BBA 4104

STRUCTURAL CHANGES RELATED TO PHOTOSYNTHETIC ACTIVITY IN CELLS AND CHLOROPLASTS

LESTER PACKER, REGINALD H. MARCHANT AND YASUO MUKOHATA
Department of Physiology, University of California, Berkeley, Calif. (U.S.A.)

(Received January 25th, 1963)

SUMMARY

The discovery that spinach chloroplasts and mitochondria *in vitro* manifest structural changes which are closely governed by phosphorylation reactions prompted the present investigation in light-grown cells of *Rhodospirillum rubrum*.

Cells of *R. rubrum* and spinach chloroplasts both manifest light-scattering increases with red-light illumination under conditions for cyclic photophosphorylation. The scattering changes are reversible in both systems by removal of the actinic light.

Dark-grown cells of *R. rubrum* cannot undergo reversible structural changes but light-grown cells and chromatophores isolated from light-grown cells can give rise to these structural changes under conditions of phosphorylation.

The requirements for scattering responses in *R. rubrum* and chloroplasts are compared and the action of certain inhibitory substances are described.

A hypothesis is presented and discussed concerning the coupling of energy-transfer systems to membrane structure in mitochondria, chloroplasts, and photosynthetic bacteria. The findings suggest a general requirement of a membrane for mechano-chemical changes linked to phosphorylation reactions.

INTRODUCTION

The primary energy-transducing systems in nature, the mitochondria and the chloroplasts of higher plants, have been found to manifest structural changes in their membranes in response to phosphorylation processes[1-4]. Both of these systems show light-scattering increases upon initiation of oxidative phosphorylation or photophosphorylation, respectively, which are reversible when the phosphorylation process is halted either by a rate-limiting substance[1,5] or by extinguishing actinic light in photosynthetic systems[3,4]. The presence of such interesting mechano-chemical events closely coupled to the energy-transfer reactions in these subcellular systems, suggested that a fruitful line of research would be to explore the presence of such systems in intact cells. Such a study[6] was carried out with animal cells, ascites-tumour cells, which manifest a Crabtree effect. Here it could be demonstrated that ascites-tumour cells manifest light-scattering increases in intact cell suspensions, which are cor-

Abbreviations: HOQNO, 2-*n*-nonyl-hydroxyquinoline-*N*-oxide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

related with the activity of mitochondrial phosphorylation systems. Control experiments were carried out for changes in cell size, by taking advantage of osmotic properties of ascites cells, and it was found that the light-scattering changes were indeed of intracellular origin. A similar approach has now been explored in the photosynthetic bacterium, *Rhodospirillum rubrum*. This organism can grow either in the light, under anaerobic conditions as a photosynthetic organism, or heterotrophically in the dark, upon organic substrates. Light-grown anaerobic cells have been found to manifest light-scattering changes in response to photosynthetic activity which are not demonstrable in cells from dark-grown cultures. The nature of the requirements for the structural changes in mitochondria, chloroplasts, and photosynthetic cells appears to be similar, and a hypothesis is presented for the coupling of energy-transfer reactions in these systems to structural changes in the membranes. The results with *R. rubrum* are of particular interest since it was discovered that chromatophores, isolated from the cells also manifest structural changes under conditions of photophosphorylation. The findings suggest a general requirement for the presence of a membrane for mechano-chemical changes linked to phosphorylation reactions.

METHODS

Spinach chloroplasts were prepared according to PARK AND PON[7] in sucrose (0.5 M), Versene (0.010 M, pH 7.4), phosphate (0.03 M, pH 7.4), and finally washed with NaCl (0.35 M), Tris–HCl buffer (0.020 M, pH 7.5), by centrifugation at 600 × *g*. The chloroplast residue was suspended in this Tris–NaCl medium. Equivalent results were obtained with chloroplasts isolated from media containing buffered 0.35 M NaCl as recommended by WHATLEY AND ARNON[8], or in 0.5 M sucrose and 0.1 M Tris (pH 8.0). Conditions for cyclic and non-cyclic photophosphorylation, assay of chlorophyll and phosphate uptake, were patterned after procedures described by WHATLEY AND ARNON[8].

Rhodospirillum rubrum was grown on a medium which contained in a stock solution DL-malic acid (175 g), L-glutamic acid (200 g), sodium citrate·2 H_2O (32 g), biotin (50 μg in 0.40 ml of 0.1 M KH_2PO_4), NH_4Cl (27 g), $MgSO_4 \cdot 7\ H_2O$ (10 g), $CaCl_2 \cdot 2H_2O$ (2.5 g), KH_2PO_4 (6 g), K_2HPO_4 (9 g), Difco yeast extract (12.5 g). The stock solution was made up to 10 l with distilled water and sterilized in bottles having a pasteur pipette inserted through the plug. For growth of anaerobic light-grown cultures, the medium was bubbled with 100% nitrogen prior to inoculation. After inoculation, the bottles were filled with medium, and stoppered, and suspended in a water-bath maintained at 25° or 30°. The bath was illuminated by 250-W bulbs located approx. 10 in from the surface of the bottles. For aerobic dark-grown cultures, the cells were grown in the same medium, with aeration, in an aluminum-foil-covered flask at 30°. Cells were harvested after approx. 40 h in the light-grown cultures, washed in distilled water and 0.01 M Tris (pH 7.8) (centrifuging at 4100 × *g*) and finally homogenized gently in 0.01 M Tris (pH 7.8) for use.

For preparation of chromatophores from *R. rubrum*, the following procedure was employed: light-grown cultures were harvested after approx. 40 h as described above. The pellet was washed with distilled water and centrifuged at 4100 × *g* for 10 min. The resulting pellet was then washed once with 0.1 M Tris buffer (pH 7.8) by centri-

fugation at 4100 × *g*. The final pellet was weighed, combined with 2–3-fold excess of alumina A-301 and ground for 3 min at 0° with a pestle and mortar. To the ground pellet, 3 ml Tris buffer (0.1 M, pH 7.8) was then added per g of pellet, and the resulting suspension centrifuged for 10 min at 10 000 × *g* in the Spinco preparative ultracentrifuge. The sediment containing the alumina and unbroken cells and large debris was discarded, and the supernatant was recentrifuged at 25 000 × *g* for 60 min. The sediment was resuspended in Tris buffer (0.1 M, pH 7.8), recentrifuged at 25 000 × g for 60 min, and then taken up in a small, 2–3 ml, volume of Tris buffer and stored in the dark at 0° until used in experiments.

The light-scattering changes were examined in washed *R. rubrum* cells, chromatophores prepared from the cells, and in spinach chloroplasts, in a Brice–Phoenix light-scattering photometer, with the photomultiplier tube positioned at 90° to the incident 546-mμ light. The 90° scattering was adjusted to read 100% on the chart paper by using a minimum intensity of 546-mμ light, and the instrument at its maximum gain. 546 mμ is near the minimum of the absorption spectra for both *R. rubrum* and chloroplast chlorophyll pigments. The material used for scattering determinations was first examined in an ordinary spectrophotometer for its transmission at 546 mμ. Scattering measurements were made with final suspensions which transmitted more than 37% light at 546 mμ, so as to rule out an interference of pigments with scattering measurements. The temperature throughout the experiment was maintained at 25° ± 0.1° by the use of a thermistor-regulated water-bath which circulated liquid through a jacketed 1-cm cell. In initial experiments, a thermistor bridge was employed to record temperature variations in the system, in the absence and presence of red-light illumination, as an index of temperature regulation. Phosphorylation was assayed in isolated *R. rubrum* chromatophores by estimation of the rate of disappearance of inorganic phosphate from the reaction mixture.

RESULTS

Typical results for the conditions necessary for demonstrating 90° light-scattering increases in *R. rubrum* induced by red light are given in Table I. The effects of phosphate, magnesium ions and several potential inhibitors on the light-scattering response are summarized. Expt. 1 shows a distinct stimulating effect of phosphate at 1 mM strength on the scattering increase and that addition of magnesium without phosphate has no effect. Expt. 2 shows the effect of vallinomycin, a known inhibitor of photophosphorylation in chromatophores isolated from *R. rubrum*[9] and of arsenate in the presence of 1 mM phosphate. Neither of these substances had a significant effect on the light-scattering increase. In the case of vallinomycin this could possibly be due to its inability to enter the cells. Finally, Expt. 3 shows the inhibitory effect of HOQNO in the presence of 1 mM phosphate. HOQNO is known to inhibit electron transport in chromatophores isolated from *R. rubrum*[10], and, in view of its effect here, may be able to penetrate the cell.

The action of *m*-chloro-carbonyl cyanide phenylhydrazone, a potent inhibitor of phosphorylation in mitochondria[11,12] and chloroplasts*, and an agent which abolishes scattering changes in chloroplasts[4] was also tested and found to inhibit

* D. O. HALL, E. JENNER AND D. Y. DEKIEWIET, unpublished results.

TABLE I

REQUIREMENTS FOR SCATTERING CHANGES INDUCED BY RED-LIGHT ILLUMINATION IN CELL SUSPENSIONS OF *R. rubrum*

The reaction system contained: Tris (0.02 M, pH 7.7), NaCl (0.035 M), and washed-cell suspension of *R. rubrum* (50% transmission at 546 mμ). See text for explanation. Numbers in parentheses indicate the number of determinations.

Expt.	*Conditions*	*Scattering increase after red light "on" (%)*
1	No addition	2.9 (8)
	Phosphate (1 mM)	16.8 (8)
	$MgCl_2$ (2 mM)	2.5 (2)
2	Phosphate (1 mM) present	
	No addition	11.0
	Vallinomycin (2 μg/ml)	9.0
	Arsenate (10 mM)	10.0
3	Phosphate (1 mM) present	
	No addition	14.0 (2)
	HOQNO (2 μg/ml)	2.0 (2)

75% of the scattering change in *R. rubrum* at a concentration of 33 μM. At 3 μM *m*-chloro-carbonyl cyanide phenylhydrazone scattering changes are about 40% inhibited.

It is also of interest that the cationic detergent, cetyl trimethyl ammonium bromide, also abolishes the scattering response with red-light illumination in *R. rubrum*. This reagent is capable of destroying the permeability properties and interferes with normal growth of bacteria[13]. At a concentration of 3 μg/ml cetyl trimethyl ammonium bromide inhibits the scattering response 75%. Treatment of *R. rubrum* cells in a 10-kc sonic oscillator (Raytheon) for 60 sec also damages cell structure, leading to a 90% inhibition of the scattering response.

The effect of phosphate on the kinetics of the scattering changes brought about under the conditions of red-light illumination in *R. rubrum* is illustrated in Fig. 1, which is a time recording of the response. In the absence of phosphate, red-light illumination induced a rapid increase in scattering of approx. 7% in a period of

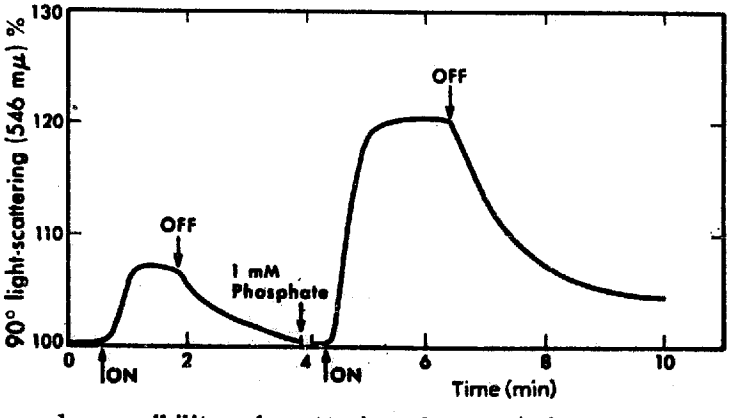

Fig. 1. Kinetics and reversibility of scattering changes induced by red-light illumination in *R. rubrum*. Conditions as in Table I.

around 30 sec. The resultant steady state of scattering change was now maintained until red light was extinguished. Thereupon the scattering response was reversed at a rate considerably slower than the formation of the increased scattering state. Reversal was brought about in a little over 2 min. At this point 1 mM phosphate was introduced into the reaction system in the dark. No scattering change occurs. When the red light is turned on again, a scattering increase is once again produced which this time is about 3 times more intense than that which occurs in the absence of phosphate. The steady state was reached in about 50 sec and, again, the response was almost completely reversed following the removal of the actinic light.

The requirement for scattering changes in cells of *R. rubrum*, chromatophores isolated from *R. rubrum*, and in spinach chloroplasts, are compared in Table II. Expt. 1 shows that light-grown cells of *R. rubrum* manifest scattering changes under the same conditions as described above. When, however, these cells are tested in the presence of high salt concentration (0.35 M NaCl), no scattering change occurs; the same result has been found with chloroplasts[3,4]. It was also of interest to test the ability of dark-grown cells and of chromatophores isolated from light-grown cells to manifest scattering changes. Expt. 2 shows that dark-grown cells are unable to manifest scattering changes with red-light illumination. However chromatophores isolated from light-grown cells also show scattering changes. In separate experiments it was shown that these chromatophores actively carry out photophosphorylation. In three separate experiments, chromatophores isolated from light-grown cells consumed an average of 0.14 μmole of phosphate per ml ($A_{800} = 0.13$; $A_{880} = 0.78$; 1-cm cuvette) in 30 min, and all three of these chromatophore preparations also showed scattering changes under the conditions shown in Table II. Exp. 3 shows that the requirements for scattering changes to be produced by red-light illumination in spinach chloroplasts are similar to those in the experiments carried out with the bacterial cells. Magnesium,

TABLE II

TEST FOR SCATTERING CHANGES INDUCED BY RED-LIGHT ILLUMINATION IN LIGHT- AND DARK-GROWN CELLS OF *R. rubrum*, BACTERIAL CHROMATOPHORES AND SPINACH CHLOROPLASTS

Conditions as in Table I except where indicated otherwise; explanation in text. Tris (0.02 M, pH 7.7) present in all experiments.

Expt.	*Conditions*	*Scattering increase after red light "on" (%)*
1	*Light-grown cells*	
	Medium: NaCl (0.035 M) + phosphate (1 mM)	9.0
	Medium: NaCl (0.35 M) + phosphate (1 mM)	0.0
2	*Medium: NaCl (0.035 M) + phosphate (1 mM)*	
	Light-grown cells	10.0
	Dark-grown cells	0.0
	Chromatophores (light-grown cells)	8.0
3	*Spinach chloroplasts; medium: NaCl (0.035 M)*	
	No addition	0.0
	$MgCl_2$ (5 mM)	1.0
	$MgCl_2$ (5 mM) + phosphate (4 mM)	1.5
	$MgCl_2$ (5 mM) + phosphate (4 mM) + ADP (1 mM)	2.0
	$MgCl_2$ (5 mM) + phosphate (4 mM) + ADP (1 mM) + NADP (3 mM)	15.0

phosphate, ADP and an electron acceptor are needed to establish the conditions for non-cyclic photophosphorylation. Under precisely these conditions, chloroplasts manifest scattering changes.

The requirement of phosphate for scattering changes induced by red light in chloroplasts is shown in Fig. 2, under conditions of cyclic photophosphorylation. At the beginning of the experiment, the chloroplasts were suspended in the presence of all the requirements for photophosphorylation with phenazine methosulfate as the electron carrier. In the absence of phosphate, several consecutive cycles of illumination with red light and its extinction were carried out before the point in the experiment shown. Cycles I, II and III gave scattering changes of 60, 24 and 18 % respectively. The fourth cycle is shown in the left-hand portion of Fig. 2. It produced a scattering change of 6 %. The results indicate the exhaustion of some essential requirement for the scattering change. Upon addition of 5 mM phosphate, the ability to manifest larger scattering changes was restored to the preparation as shown by the subsequent

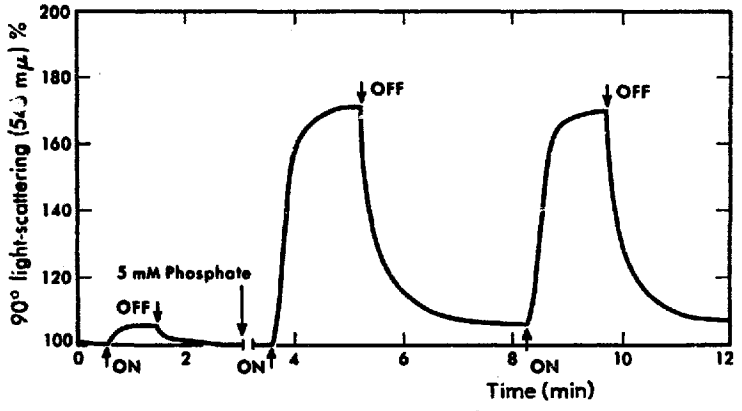

Fig. 2. Effect of phosphate on scattering changes induced by red-light illumination in spinach chloroplasts. The reaction system contained Tris (0.02 M, pH 7.7), NaCl (0.035 M), $MgCl_2$ (0.005 M), ADP (1 mM), ATP (1 mM), ascorbate (2.5 mM), phenazine methosulfate (20 μM) and chloroplasts (2 μg/ml chlorophyll). Explanation in text.

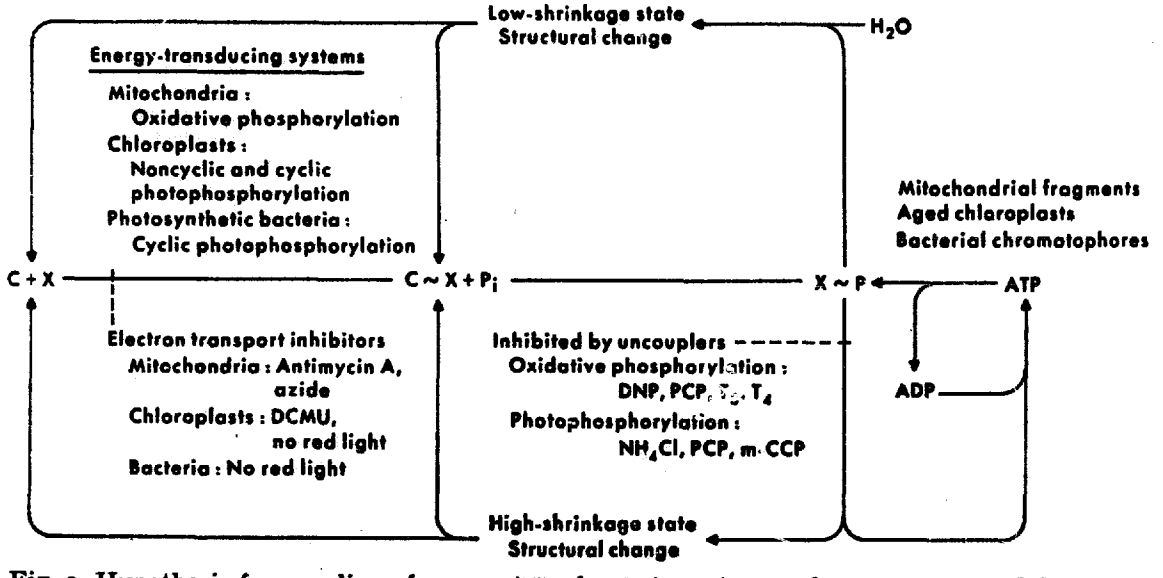

Fig. 3. Hypothesis for coupling of energy-transfer systems to membrane structure. PCP, pentachlorophenol; T3, 3′,5′,5-triiodothyronine; T4, L-thyroxin; *m*-CCP = *m*-chloro-carbonyl cyanide phenylhydrazone.

two cycles of red light on/off where scattering changes of 71 and 60 % were produced, respectively. It is concluded that the requirements of the scattering changes for phosphate are remarkably similar in light-grown cells of *R. rubrum* (*cf.* Fig. 1) and spinach chloroplasts when both are incubated under conditions of cyclic photophosphorylation.

DISCUSSION

The photophosphorylation process in the photosynthetic bacterium *R. rubrum*, and in chloroplasts isolated from spinach leaves appear to be coupled to changes in structure in membranes of these systems. These phenomena can be observed if the following conditions are met: (a) Examination of chloroplasts and cells for scattering changes are made at very low pigment concentrations so that the presence of pigments and/or their reactions will not mask or interfere with scattering measurements. (b) Establishing the necessary conditions for photophosphorylation; these conditions are relatively simple in *R. rubrum* chromatophores which carry out a cyclic type of photophosphorylation for which no cofactors are needed except phosphate acceptors, appropriate reducing conditions, and actinic light, as pointed out by HORIO AND KAMEN[14]. In chloroplasts, the requirements are slightly more exacting, cyclic photophosphorylation requires phosphate acceptors, appropriate reducing conditions, actinic light and an electron carrier; in non-cyclic phosphorylation the appropriate electron acceptor must be added in place of the electron carrier[8]. Establishing these conditions for photophosphorylation also provides the requirements for their associated structural changes as illustrated in Figs. 1 and 2 for bacterial cells and chloroplasts respectively. Examination of these photosynthetic systems under conditions which are unfavorable for the photophosphorylation process abolishes the associated structural changes. For example, merely removing the actinic light, or adding an inhibitor of the photosynthetic electron-transport process such as HOQNO or DCMU abolishes the structural change characteristically associated with photophosphorylation. Damage of the membrane systems, as by treatment in a sonic disintegration apparatus, or with a detergent like cetyl trimethyl ammonium bromide, also destroys the ability to manifest the red-light-induced structural changes (*cf.* RESULTS and ref. 4).

The remarkable similarity of structural changes in photosynthetic systems to those brought about in association with oxidative phosphorylation of mitochondria indicates that some basically similar mechanism may operate for mechano-chemical coupling in primary energy-transducing systems. The scheme illustrated in Fig. 3 is a suggested hypothesis for the relation of the biochemical processes of energy-transfer to changes in membrane structure. It is visualized that the various energy-transducing systems generate intermediates through electron transport, *i.e.* carrier $\sim X$, and $X \sim P$ in accordance with present theories of the mechanism of oxidative and photophosphorylation[15-17]. The energy of these intermediates can then be either conserved, *i.e.* synthesize ATP, or alternatively be used to drive an associated mechano-chemical change. This mechano-chemical change leads to shrinkage in mitochondria and to some as yet unspecified structural change* in the photosynthetic systems. Any condition which retards the formation of the intermediates such as inhibitors of electron transport, or which promotes hydrolysis of the intermediates

* A report on this subject is in preparation.

(heat production) such as uncoupling agents, favors a low shrinkage state in mitochondria and a changed structural state (measured at a lower scattering intensity) in photosynthetic systems. In previous reports[3,4] it has been observed that ATP is required to maintain the structural state associated with phosphorylation if the mitochondria or chloroplasts are damaged. In damaged membrane systems it may be difficult to maintain high concentrations of energy-linked intermediates because of their hydrolysis under these conditions, and it may be that the requirement of externally added ATP in these cases is to maintain levels of the intermediates sufficient to drive the associated mechano-chemical changes.

Finally one further point may be made concerning the ability of chromatophores to manifest scattering changes under conditions of cyclic photophosphorylation. Recent research on the ultrastructure of photosynthetic bacteria suggest that "chromatophores" do not really exist *in vivo*, but are part of the cell membrane and its infoldings. Isolated chromatophores must then be regarded as artifacts of the preparative procedures employed to break open the cells[18]. Viewed in this way, it may be tentatively assumed that the association of the photosynthetic mechanism with the membrane in the intact cell may be the minimum requirement for a coupled mechano-chemical change. This interesting question would appear to warrant further study.

ACKNOWLEDGEMENT

This investigation was supported by the National Science Foundation.

REFERENCES

1 L. PACKER, *J. Biol. Chem.*, 235 (1960) 242.
2 L. PACKER, in T. W. GOODWIN AND O. LINDBERG, *Biological Structure and Function*, Vol. II, Academic Press, London, 1961, p. 85.
3 L. PACKER, *Biochem. Biophys. Res. Commun.*, 9 (1962) 355.
4 L. PACKER, *Biochim. Biophys. Acta*, 75 (1963) 12.
5 L. PACKER, *J. Biol. Chem.*, 236 (1961) 214.
6 L. PACKER AND R. H. GOLDER, *J. Biol. Chem.*, 235 (1960) 1234.
7 R. B. PARK AND M. G. PON, *J. Mol. Biol.*, 3 (1961) 1.
8 F. R. WHATLEY AND D. I. ARNON, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. VI, in the press.
9 H. BALTSCHEFFSKY AND B. ARWIDSSON, *Biochim. Biophys. Acta*, 65 (1962) 425.
10 H. BALTSCHEFFSKY, *Biochim. Biophys. Acta*, 40 (1960) 1.
11 P. G. HEYTLER AND W. W. PRICHARD, *Biochem. Biophys. Res. Commun.*, 7 (1962) 272.
12 G. R. SCHONBAUM, L. PACKER AND C. R. BOVELL, *Arch. Biochem. Biophys.*, in the press.
13 OBAYASHI, *J. Gen. Appl. Microbiol. Tokyo*, 7 (1961) 233.
14 T. HORIO AND M. D. KAMEN, *Biochemistry*, 1 (1962) 144.
15 B. CHANCE, *J. Biol. Chem.*, 236 (1961) 1569.
16 A. L. LEHNINGER, *Federation Proc.*, 19 (1960) 952.
17 E. C. SLATER, *Australian J. Exptl. Biol. Med. Sci.*, 36 (1958) 51.
18 G. COHEN-BAZIRE AND R. KUNISAWA, *J. Cell. Biol.*, 16 (1963) 401.