PHOSPHORUS TURNOVER AND PHOTOSYNTHESIS[1]

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WITH ONE FIGURE

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The participation of phosphorus in biological oxidation-reduction reactions of the type found in glycolysis: ADP + PO_4H^- + 3-phosphoglyceraldehyde + DPN^+ = 3-phosphoglycerate$^-$ + $2H^+$ + DPNH + ATP, has suggested theories in which similar reactions are proposed for photosynthesis (LIPMANN **6**, RUBEN **8**, EMERSON, STAUFFER, and UMBREIT **2**). In these theories the reducing power of photosynthesis is utilized not only for reduction of carbon dioxide but also, by means of coupled oxidations, for the generation of high-energy phosphate bonds, or, in the last reference, directly for the generation of high-energy phosphate. Since in these theories acyl phosphate is formed from inorganic phosphate, the concepts are amenable to test without isolation of particular intermediates, by means of radioactive phosphorus. It would be expected that the rate of conversion of inorganic phosphate to organic phosphate would be greater in light than in the dark. We have investigated this possibility under a variety of conditions. The following experiments are reported:

a) WITH GRANA. The use of grana, which are capable of accomplishing the photochemical reduction of a variety of substrates, e.g. of quinones (WARBURG **9**, FAN *et al.* **3**, ARONOFF **1**), permits a test of the theory of EMERSON *et al.* It is to be expected that the direct utilization of light should result in the formation of organic phosphate.

b) WITH LEAVES. There is some evidence to indicate that the light reaction involving the fixed carbon dioxide occurs within the chloroplast (FRENKEL, **4**). This suggests that the chloroplasts, isolated from lighted and darkened halves of a leaf will show differences in rate of formation of organic phosphate.

c) WITH ALGAE. Diverse aspects were investigated; light vs. dark, the presence and absence of carbon dioxide and of oxygen.

Method

The P* obtained as carrier-free $HPO_4^=$ had a specific activity of approximately 35 $\mu c/\gamma$, the concentration of the P* unfortunately varying from sample to sample and commonly being as high as 10 γ/ml. Experimental total counts of P* were of the order of $10^6 \rightarrow 10^7$, involving aliquots of $0.1 \rightarrow 0.2$ ml. of the P* solution. The amount of absorption of P* into e.g. 100 c.mm. algae during a normal run of ca. 15 minutes is of the order of

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0.1 → 5% depending upon particular conditions. All experiments with algae were performed with the usual manometric equipment, P* being tipped in after equilibration. The determination of the activity in the algae was accomplished, after quantitative removal from the reaction vessels, by centrifugation (complete within 2.5′, 2°–3° C.), and washing twice with cold distilled water, and finally being made to volume 2, 5 or 10 ml. (depending on activity of P* or number of cells) with cold M/10 KH_2PO_4. An aliquot of this suspension was pipetted onto a counting plate, made by glueing filter paper onto an aluminum disc with rubber cement. (In preparation

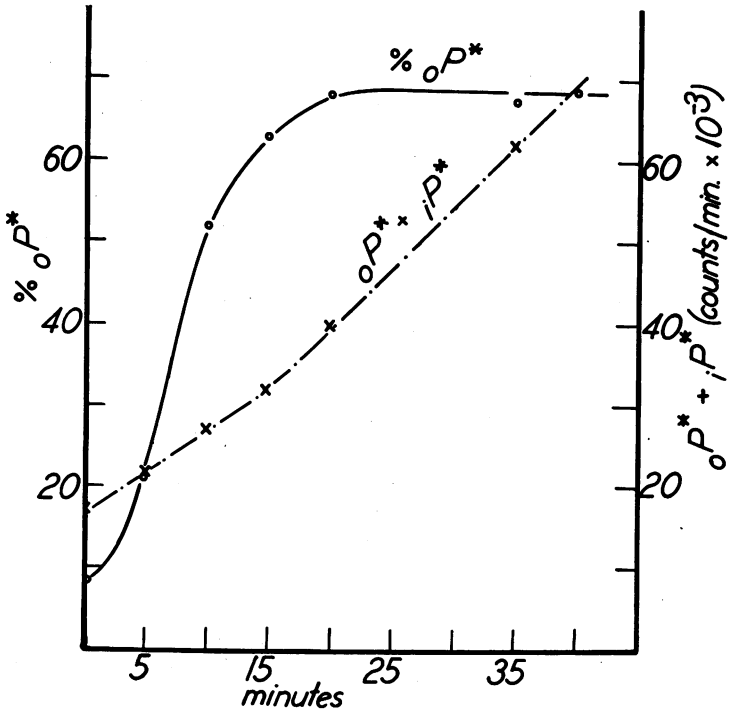

FIG. 1. The rate of conversion of inorganic to organic phosphorus by aerobic algae (for explanation see text).

the use of the rubber cement should not be minimized, and pressure should be applied during drying.) With our activities and cell numbers, counts on a plate usually were of the order of 100 → 1000/minute.

The method is based on the observation that two washings of cells with distilled water remove external radio-phosphate completely (less than 0.01%) and further washings do not remove additional phosphate from the cells. The suspension of the cells in M/10 $H_2PO_4^-$, for a period of 8–10 minutes, results in a semi-quantitative exchange between the relatively concentrated external inactive phosphate and the intracellular inorganic radio-phosphate. Different cultures appeared to require varying pH's, up to 6.5 for quantitative removal of $_iP^*$ (inorganic radio-phosphate). Centrifuga-

tion and resuspension of the cells in $H_2PO_4^-$ does not cause appreciable additional removal of phosphate. However, periods longer than the 8–10 minutes diminish the apparent organic P* ($_oP^*$) since there is continual interchange ($_oP^* \leftrightarrows {}_iP^*$) between external P ($_eP$) and $_iP^*$. The extension of the suspension period e.g. from 10′ → 30′ results in an apparent diminution of $_oP^*$ from 80% (at equilibrium, $_oP/{}_iP = 4$) → 65% (fig. 1). This is complicated by the $_iP \rightarrow {}_oP$ change, but the use of cold solutions diminishes the rate of these processes. Results within a particular experiment are believed to be valid to within 10%.

The total P* ($_tP^*$) having thus been obtained, the inorganic P* ($_iP^*$) may be determined from the supernatant after the suspension has equilibrated with the cold $H_2PO_4^-$ for ~ 10′. Determination of $_oP^*$ from the cells is preferable, however, since the "geometry" of the counting plate is more comparable to that for the total.

In either theory under consideration of the relation of phosphate to photosynthesis, an increased rate of conversion to organic acyl-phosphate compounds is expected. Were it not for limitations of permeability, one could measure the effect merely by determination of total P* uptake, since this would be increased under the influence of light. Actually, by far the slower of the two processes is that of the initial entrance of P into the cell. Considerations discussed below will show that although it is desirable to be aware of both rates, a) the rate of entry of external radio-phosphorus ($_eP^*$) into the cell, becoming internal inorganic radio-phosphorus ($_iP^*$), and b) the rate of conversion of $_iP^*$ to internal organic radio-phosphorus ($_oP^*$), the critical quantity is the rate of conversion of $_iP^*$ to $_oP^*$.

Experiments

Grana

Grana were prepared from spinach leaves, purchased locally, via a method similar to that used by Aronoff (**1**) for the chloroplasts, except for the further centrifugation in a high-speed centrifuge (20,000 g., 10′, T = 2.5° C.). Almost all of the P* was removed by washing with KH_2PO_4 ($_oP$-compounds are very soluble in the grana matrix). Thus, no conversion to $_oP$ had occurred, and the equality of P* uptake in both light and dark indicated no effect of light (table I). The theory of Emerson *et al.* (**2**) is thus not confirmed.

Leaves

Tobacco leaves, used for chloroplast preparation, were obtained from plants grown in a greenhouse in nutrient solution, during summer months. Chloroplasts were prepared by pressing leaves with a pestle in a mortar containing ice-cold M/20 $KHCO_3$, followed by differential centrifugation, and washing with distilled water.

An excised tobacco leaf was permitted to absorb P* in the dark, followed by an equal period of distilled water absorption, including three changes of

TABLE I

EFFECT OF LIGHT ON P* UPTAKE DURING PHOTOCHEMICAL REDUCTION BY GRANA*

SUBSTRATE	C/M P*	GAS EXCHANGES
xs quinone, light	3620	+ 86 cmm.
xs quinone, dark	3860	− 4 cmm.
No substrate, light	3660	+ 12 cmm.
No substrate, dark	3660	+ 6 cmm.

* For grana preparation, see text. Grana washed once and resuspended in distilled H_2O. Atmosphere, nitrogen.

water. Half the leaf was then illuminated, the stem being immersed in distilled water. From table II we note that the leaf punches show slightly more activity in the illuminated than in the dark side, but the chloroplasts, isolated from each half (2° C., as simultaneously as possible) possessed not only approximately equal activity, but they indicate no preferential conversion of ${}_1P^* \rightarrow {}_0P^*$.

If one accepts the percent of total volume occupied by the chloroplasts of a tobacco leaf as equal to a spinach leaf, i.e. 17% (MENCKE 7), then the specific activities of the chloroplasts were only ca. 1/10 that of the cytoplasm. If the leaf is half-illuminated directly while absorbing P*, a much higher P* ($\sim$ 2.5 times) is found on the illuminated than the dark side, although the ratio of chloroplast P*/total P* is equal for each half. This effect is presumably due to transpirational differences.

It is often stated, even among physiologists, that the use of algae is preferable to leaves, since they are more uniform, statistically, than different leaves. The comparison is not valid, since the chloroplasts within a single leaf are probably as uniform as the algae within a culture, and it is doubtful whether two algal cultures are more identical than opposite leaves. Furthermore, algae are complicated by a completeness and complexity of physiology not present within a more specialized organ such as a leaf. Thus, although a variety of experiments have been done with algae, differences in behavior of different cultures have made comparisons in experiments difficult, and the data offered are to be considered only as preliminary.

TABLE II

EFFECT OF LIGHT ON P* UPTAKE IN TOBACCO CHLOROPLASTS*

MATERIAL	LIGHT	DARK
Leaf	373.000	340,000
Total P* in chloroplasts	5,000	6,200
${}_0P^*$ in chloroplasts	570	620

* Leaf used ½ hour after cutting. Area = 300 cm.^{2} Stem immersed in 5 cc. distilled H_2O containing 9.0×10^6 "counts" P*. In dark 45′, after which P* solution is replaced with 20 cc. distilled H_2O, let stand 15′ and twice repeated. One half covered with Al foil. Leaf illuminated 30′ with 300 W reflector spot lamp. Illumination intensity at leaf ca. 10,000 lux. FS 311 infra-red absorbing filter between source and leaf.

TABLE III

EFFECT OF LIGHT ON P* UPTAKE BY ANAEROBIC ALGAE. 0.120 cc. ALGAE. $_eP^* = 0.6\gamma$, C/M $= 6.0 \times 10^6$. t = 23′. GAS PHASES $N_2 + 4\%$ CO_2. MEDIUM 0.1 M $KHCO_3$

	TOTAL P	$_oP^*$	% $_oP^*$
Light	101,000	38,000	38%
Dark	102,000	38,000	38%

ALGAE

The primary comparison to be made is that of light vs. dark. Furthermore, since respiration is known to involve considerable phosphorylation, while photosynthesis requires only minute amounts of oxygen, most experiments were anaerobic.

Chlorella vulgaris, grown in Emerson's solution over two 100 W white fluorescent lights (10 cm. from culture, fan circulation of air between lights) were generally 3–4-day-old cultures, which had been stored after growth at 2–3° C. until use. Because of variabilities in growth and duration of storage, the algae proved to be rather unpredictable both as to length of induction period for photosynthesis and, to a lesser extent, respiration. This variability appeared to affect both rate and extent of P* pickup so that experiments were directly comparable only if algae had been aliquoted from the same stock. Further work undoubtedly requires algae cultured and used under identical conditions. A simplified method for continuous culture has been developed and will be published shortly. A typical experiment testing the effect of light is summarized in table III. It is apparent that no effect of light is found.

Furthermore, even in the presence of oxygen, which could conceivably be specifically coupled with the phosphorylations, a short-run correlation could not be found, as is evident from table IV. Identical experiments with cultures in the presence of oxygen (or for protracted periods [60′] in its absence) indicated that 20 minutes was generally required for equilibration between $_iP$ and $_oP$ (i.e., in algae, $_iP/_oP = \frac{1}{4}$, so that when the % ratio is 20/80, complete equilibration has occurred and the continuation of the experiment beyond this time is of no further value (e.g. fig. 1).

It is, however, apparent from table III that the permeability is the limiting factor, since less than 2% of the original activity had entered the cell. To calculate the distribution between cells and medium at equilibrium,

TABLE IV

EFFECT OF LIGHT ON P* UPTAKE BY AEROBIC ALGAE. 0.100 cc. ALGAE. $_eP^* = 0.6\gamma$, C/M $= 3 \times 10^6$. t = 15′. GAS PHASES, AIR + 4% CO_2

	TOTAL P	$_oP^*$	% $_oP^*$
Light	16,300	8,200	50%
Dark	17,300	8,700	49%

assume that P enters only by exchange. Equilibrium will then occur when the specific activity of ${}_eP^* = {}_iP^* + {}_oP^*$. The average composition of algal P may be assumed to be $\sim 0.1\%$ of the wet weight, so that 120 mg. of algae contains 120 γ of P. The external radioactive solution contained 0.60 γ P, so that at equilibrium, the "count" in the external solution should be $\left(\frac{0.60}{0.60+120}\right)(6.0\times10^{-6}) = 5.0\times10^{-4}$, while the cells should contain (120/120.6) $(6.0\times10^{7}) = 6.0\times10^{7}$. It is obvious that the cells are far from equilibrium with the external solution and that the permeability is by far the slower rate.

There is a very definite effect of oxygen in the dark, as shown in table V. The data for ${}_oP^*$ appear to be somewhat high and indicate in this instance incomplete removal of ${}_iP^*$. It is to be noted that not only does the presence of oxygen accelerate the rate of conversion of ${}_iP^* \rightarrow {}_oP^*$, but also the permeability of the cell, i.e., ${}_eP \rightarrow {}_iP$. Although anaerobiosis does not always affect the rate of absorption of ions through cell membranes, there are numerous instances of this as reported by HOAGLAND (5), where it is believed to occur.

TABLE V

EFFECT OF OXYGEN ON P* UPTAKE BY ALGAE. 0.150 cc. ALGAE. ${}_eP^* = 6\gamma$, C/M = 10^6. t = 30′. GAS PHASES, $N_2 + 4\%$ CO_2; ARGON, AIR − CO_2

	TOTAL P	${}_oP$	% ${}_oP$
$N_2 + 4\%$ CO_2	23,900	11,000	46%
Argon	26,800	12,000	45%
Air − CO_2	142,000	127,000	89%

An attempt was therefore made to determine the relation between O_2 assimilation and P fixation. Duplicate flasks were used, one for the determination of respiratory pickup of P, another for fermentative, i.e., in the presence of argon. Respiratory rate was determined in a third vessel. Aliquots were made from the flask containing air + P* at 5′ intervals and ${}_oP^*$ determined. At the conclusion of the experiment, a similar analysis was made for the vessel containing argon.

One may calculate the relation between O_2 consumption + P conversion from the rate-formula given herewith. It has been shown earlier that the rate of entry of P* from the external medium into the cells is very much slower than its rate of conversion within the cell to organic P*. Therefore, neglecting ${}_eP^* \rightarrow {}_iP^*$, we may write the equation for the rate of formation of ${}_oP^*$ from ${}_iP^*$ as:

$$\frac{d\,{}_oP^*}{dt} = k\left(\frac{{}_iP^*}{{}_iP} - \frac{{}_oP^*}{{}_oP}\right) \qquad (k = \text{constant})$$

where, at t = o, ${}_iP^* = {}_oP^* = 0$; furthermore ${}_iP = {}_iP^* + {}_oP^*$ at all times. Ob-

viously, when the *specific activities* ($_iP^*/_iP$ and $_oP^*/_oP$) of the two fractions are equal, $d_oP^*/dt = 0$. The solution of the differential equation is

$$_oP^* = \frac{_oP_iP}{_oP + _iP}\left[1 - e^{-k\left(\frac{_oP + _iP}{_iP_oP}\right)t}\right]$$

which may be rewritten as

$$-1n\ (1 - u_oP^*) = ukt. \text{ where } u = \frac{_oP + _iP}{_oP_iP}$$

From the curve (fig. 1) it is seen that half of the conversion of $_iP^* \rightarrow _oP^*$ (i.e., $_oP^* = 40\%$) occurs at 7.5 minutes. Substitution in the above, after utilization of the relation, $_iP = _iP^* + _oP^*$, results in the evaluation of $k = 14.5\%/min.$

The 290 cmm. of cells are estimated to contain 290 γ P of which 80% or 232 γ, are believed to be organic (EMERSON *et al.* **2**). This is equivalent to $232/290 = 0.80$ γ $_oP$/cmm. cells, or 0.0258 micromoles/cmm. cells. The fraction formed is $14.5\% = 0.0038$ micromoles/min./cmm. cells. The rate of O_2 assimilation is 0.015 cmm. O_2/min./cmm. cells. This is equivalent to $0.015/22.4 = 0.0067$ micromoles/min./cmm. cells. The ratio of O_2/P is thus 67/38 or approximately one atom 0/three atoms P.

Discussion

It is evident from the experimental data presented that there is no direct connection between light and the gross formation of organic phosphorus compounds. Thus the scheme of Ruben-Lipmann is not applicable in its largest interpretation. There still remain two possible interpretations by which phosphorus might still play a direct part in photosynthesis. If an organic phosphorus fraction constituting less than 10% of the total organic phosphorus were involved in the photochemical interchange with inorganic phosphorus, the present method would not have been able to detect an acceleration of this interchange by light. The second possibility is that the light accomplishes transformation of organic phosphorus from one form to another without passing through the inorganic state.

Summary

Using radioactive phosphorus, no direct connection between *gross* formation of organic phosphorus compounds and photosynthesis or photochemical reductions has been found to occur.

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