action of hydrolytic enzymes such as galactolipases may bring about modification of the physicochemical properties of the photosynthetic membrane and concomitant inhibition of photochemical reactions, cf. SIEGENTHALER (1972) and WINTERMANS et al. (1969).

(b) release of free (un)saturated fatty acids which induces swelling and inhibits the energy-linked reactions, cf. SIEGENTHALER (1970, 1972, 1973, 1974) and HARNISCHFEGER (1972).

(c) peroxidation of lipids and fatty acids, cf. HOSHINA et al. (1975) and HEATH & PACKER (1965). Reduction of photochemical activity of chloroplasts *in vitro* due to ageing can be suppressed to a great extent if a number of precautions are taken, *e.g.* (1) the hydrogen ion buffer and pH selected should be those most suited for the reaction studied, cf. GOOD et al. (1966), GOOD & IZAWA (1972) and SIEGENTHALER (1973); (2) chloroplasts should be isolated and stored in media containing an appropriate concentration ($\pm$ 0.4 M) of sucrose, cf. JAGENDORF & AVRON (1958). Under these conditions the rate of release of free fatty acids is slower, cf. WINTERMANS et al. (1969); (3) dense chloroplast preparations should be isolated and stored in media supplemented with BSA and kept in the dark at 0°C during storage, cf. FRIEDLANDER & NEUMANN (1968, SIEGENTHALER (1972), WASSERMAN & FLEISCHER (1968) and WINTERMANS et al. (1969). Albumin has been reported to bind unsaturated fatty acids released endogeneously during isolation and storage of chloroplasts, cf. FRIEDLANDER & NEUMANN (1968) and SIEGENTHALER (1972).

However, as will be shown here, despite these precautions chloroplast preparations show a reduction of photophosphorylation activity due to ageing. The rate of activity loss depends strongly on the type of photophosphorylation. These activity losses always have to be taken into account and appropriate corrections made, especially in the case of quantitative experiments which take many hours to perform *e.g.* measurement of photophosphorylation action spectra.

2. MATERIAL AND METHODS

Spinach (*Spinacia oleracea* var. Noorman) was cultivated in a growth chamber as described by GUNTHER & WAGNER (1971), in an air conditioned room, temperature 16–20°C, relative humidity 60–70%. The plants were illuminated by Philips TLF 33 or TL 34 fluorescent tubes having a light intensity of approx. 15 $mW \cdot cm^{-1}$. The light-dark cycle was 12 hours light, 12 hours dark. Four to six week old spinach leaves were washed with distilled water, deribbed and homogenized at 0–4°C in a Braun multipress, containing a piece of filter paper

for the removal of cell debris, in a medium containing 0.4 M sucrose, 0.01 M sodium chloride, 1 g/l defatted bovine serum albumin and 0.05 M Tricine adjusted to pH 7.8 with sodium hydroxide.

Chloroplasts were collected by centrifuging at 1500 × g for 10 minutes in a Sorvall RC-2B refrigerated centrifuge. They were washed once in the grinding medium and stored on ice in the dark in the same medium. Total chlorophyll was estimated according to BRUINSMA (1961) in a Shimadzu UV-200 spectrophotometer.

Only reagent-grade chemicals were used.

$Na_2H^{32}PO_4$ was purified according to SAHA et al. (1970) on Norit, pre-equilibrated with sodium orthophosphate.

Phosphorylation experiments were performed in air at 25 ± 0.1 °C in semi-micro cuvettes with light path 1 cm. The temperature of the cuvettes was kept constant by a thermostat. The schematic lay-out of the spectrograph used for irradiation of the samples is shown in *fig. 1.*

Light from an Osram XBO 1600 W xenon arc lamp is focused on the slit of a Jarrell Ash 0.25 m Ebert grating monochromator by means of a condenser. The lenses L_1 and L_2 yield the geometry of the monochromatic beam required for the cuvette. Beamsplitter M_1 directs a fraction, approximately 10%, of the light to a Siemens solar cell. The output of the solar cell, approximately 100 mA with a load of 1 ohm, is fed back to the Heinzinger TNX 1600 power supply for stabilization purposes. The reaction time of the solar cell combined with the amplifier is 100 μs. In this way the variations in the total photonflux are reduced to values ≤ 0.1%.

Quantitative measurement of the monochromatic light intensity was achieved by means of a calibrated CA-1 compensated thermopile from Kipp and Zonen, Delft, which receives light from the beamsplitter M_2. If necessary M_2 can be removed during exposure. Calibration of the thermopile was performed by Kipp and Zonen, Delft, The Netherlands. The exact amount of photons incident in the cuvette was measured by means of a chemical actinometer.

For a detailed description of the light intensity measurements, cf. VAN GINKEL (1975).

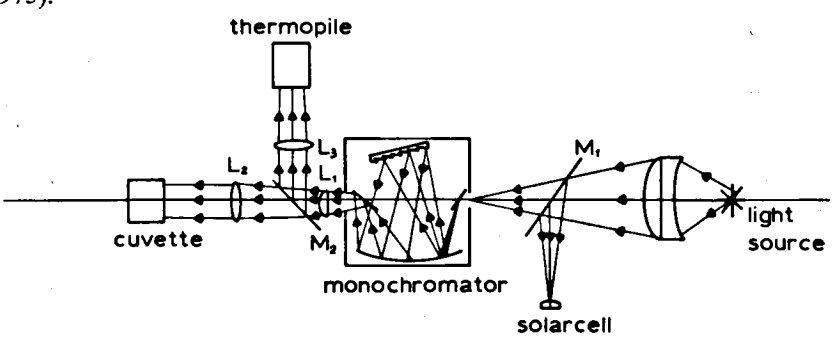

Fig. 1. Schematic lay-out of the spectrograph. L = lens; M = beamsplitter.

The standard reaction mixture, volume 530 µl, contained: 1.5 mM Na_2HPO_4 (carrying approx. $1–15 \times 10^6$ desintegrations/min of ^{32}P), 1.5 mM ADP, 50 mM Tricine/NaOH pH 8.5, 1 mg/ml BSA, 37.5 mM sucrose, 4.5 mM $MgCl_2$, 0.9 mM NaCl and spinach chloroplasts. The concentration of chlorophyll and other components is indicated in the relevant figures. Irradiation time for the samples was two minutes. The $AT^{32}P$ formed was estimated based on residual radioactivity upon extraction of the remaining orthophosphate as phosphomolybdate according to the method of AVRON (1960) which we modified slightly. Distilled water was added instead of acetone. The amount of $AT^{32}P$ formed was determined by measuring the Čerenkov radiation of ^{32}P in a Packard 2425 liquid scintillation counter according to the method of ASADA et al. (1972). For a detailed description of the method, see VAN GINKEL (1975).

The liquid scintillation counter was calibrated with a ^{32}P source bought from the Laboratoire primaire de métrologie des rayonnements ionisants, Gif-sur-Yvette, France; the activity of this source had an error of 0.4%. Counting was sustained until the standard deviation was less than 1%. Corrections for quenching according to the channels-ratio method, cf. VAN GINKEL (1975), HERBERG (1965), STUBBS & JACKSON (1967) and for radioactive decay were made by means of a Diehl Algotronic calculator. All volumetric manipulations were checked by weighing on analytical balances, cf. VAN GINKEL & VAN OOIJEN (1974) and VAN GINKEL (1975). Each plot in the figures shown represents the average of five experiments, the standard deviation being indicated by bars.

3. RESULTS AND DISCUSSION

Our results relating to the influence of ageing on non-cyclic photophosphorylation shown in *figs. 2* and *3* demonstrate that there is a rapid reduction of phosphorylation activity immediately after isolation of the chloroplasts, whereas the reduction rate decreases considerably after 3–4 hours. The reversed shape of the curves resemble the curves published by WINTERMANS et al. (1969) in which release of free fatty acids is plotted as a function of chloroplast age. A connection between fatty acid release and decrease of photophosphorylation activity seems obvious and has been suggested by many investigators, e.g. SIEGENTHALER (1969, 1970, 1972), FRIEDLANDER & NEUMANN (1968) and WINTERMANS et al. (1969). As indicated in the captions for figures 2 and 3 we used rather low concentrations of chloroplast fragments.

WASSERMAN & FLEISCHER (1968) have reported that non-cyclic photophosphorylation requires high chloroplast fragment concentrations and the presence of BSA in the isolation and storage medium to maintain best stability. However, with lower fragment concentrations in media containing sucrose, release of free fatty acids is enhanced as compared to higher fragment concentrations, but still lower than in salt solutions, cf. WINTERMANS et al. (1969). SIEGENTHALER (1973) has shown that free unsaturated acids inhibit non-cyclic photophosphorylation in PS-2 with ferricyanide as electron acceptor more

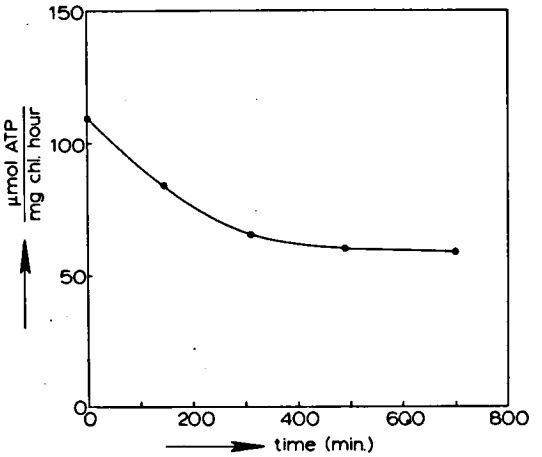

Fig. 2. Effect of chloroplast storage on the rate of non-cyclic photophosphorylation with $K_3Fe(CN)_6$ as electron acceptor. Light intensity: 4.60 nEinstein·cm^{-2}·s^{-1}; wavelength 680 nm; halfwidth 6.4 nm; temperature 25 ± 0.1 °C; 0.45 mM $K_3Fe(CN)_6$; 5 µg chl/ml. Standard reaction mixture as described in Material and Methods.

strongly than PS-1 catalyzed cyclic photophosphorylation (measured with PMS reduced by high intensities of white light).

In a more recent study SIEGENTHALER (1974) suggests that inhibition of unsaturated fatty acids is located on the oxidizing side of PS-2. In view of these experimental data the observed decrease of photophosphorylation activity

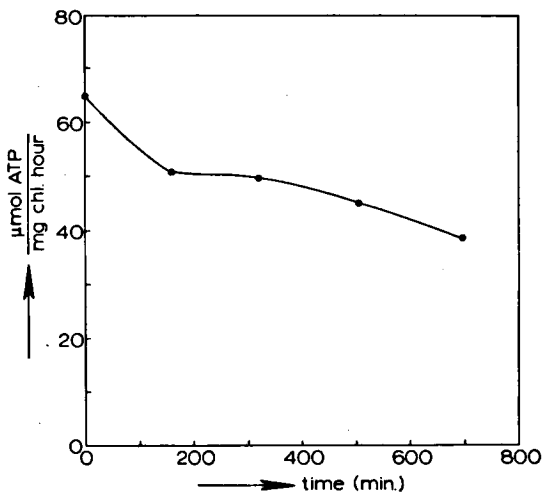

Fig. 3. Effect of chloroplast storage on the rate of non-cyclic photophosphorylation with NADP and ferredoxin as electron acceptors. Light intensity: 3.03 nEinstein·cm^{-2}·s^{-1}; wavelength 680 nm; halfwidth 6.4 nm; temperature 25 ± 0.1 °C; 9.0 mM NADP; 45 µg ferredoxin/ml; 6 µg chl/ml. Standard reaction mixture as described in Material and Methods.

in *figs. 2* and *3* is understandable although it must be mentioned that reduction of activity is very much lower in our case than was found by SIEGENTHALER (1969) and WINTERMANS et al. (1969) probably owing to the storage temperature, pH and the medium we used.

Our results relating to PMS-catalyzed photophosphorylation shown in *figs. 4–6* are more difficult to interpret. Under the conditions in which we measured photophosphorylation with PMS: aerobic, red light, no reductant, PMS acts as electron acceptor for PS-2 (Hill oxidant) and as electron donor for PS-1 provided PMS is reduced by the photosynthetic electron transport from water via the electron transport chain, cf. HAUSKA et al. (1970), JAGENDORF & MARGULIES (1960) and TREBST & ECK (1961).

However, electron transport is much less sensitive than photophosphorylation to free unsaturated fatty acids or ageing, cf. SIEGENTHALER (1972, 1973) and WINTERMANS et al. (1969). Therefore, PMS-catalyzed photophosphorylation shown in *fig. 4* may be considered to consist of the sum of non-cyclic and cyclic photophosphorylation in which the contribution of the latter increases progressively with increasing chloroplast age until photosynthetic electron transport is affected. This is probably the reason why phosphorylation activity with PMS in *fig. 4* is hardly affected by chloroplast age, for there is much experimental evidence to show that PS-1 (cyclic photophosphorylation) is more stable and resistant to ageing than PS-2, cf. TREBST (1970, 1974). However, upon addition of low concentrations of DCMU, see *figs. 5* and *6*, electron transport from water via PS-2 to PS-1 is inhibited, cf. IZAWA & GOOD (1972) and photosynthetic reduction of PMS is inhibited as well. In the latter case non-cyclic photophosphorylation of PS-2 catalyzed by oxidized PMS becomes rate-limiting and the influence of chloroplast ageing should influence this photophosphorylation activity to the same extent as non-cyclic photophosphorylation with other electron acceptors (ferricyanide or NADP-ferredoxin, see *figs. 2* and *3*.

The results shown in *figs. 5* and *6* confirm this reasoning. As mentioned

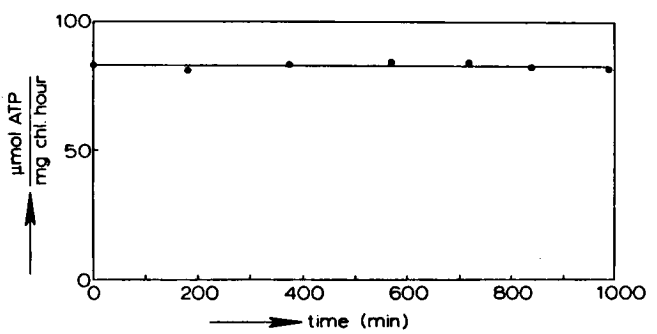

Fig. 4. Effect of chloroplast storage on the rate of PMS-catalyzed photophosphorylation. Light intensity 7.39 nEinstein·cm^{-2}·s^{-1}; wavelength 680 nm; halfwidth 6.4 nm; temperature 25 ± 0.1 °C; 19 µM PMS; 6.0 µg chl/ml. Standard reaction mixture as described in Material and Methods.

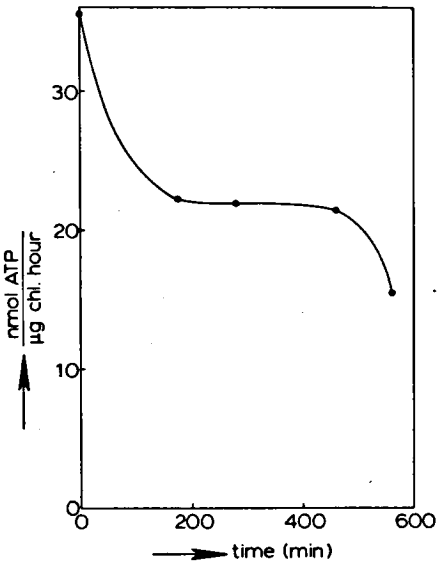

Fig. 5. Effect of chloroplast storage on the rate of PMS-catalyzed photophosphorylation poisoned with 1.75×10^{-8} M DCMU. Light intensity: 7.39 nEinstein·cm^{-2}·s^{-1}; wavelength 680 nm; halfwidth 6.4 nm; temperature 25 ± 0.1 °C; 18 µM PMS; 4.7 µg chl/ml. Standard reaction mixture as described in Material and Methods.

already there is much experimental evidence to show that PS-1 is much more stable and resistant to ageing than PS-2. Electron flow and photophosphorylation of PS-1 is also much less affected by unsaturated fatty acids than those of PS-2, cf. SIEGENTHALER (1973). This stability and resistance to ageing can also

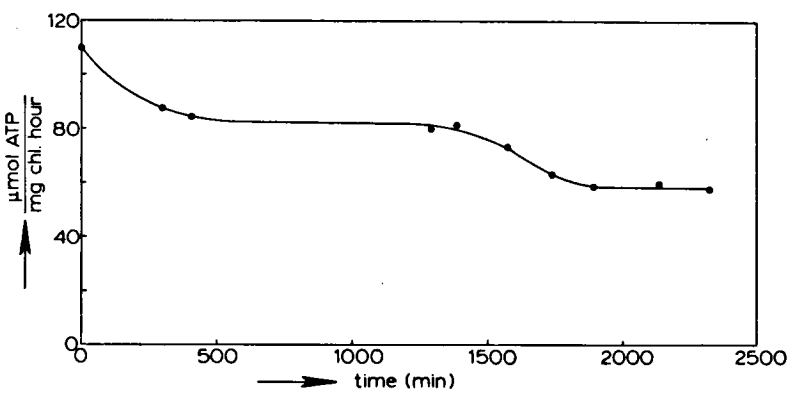

Fig. 6. Effect of chloroplast storage on the rate of PMS-catalyzed photophosphorylation poisoned with 1.75×10^{-7} M DCMU. Light intensity: 3.94 nEinstein cm^{-2}·s^{-1}; wavelength 680 nm; halfwidth 6.4 nm; temperature 25 ± 0.1 °C; 22 µM PMS; 7.3 µg chl/ml. Standard reaction mixture as described in Material and Methods.

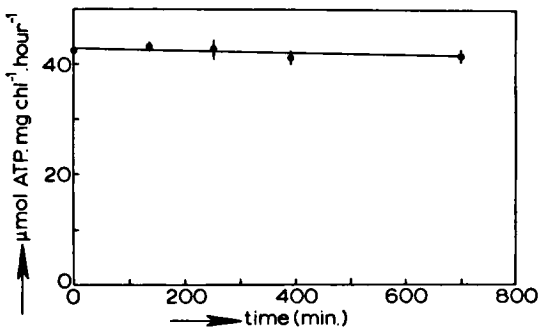

Fig. 7. Effect of chloroplast storage on the rate of PS-1 catalyzed photophosphorylation with reduced DCPIP as electron donor and methylviologen as electron acceptor. Light intensity: 14.0 nEinstein·cm^{-2}·s^{-1}; wavelength 680 nm; halfwidth 6.4 nm; temperature 25 ± 0.1 °C; 6.7 µg chl/ml; 0.2 mM DCPIP; 1.8 mM ascorbate; 0.2 mM methylviologen; 8.5 µM DCMU; 2.5 mM Na_2HPO_4; 1.6 mM ADP; other components as described in Material and Methods.

be seen in *fig. 7*, which shows PS-1 catalyzed photophosphorylation as a function of chloroplast age.

REFERENCES

ASADA, K., M. TAKASHI & M. URANO (1972): Phosphorylation assay in liquid scintillation counter using Čerenkov radiation of ^{32}P: Application to photophosphorylation. *Anal. Biochem.* **48**: 311–315.

AVRON, M. (1960): Photophosphorylation by Swiss-chard chloroplasts. *Biochim. Biophys. Acta* **40**: 257–272.

BRUINSMA, J. (1961): A comment on the spectrophotometric determination of chlorophyll. *Biochim. Biophys. Acta* **52**: 576–578.

DEAMER, D. W., A. R. CROFTS & L. PACKER (1967): Mechanisms of light-induced structural changes in chloroplasts. I. Light-scattering increments and ultrastructural changes mediated by proton transport. *Biochim. Biophys. Acta* **131**: 81–96.

FRIEDLANDER, M. & J. NEUMANN (1968): Stimulation of photoreactions of isolated chloroplasts by serum albumin. *Plant Physiol.* **43**: 1249–1254.

GINKEL, G. VAN (1975): Solar energy conservation in a green plant as studied by detailed action spectra of photophosphorylation. *Doctoral thesis*, Utrecht.

– & J. A. C. VAN OOIJEN (1974): Systemen voor het pipetteren en doseren. *Labinstrumenten* **10**, no. 12: 465–469.

GOOD, N. E. & S. IZAWA (1972): Hydrogen ion buffers. In: *Methods in Enzymology* (A. SAN PIETRO, ed.) Academic Press, New York and London, Vol. **24B**: 53–68.

—, G. D. WINGET, W. WINTER, T. N. CONNOLLY, S. IZAWA & R. M. M. SINGH (1966): Hydrogen ion buffers for biological research. *Biochemistry* **5**: 467–477.

GUNTHER, P. P. & R. H. WAGNER (1971): An inexpensive growth chamber for research and teaching in plant science. *Bioscience* **22**: 32.

HARNISCHFEGER, G. (1970): Changes in appearance, volume and activity during the early stages of disintegration in isolated chloroplasts. *Planta (Berl.)* **92**: 164–177.

— (1972): Photosensitized inhibitor formation in isolated ageing chloroplasts. *Planta (Berl.)* **104**: 316–328.

HAUSKA, G. A., R. E. MCCARTY & E. RACKER (1970): The site of photophosphorylation

associated with photosystem I. *Biochim. Biophys. Acta* **197**: 206–218.
HEATH, R. L. & L. PACKER (1965): Effect of light on lipid peroxidation in chloroplasts. *Biochem. Biophys. Res. Commun.* **19**: 716–720.
HERBERG, R. J. (1965): Channels ratio method of quench correction in liquid scintillation counting. *Packard Technical Bulletin*, Packard Instrument Company, U.S.A. **15**: 1–8.
HOSHINA, S., T. KAJI & K. NISHIDA (1975): Photoswelling and light-inactivation of isolated chloroplasts. I. Change in lipid content in light-aged chloroplasts. *Plant Cell Physiol.* **16**: 465–474.
IZAWA, S. & N. E. GOOD (1972): Inhibition of photosynthetic transport and photophosphorylation. In: *Methods in Enzymology* (A. SAN PIETRO, ed.). Academic Press, New York and London, Vol. **24B**: 355–377.
JAGENDORF, A. T. & M. AVRON (1958): Cofactors and rates of photosynthetic phosphorylation by spinach chloroplasts. *J. Biol. Chem.* **231**: 277–290.
— & M. MARGULIES (1960): Inhibition of spinach chloroplast photosynthetic reactions by p-chlorophenyl-1, 1-dimethyl urea. *Arch. Biochem. Biophys.* **90**: 184–195.
SAHA, S., S. IZAWA & N. E. GOOD (1970): Photophosphorylation as a function of light intensity. *Biochim. Biophys. Acta* **223**: 158–164.
SIEGENTHALER, P. A. (1968): Synergetic effect of light and aging on the swelling and photochemical activities of isolated chloroplasts. *Experientia* **24**: 1198–1199.
— (1969): Vieillissement de l'appareil photosynthétique. I. Effet synergique de la lumière et du vieillissement in vitro sur les changements de volume de chloroplastes isolés d'épinard. *Plant Cell Physiol.* **10**: 801–810.
— (1970): Chloroplast aging in vitro and relationship to fatty acids and polyphenoloxidase activity. *Experientia* **26**: 1308–1310.
— (1972): Aging of the photosynthetic apparatus. IV. Similarity between the effects of aging and unsaturated fatty acids on isolated spinach chloroplasts as expressed by volume changes. *Biochim. Biophys. Acta* **275**: 182–191.
— (1973): Change in pH dependency and sequential inhibition of photosynthetic activity in chloroplasts by unsaturated fatty acids. *Biochim. Biophys. Acta* **305**: 153–162.
— (1974): Inhibition of photosystem II electron transport in chloroplasts by fatty acids and restoration of its activity by Mn^{2+}. *FEBS letters* **39**: 337–340.
STUBBS, R. D. & A. JACKSON (1967): Channels ratio colour quenching correction in Čerenkov counting. *Int. J. appl. Radiat. Isotopes* **18**: 857–858.
TREBST, A. (1970): Neue Reaktionen und Substanzen im photosynthetischen Elektronentransport. *Ber. Dtsch. Bot. Ges.* **83**: 373–398.
— (1974): Energy conservation in photosynthetic electron transport of chloroplasts. *Ann. Rev. Plant Physiol.* **23**: 423–458.
— & H. ECK (1961): Untersuchungen über die Beteiligung des Sauerstoffs in photosynthetischen Reaktionen mit Hilfe von Hemmstoffen. *Z. Naturforschg.* **16B**: 455–461.
WASSERMAN, A. R. & S. FLEISCHER (1968): The stabilization of chloroplast function. *Biochim. Biophys. Acta* **153**: 154–169.
WINTERMANS, J. F. G. M., P. J. HELMSING, B. J. J. POLMAN, J. VAN GINSBERGEN & J. COLLARD (1969): Galactolipid transformations and photochemical activities of spinach chloroplasts. *Biochim. Biophys. Acta* **189**: 95–105.