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PHOSPHORUS AND PHOTOSYNTHESIS I. DIFFERENCES IN THE LIGHT AND DARK INCORPORATION OF RADIOPHOSPHATE

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Abstract

The distribution of radioactivity in metabolites of photosynthetic algae exposed to $KH_2P^{32}O_4$ in light and dark has been determined by paper chromatography and radioautography. The large relative increase of adenosine triphosphate in the dark and of 3-phosphoglyceric acid in the light is discussed in relation to the mechanism of phosphorylation of photosynthetic intermediates. Evidence is given indicating that adenosine triphosphate is the first isolable product formed from inorganic phosphate, and that uridine diphosphate glucose and adenosine diphosphate are active in phosphate metabolism.

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Since the majority of significant photosynthetic intermediates are phosphorylated,[2,3,4,5] one might expect that there would be differences in the steady-state distribution of phosphate among intermediate metabolites in green plants depending on whether they are photosynthesizing or merely respiring. Furthermore, there might be differences in the relative rates at which entering phosphate is distributed among these same intermediates. However, fractionation methods[6,7,8,9,10,11] that have heretofore been employed in such studies have not permitted much chemical characterization of the fractions. In addition, some of the procedures used, i.e., strong acid extractions,[6,7,8,10,11] produce irreversible chemical changes in the materials.

We have, therefore, initiated a series of investigations of short-time radiophosphate assimilations using paper chromatography to separate the intermediates, followed by radioautography in conjunction with co-chromatography to locate and identify them. This method permits the simultaneous analysis for a greater number of specific compounds with less chemical change than any technique previously employed.[12]

Experimental Procedure

Algae (Scenedesmus obliquus) were grown under controlled conditions,[13,14] centrifuged from the growth medium, and resuspended as a 1% by volume suspension in distilled water.[15] The experiments were carried out in the apparatus shown in Figure 1.

In the light experiments,[16] the vessel was illuminated by two General Electric photospots No. RSP2 placed at 32 cm. from the vessel, so as to give 11,000 foot candles on each side, as measured with a Weston Photocell.

Water-cooled glass infra-red absorbing filters were placed between the lamps and the vessel to prevent overheating of the latter. The temperature therein was controlled by a surrounding water jacket at 23° C. In the thirty-second experiments two General Electric Reflector Spots (300 watt) were substituted for the photospots, reducing the light intensity to 6,000 foot candles.

In the dark experiments the water jacket was filled with a black ink solution which effectively excluded all light from the vessel. The light intensity on the outside of the jacket was less than 7 foot candles.

In all light experiments the algae were preilluminated for thirty minutes with a stream of air bubbling through the suspension. In all dark experiments, the illumination was stopped after fifteen minutes and the suspension kept in the dark with air bubbling through for an additional fifteen minutes to stop all residual photosynthetic activity.[13] At the end of the adaptation period, the bubbler was removed and radiophosphate was rapidly added. The amount of carrier-free P^{32} added as KH_2PO_4 varied from 0.1 to 0.75 millicuries depending on the experiment. The air bubbler was then immediately reinserted in the vessel to aid in mixing.

Thirty-second experiments. - After the orthophosphate was added and the bubbler reinserted, the algae were allowed to remain in contact with the radiophosphate in light or dark for approximately 30 seconds. The suspensing medium was then rapidly filtered through a large, fine sintered glass filter funnel. The algae, which remained on the filter, were killed by the rapid addition of boiling absolute ethanol.[15] The time of the experiment was measured from the addition of the radiophosphate until the ethanol was added (± 1 sec.). The killing solution was filtered off and collected in a second

flask. 80% boiling ethanol was slowly added to the dead algae and filtered into the same flask. This was followed by a hot 20% ethanol extraction of the cell hulks, which were then discarded.

The combined extracts were concentrated under vacuo at room temperature to 2.0 ml. Aliquots of these concentrates were placed on Whatmann No. 1 filter paper,[3] washed in acetic or oxalic acids.[17] The papers were run two dimensionally in phenol saturated with water and butanol/propionic acid/water solvents (standard solvents). Radiograms were taken of the dried papers with Eastman Kodak "No-Screen" X-ray film,[3] and the tracer-containing compounds on the chromatogram located by placing the X-ray film over guide marks on the paper and counting the activity through the X-ray film. % based on total organic P^{32}-activity were calculated, duplicate papers giving values with an average deviation of ± 1.0. Absolute amounts of activity fixed were not used in this work.

In the longer term experiments the exogenous radiophosphate was filtered from the cells through a celite filter pad, which was thereafter washed with 100 ml. of water to aid the removal of the exogenous radiophosphate from the algae.[6] Aliquots of the combined extracts, obtained as above, and of the resuspended cell hulks were counted on aluminum plates.[13] In all counting a thin mica-windowed, helium-filled, Scott Geiger-Mueller tube was used.[13]

In the one-hour experiments, the algae were exposed for sixty minutes in the light with a relatively small amount of radiophosphate (approximately 0.25 millicuries) at which time a sample was taken. The light was then turned off and after an additional fifteen minutes in the dark, another sample was removed. In a separate experiment the light and dark conditions were reversed.

Identifications: Spots which were suspected of being sugar monophosphates (HMP), 3-phosphoglyceric acid (PGA), and orthophosphate (OP), from their R_f

values in the standard solvents[3,4,16] were cut out, eluted, and rerun separately in a solvent consisting of 2 gm. picric acid, 80 ml. t-butanol, 20 ml. water (picric acid solvent[17]), one-dimensionally with 100 micrograms of authentic carriers of OP, PGA, fructose-6-phosphate (F-6-P), and glucose-6-phosphate (G-6-P). After radioautography the paper was sprayed for phosphate and the characteristic blue color developed with an ultraviolet light and intensified with H_2S.[17,18] Exact coincidence of location and shape of the radioactive spot and the phosphate spot was considered necessary and sufficient proof of identity.

The nucleotide areas were likewise eluted and rechromatographed with carrier adenosine triphosphate (ATP), adenosine-5'-phosphate (AMP), uridine diphosphate glucose (UDPG),[19] and uridine diphosphate (UDP)[19] in 7.5 vol. 95% ethanol and 3 vol. molar ammonium acetate solvent.[20] Samples were also subjected to seven-minute hydrolysis in N HCl at 100° C. and rerun in the standard solvents with carrier AMP, uridine-5'-phosphate (UMP),[19] and ribose-5'-phosphate. In the case of ATP, AMP, UDPG, UDP and UMP exact coincidence of radioactivity with ultraviolet absorptive spots of the carrier, observed by viewing the absorption of ultraviolet light by the chromatogram, was taken as proof of identity.[21]

Phosphodihydroxyacetone (PDHA) was identified by cochromatography with the corresponding area from a carbon-labeled chromatogram. The resulting single spot containing both P^{32} and C^{14} activity was phosphatased and rechromatographed, the carbon activity corresponding to carrier dihydroxyacetone while the phosphorus went to OP. Phosphoenolpyruvic acid was identified solely by its R_f value in the standard solvents.[3]

In the exchange experiments,[22,23] 5×10^{-10} moles of carrier-free radiophosphate were added to 500 micrograms of authentic samples of PGA, G-6-P, F-6-P, AMP, ATP, phosphoglycol, and glycerol-phosphate, in a total volume of 0.5 ml. The initial pH of the solutions was approximately 3 and the solutions were kept at room temperature for approximately 312 hours, samples being drawn out and chromatographed in the picric acid solvent at appropriate intervals. ATP and glycerol-phosphate seem to have hydrolyzed under these conditions.

Results

Compounds isolated: The radioactivity on the chromatograms (Figure 2) was separated into at least 10 distinct areas, consisting of OP, PGA, HMP, sugar diphosphates (DIP), three nucleotide areas, and AMP, PDHA, and PEPA, the latter three appearing in only small amounts.

In the short time experiments, nucleotide area I was shown to consist of nearly 100% ATP, while nucleotide area II consisted principally of UDPG.[4] Hydrolysis shows most of the activity in the two terminal phosphate groups of ATP and in the phosphate adjacent to the glucose of UDPG.

Nucleotide area III gave AMP and ribose-5'-phosphate upon hydrolysis and exhibited R_f values intermediate between ATP and AMP. It is therefore considered to be adenosine-diphosphate (ADP).[24]

The HMP area was shown to contain F-6-P, G-6-P, G-1-P and an unidentified spot which had the chromatographic characteristics of either mannose-6-phosphate (M-6-P) or sedoheptulose phosphate (SP).[4,15]

There is evidence of labile combinations of both OP and PGA which partially decompose in the butanol/propionic/water direction, giving rise to a spot of OP and of PGA distinctly separated from the normal OP and PGA. A reasonable explanation is that this phenomenon is caused by the presence of

1,3-diphosphoglyceric acid (DIPGA), but at present, we are unable to rule out all other possible interpretations.

Total incorporation, soluble and insoluble fractions: As it was necessary to use carrier-free radiophosphate to obtain measurable amounts of radioactivity in the various intermediates, unavoidable isotopic dilution at such concentrations ($\sim 10^{-5}$ M) with inactive phosphate was highly probable. Under these conditions, total incorporation rates cannot be significant.

An appreciable amount of activity (20-40% of the total activity fixed) remained in the cells after extraction with ethanol and water.[6,11] Work is in progress to fractionate these insolubles which presumably contain metaphosphates, phospholipids, phosphoproteins, and nucleic acids.[6,11] Our lack of information about this fraction may cast some doubt on the validity of a kinetic study. However, since many of the intermediates previously shown to be important in metabolism are soluble under the conditions used,[3,4,25] an investigation of the relative rates of incorporation of phosphate into these compounds is of considerable interest.

Kinetics

Thirty-second experiments (Series IV): The purpose of this series was to determine the first isolable intermediate in phosphate metabolism.[6] If there is but one mechanism for the uptake of phosphate, the first compound formed should contain 100% of the incorporated radiophosphate as the exposure time approaches zero. The results of the short time experiments are given in Table I.

Table I

Compound	% of total activity in organic compounds	
	37 sec. dark	29 sec. light
PGA	2.6	17.1
HMP area	5.4	18.5
ADP	8.8	15.1
DIP area	11.4	39.2
ATP	72.0	9.8

Because of the relatively large amount of radioactive OP on the chromatograms of this series, the percentages given are not as accurate as in the longer term experiments, and in particular the DIP area is likely to be abnormally high because of streaking of the large amount of radioactive OP present. However, we may conclude that in the dark ATP seems to be the first isolable compound,[26] whereas in the light no single intermediate assumes such prominence, with the possible exception of some very labile compound,[27] i.e. DIPGA, etc. The more even distribution of activity among the fractions in the light indicates a more rapid turnover rate than in the dark,[10,11] in agreement with previous observations. It is of interest to note that in thirty seconds all of the intermediates observed in one-hour exposures are present, necessitating even shorter exposure times to differentiate among them with regard to their order of appearance.

One to twenty-five minute experiments (Series I and II): These experiments differed from the above in that exogenous phosphate was washed from the cells before killing, whereas in the thirty second experiments, time did not permit this operation. The resulting decrease in radioactive OP in these

series gave more accurate values for activity in the organic compounds. The results are given in Figures 3-5.

In the dark, ATP continues to show the characteristics of a primary product, while PGA is the most slowly labeled reservoir. In photosynthesizing algae, however, PGA becomes labeled very rapidly, while ATP is not as prominent as in the dark.[28] After a couple of minutes, with the exception of the above compounds, most of the intermediates are essentially the same in light and dark. This does not indicate that the steady state reservoir concentrations of these intermediates are identical in both light and dark since we have no specific activity data. No effort was made to obtain such data because of the possibility of multiple reservoirs of the same compound located at different sites within the cell and temporally separated from each other.[12,15]

In connection with the HMP area it might be pointed out that the ratio of activity in the three major components is the same within counting errors for all of the six points (Series I) in light and dark, as shown in Table II. These percentages indicate that there is either a very rapid equilibrium between the hexose phosphates in the plant or that phosphate is being incorporated into the monophosphate reservoirs at proportional rates, which if so would rule out their consecutive formation. The agreement between our values and the equilibrium values[29] suggests that the former mechanism is operating and in addition that the unknown spot is mostly mannose-6-phosphate. Ion exchange studies give evidence for the presence of G-1-P (approximately 6%) in this HMP area.[30]

Table II

Fractionation of HMP area
% of HMP activity

Compound	Dark 2 min.	Dark 11 min.	Dark 26 min.	Light 2 min.	Light 11 min.	Light 26 min.	Slein[29]
Glucose-6-P	59.	59.	59.	60.	59.5	56.	57.4
Fructose-6-P	18	19.5	21.	19.	21.	20.5	25.5
Mannose-6 or Sedoheptulose-P	23.	21.	20.5	20.	19.5	23.5	(17.1)*

(*) Mannose-6-P only.

One-hour experiments (Series III): In this series algae were exposed to less radiophosphate for longer periods. Since the activity fixed was the same in seventy-five minutes as in sixty minutes, it was evident that incorporation had ceased at least by one hour and that the amount of radiophosphate within the cell was time-invariant. Presumably, the specific activity of all actively participating reservoirs is the same by this time, and the % values (Table III) for the series are proportional to reservoir sizes.

Table III

Relative Reservoir Sizes

	60 min. light	60 min. light + 15 min. dark	60 min. dark	60 min. dark + 15 min. light
PGA	32.9	30.0	29.4	39.0
HMP	7.2	5.8	6.5	7.0
ATP	17.6	29.7	27.5	25.3
UDPG	14.0	10.8	6.9	9.9
DIP	19.2	13.5	16.7	9.3
ADP	9.1	10.2	7.0	9.6

Exchange experiments: A question might be raised as to whether the observed distribution of activity corresponds to that existing within the organism immediately preceding the killing.[6,11] Certainly, highly labile phosphates such as acyl phosphates would never be observed on paper[31,32] except insofar as they might be a source of OP. The observed activity in OP (approximately 30% of the total on the chromatogram) cannot be significant since the intracellular OP is combined with a variable amount of exogenous and adsorbed OP,[6] and at present it is impossible to differentiate among them.

To test the possibility of non-enzymatic exchange between radioactive OP and the inactive organic phosphates, small amounts of radiophosphate were incubated with relatively large amounts of selected organic phosphates, present in algal extracts and stable to chromatography.

Table IV

Non-enzymatic exchange in two weeks (312 hours)

Compounds	Activity org. P/ Activity OP	Concentration org. P/ Concentration OP	Fraction isotopic equilibration
PGA	$< 1.6 \times 10^{-3}$	5.4×10^{3}	$< 3.0 \times 10^{-5}$
G-6-P	$< 1.3 \times 10^{-3}$	3.8×10^{3}	$< 3.5 \times 10^{-5}$
P-glycol	$< 1.5 \times 10^{-3}$	7.0×10^{3}	$< 2.0 \times 10^{-5}$
F-6-P	$< 1.3 \times 10^{-3}$	3.8×10^{3}	$< 3.5 \times 10^{-5}$
AMP	$< 3.0 \times 10^{-3}$	2.8×10^{3}	$< 1.1 \times 10^{-5}$

ATP (unstable under conditions used)

From Table IV, it is evident that no appreciable exchange of this type has occurred.

Another exchange experiment involved the addition of radiophosphate to non-radioactive algal extract within one second after the killing operation. Chromatography showed but one spot, which was OP. In addition, the possibility of slow exchange among the organic phosphates not involving OP was checked, by rechromatographing identical aliquots of an extract one month after the experiment (the algal extracts were kept frozen during the period) and redetermining the distribution of radioactivity. The differences observed were not greater than those observed between identical samples chromatographed at the same time, ruling out this type of exchange. The possibility of very rapidly equilibrating exchange of this type still remains. Enzymatic non-metabolic exchange prior to or during the killing operation could not be checked.

Discussion

An analysis of the information which can be obtained from percentage appearance curves is a prime requisite to sound interpretation of the results. The first piece of information is that in the long-term series the percentage values are proportional to reservoir sizes. Additional information may be deduced from the shapes of the curves in shorter times. These curves may be misleading if they are confused with total activity appearance curves. The behavior of the slope of each curve is indicative of the position and function of the compound in a metabolic pattern.

A zero slope for a particular compound indicates that the activity is being incorporated into the reservoir at a rate proportional to the total organic incorporation rate (TOIR). This does not mean that the total activity in the reservoir is time invariant. If there is more than one path or mechanism for the entrance of ortho phosphate into metabolism, the rates of entrance into

this path must be proportional to TOIR. A general way in which this may arise is the following: The reservoir is in rapid equilibrium with all intermediates between it and the entrance point, and the entrance is the rate determining step. Furthermore, the specific activity of the reservoir must be low compared with that of the ortho phosphate in order that essentially all the radioactivity which enters the reservoir remains in it.

A positive slope for a compound indicates that the rate of incorporation into the reservoir is accelerating with respect to the TOIR. In general there must be large reservoirs between it and entering phosphate through which the latter must pass in at least one relatively slow step.

A negative slope indicates that the rate of incorporation into the reservoir is decelerating with respect to the TOIR. This does not mean that the total activity in the reservoir is decreasing with time. The same general considerations apply as for a zero slope, with the difference that the specific activity of the reservoir is appreciable compared with the ortho phosphate.

The fact that PGA becomes labeled more rapidly in the light than in the dark strongly suggests that there is a close relationship between ortho phosphate incorporation and photosynthesis.[10] In addition, the fact that the ATP percentage is lower in the light than in the dark further suggests that the incorporation of phosphate into photosynthetic intermediates probably proceeds via ATP.[7,28,33] In contrast to ATP and PGA the distribution of radioactivity among the other intermediates seems unaffected by light and dark.

The rapid labeling of UDPG shows that it is actively participating in phosphate metabolism in green plants. Among the postulated functions of UDPG is its role as a co-factor in the interconversion of glucose and galactose.[4,34,35] The fact that the hexose monophosphates appear to be in rapid equilibrium,

together with the fact that that UDPG is simultaneously labeled with them, suggests that it might be involved in other hexose interconversions, namely among glucose, fructose, and mannose, as well as in the synthesis of polysaccharides.[4] This conclusion is supported by the fact that a mannose-containing nucleotide has been found in this area.[4]

The rapid labeling of ADP indicates that it, too, is actively participating in phosphate metabolism. There are very few metabolic reactions in which the terminal phosphate of ADP is utilized as an energy donor. However,[12,36,37] ATP may react in such a way as to give AMP and pyrophosphate, and the stepwise regeneration of ATP from the AMP and ortho phosphate would yield ADP labeled in the terminal phosphate.

It is of interest to relate the results with the known elements of respiration and photosynthesis.[4] Figure 6 represents a modified respiratory and a photosynthetic scheme into which some of our previous suggestions have been incorporated. Thus in the dark a phosphorylase reaction originating with sucrose and polysaccharides would bring label into the HMP group of compounds, including UDPG, which are in rapid reversible equilibrium with each other, as evidenced by the zero slope for the percent appearance curves.

The very rapid labeling of ATP in the dark might be brought about through the oxidative phosphorylating reaction of 3-phosphoglyceraldehyde, accounting for the possible early appearance of DIPGA labeled first in the number one phosphate. There are, of course, other routes by which ortho phosphate may appear in ATP. The negative slope of the percentage appearance curve may be taken to indicate that this reservoir is saturated more rapidly than any other so far observed by these methods.

The labeling of the phosphate of PGA in the dark occurs at an accelerating rate as evidenced by the positive slope of the percentage curve, and this

is accounted for in such a scheme by the requirement of the prior labeling of ATP and fructose 1,6-diphosphate, followed by a slow step.

In the light, the negative slope of ATP again indicates its early saturation, but its lower level suggests that the labeled high energy phosphates so produced are in greater demand for their function in CO_2 fixation in photosynthesis,[27,28,38,39,40] leading to the production of PGA. This would account for the rapid labeling of PGA in the light.

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Captions to Figures

Fig. 1 - Experimental apparatus for exposing algae to radiophosphate.

Fig. 2 - Radiogram of chromatogram of 10 min. dark exposure of Scenedesmus to radiophosphate. Abbreviations used are as follows: OP, ortho phosphate; PGA, 3-phosphoglyceric acid; HMP, hexose monophosphate area; DIP, sugar diphosphates; Nucleotide I, predominantly adenosine triphosphate; Nucleotide II, predominantly uridine diphosphate glucose; Nucleotide III, adenosine diphosphate; AMP, adenosine monophosphate; PDHA, phosphodihydroxy acetone; PEPA, phosphoenolpyruvate.

Fig. 3 - % of adenosine triphosphate as a function of time. O, in light; ● in dark. Ordinate is the % of total activity in soluble organic compounds. Data is from series II.

Fig. 4 - % of 3-phosphoglyceric acid as a function of time. O, in light; ●, in dark. Ordinate is the % of total activity in soluble organic compounds. Data is from series II.

Fig. 5 - a, % of adenosine monophosphate; b, % of uridine diphosphate glucose; c, % of hexose monophosphates; d, % of sugar diphosphates as function of time. Ordinate is the % of total activity in soluble organic compounds. Data is from series II.

Fig. 6 - Schematic representation of the relationship between ortho phosphate and certain organic phosphates. Double lines indicate phosphate transfer; dotted lines, a change in the carbon skeleton; single lines, other types of transformation. "TCA" denotes the tricarboxylic acid cycle and "Light Reaction" indicates methods of converting ortho phosphate to high energy phosphate in the form of ATP by means dependent upon photo-chemical reactions and not involving the reduction of CO_2 followed by reoxidation of the products.

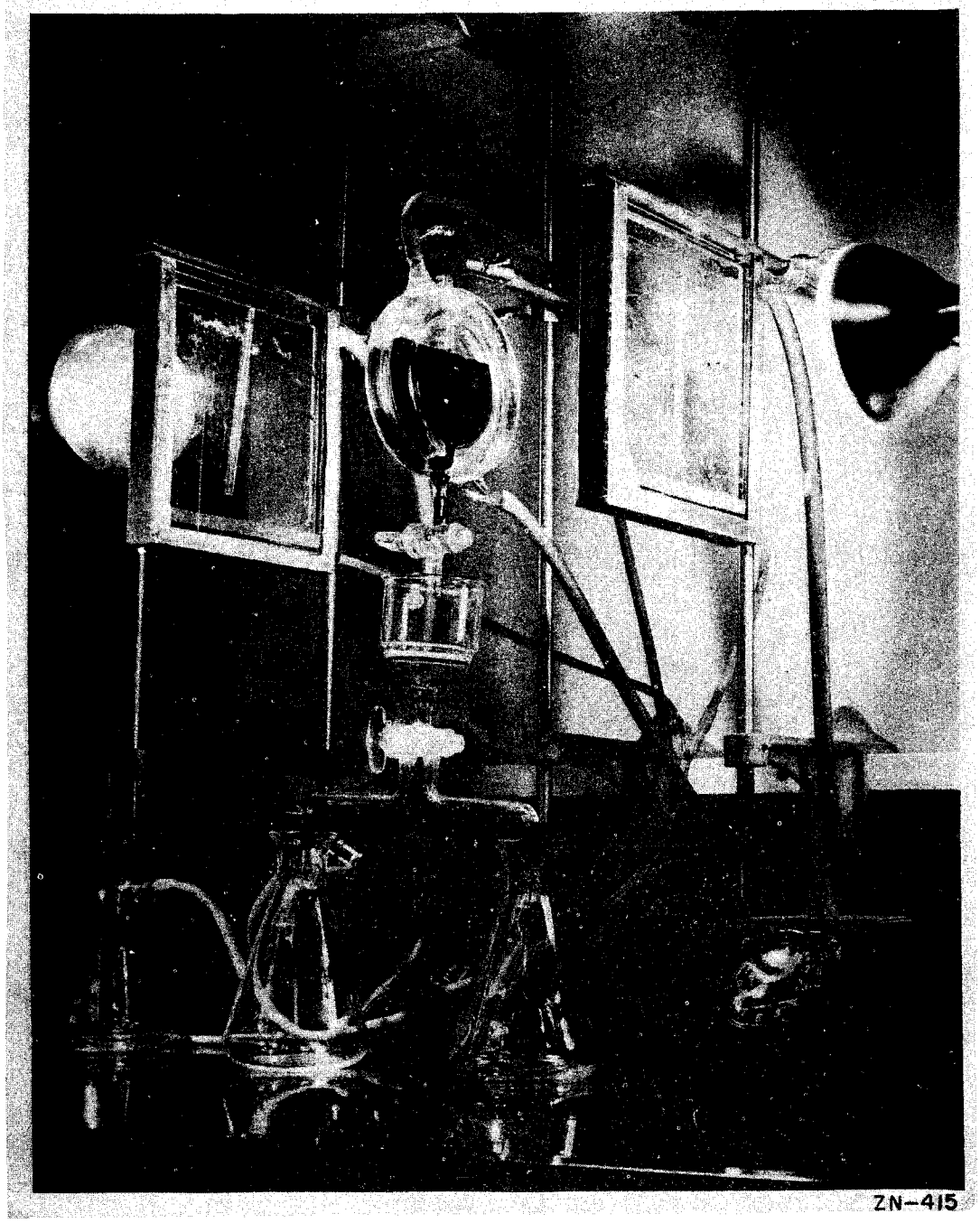

Fig. 1

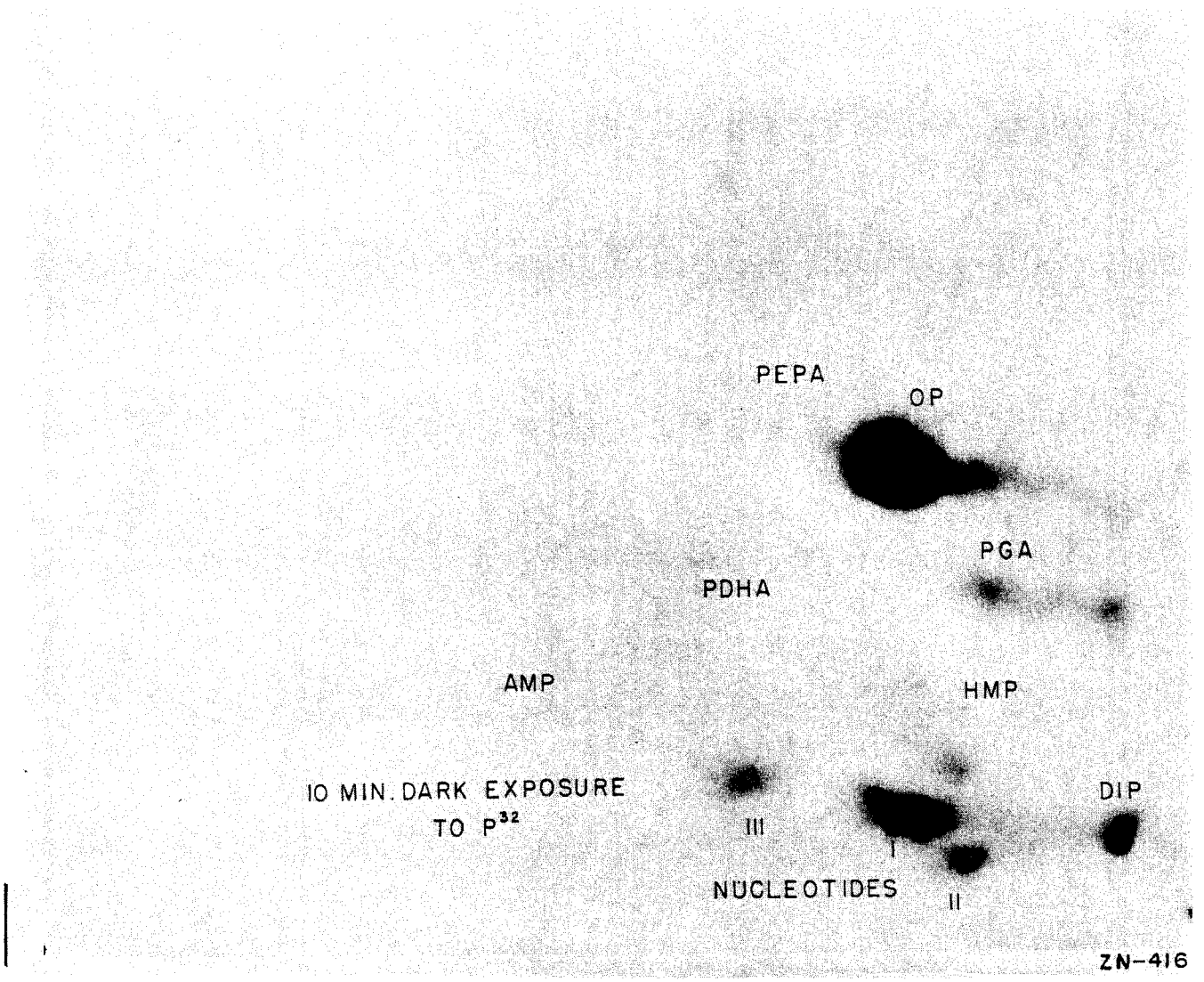
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Fig. 2

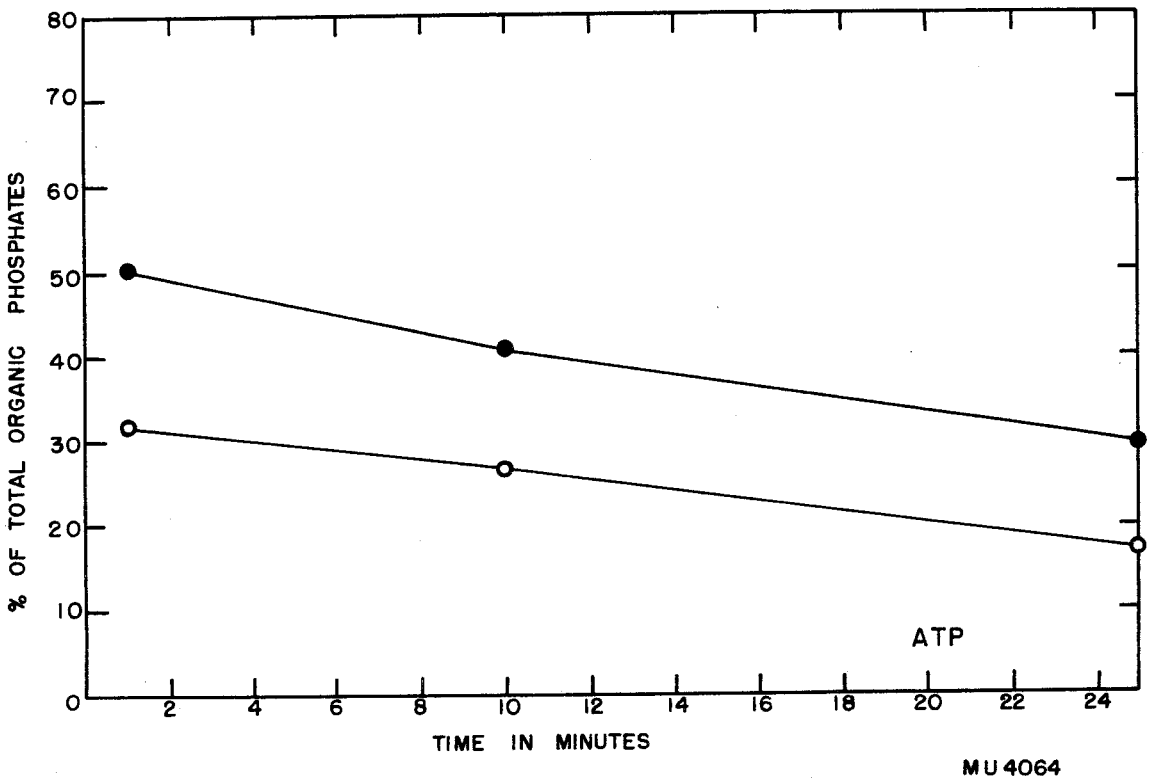
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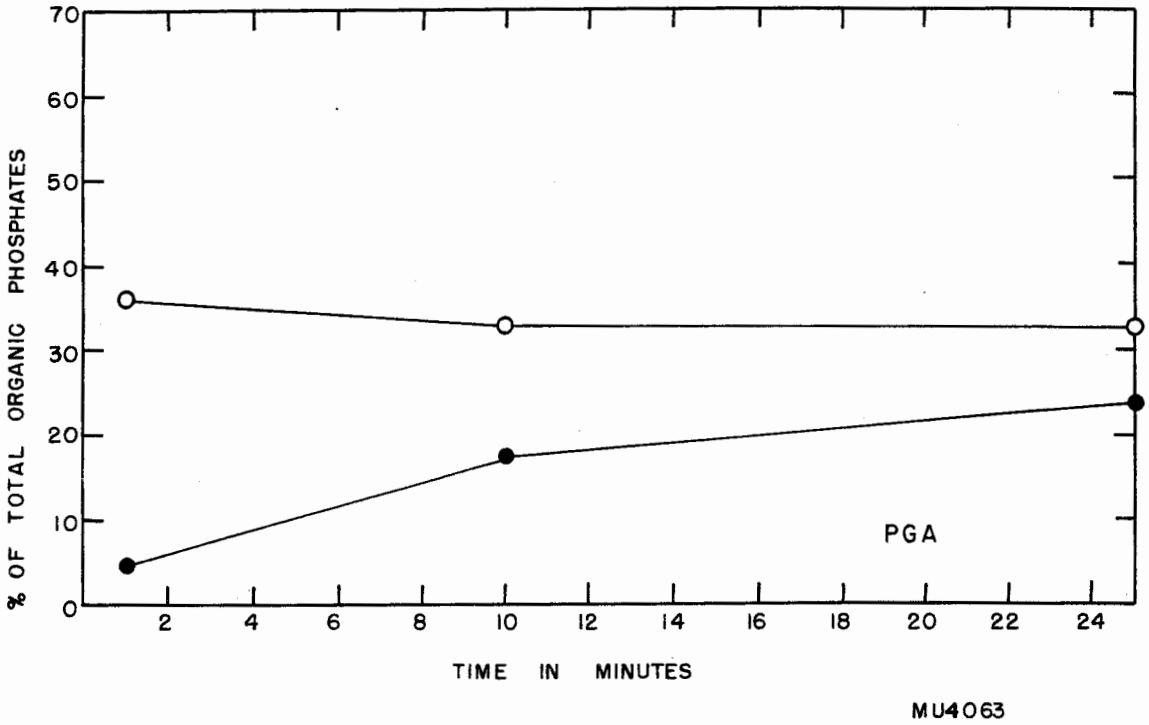
Fig. 3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MU4063

Fig. 4

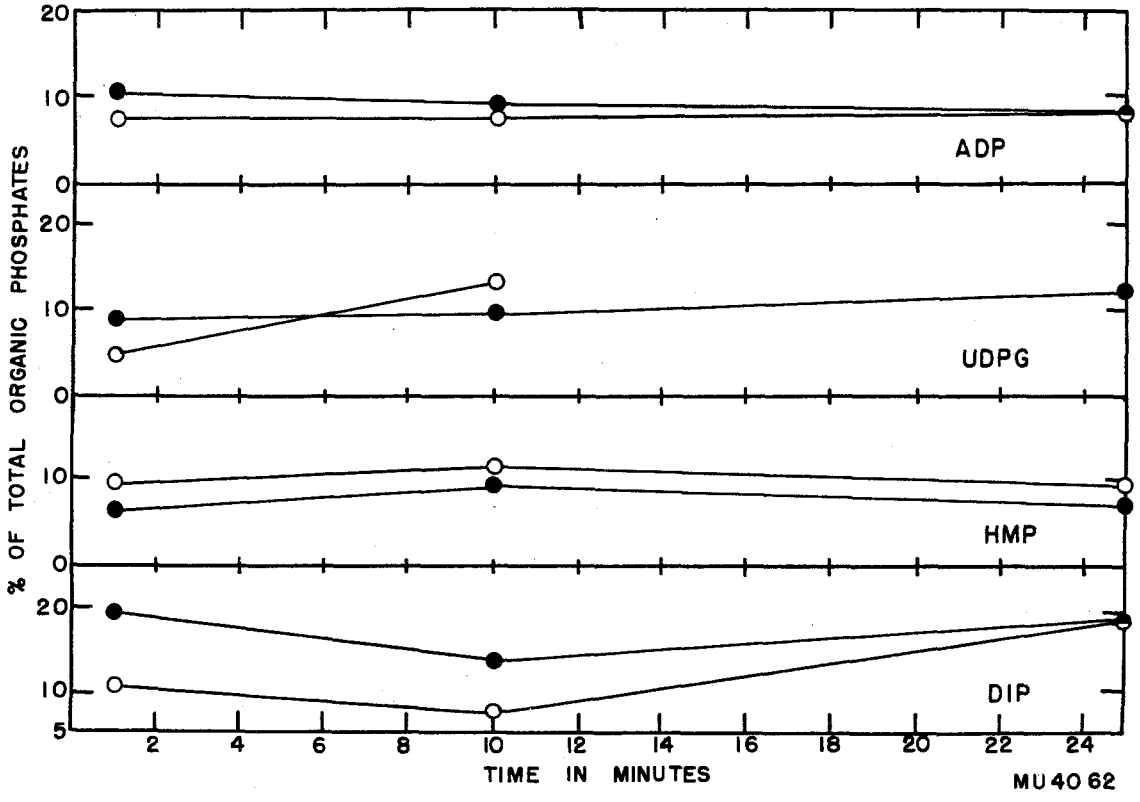
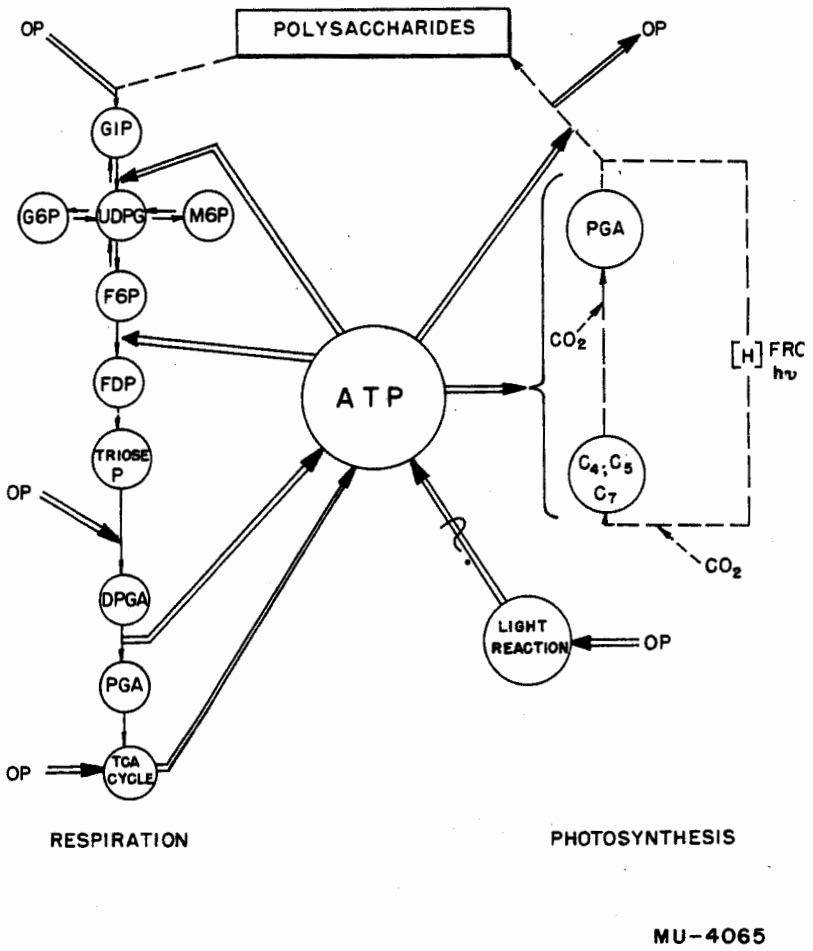

% OF TOTAL ORGANIC PHOSPHATES
20
10
0
ADP
UDPG
HMP
DIP
5
2
4
6
8
10
12
14
16
18
20
22
24
TIME IN MINUTES
MU 40 62

Fig. 5


POLYSACCHARIDES
OP
OP
GIP
G6P
UDPG
M6P
F6P
FDP
TRIOSE P
OP
DPGA
PGA
OP
TCA CYCLE
ATP
PGA
CO_2
C_4; C_5 C_7
[H] FRC hν
CO_2
LIGHT REACTION
OP
RESPIRATION
PHOTOSYNTHESIS

MU-4065

Fig. 6