Johnsson and Högsberg.[11] However, Geschwind, Reinhardt and Li[12] do not accept this conclusion. It has been observed here that, although quantitative values are not available, highly purified corticotropin-B caused melanophore expansion in excised frog skin, even at low concentrations where crude corticotropin was ineffective. The details of these studies will be reported elsewhere.

The recent preparations here[1] and elsewhere[13,14] of unhydrolyzed corticotropin concentrates many times more active than the protein described in 1943 as the adrenocorticotropic hormone[15,16] have inevitably raised questions as to the nature of the reportedly pure protein preparation. The ease with which corticotropin can be removed from the "protein" can be interpreted by a hypothesis that the "protein" consists of an aggregate of proteinaceous units having a more or less characteristic composition, but still capable of easy separation into the component parts as, for example upon dialysis, ultra-filtration, acetic acid extraction or adsorption on oxycellulose or resin. A second interpretation is that the "protein" preparation considered homogeneous actually contained 1% or less of the active principle as a trace constituent, an amount impossible to detect in material of this nature by the physical criteria which were used.

Experimental

Acid Hydrolysis Prior to Analytical Separation of Amino Acids.—Samples weighing 8 to 10 mg. were dissolved in about 0.05 ml. of double-distilled water, followed by the addition of twice redistilled constant boiling hydrochloric acid. The mixtures were boiled under reflux for 18 hours in an atmosphere of nitrogen. Excess hydrochloric acid was removed by repeated evaporation *in vacuo* under nitrogen, and the residues finally dried *in vacuo*. The dried residues were taken up in the buffer of *p*H 3.42 described by Moore and Stein[2] containing thiodiglycol and Versene. One-half ml. aliquot portions containing the equivalent of between 2.0 and 2.5 mg. of unhydrolyzed material were used for chromatography. Total nitrogen in the hydrolyzates was determined by a modification of the Nessler reaction.[17]

Performic Acid Oxidation of Corticotropin-B.—A performic acid solution was prepared by adding 0.5 ml. of 30% hydrogen peroxide solution to 4.5 ml. of 88% formic acid and allowing the mixture to stand at room temperature for 25 minutes. A 10-mg. quantity of corticotropin-B was dissolved in 5 ml. of this solution and the reaction mixture was kept at −5° for 45 minutes. It was then diluted with 20 ml. of water and lyophilized. The solution in water and lyophilization were repeated. The product, which had a bioactivity of less than 25 u./mg. was hydrolyzed with acid as described above. Only a trace of cysteic acid was found in the hydrolyzate.

Acknowledgment.—The authors are indebted to Mr. R. N. Boos and his associates for the microanalyses, to Dr. N. R. Trenner and co-workers for the infrared spectra and to Mr. Arthur Rosenberg for the ultracentrifuge determinations. They wish to thank Dr. C. A. Winter of the Merck Institute for Therapeutic Research for his studies on the adrenal weight and melanophore-expanding properties of corticotropin-B. The assistance of Messrs. D. E. Ayer and M. A. P. Meisinger in the preparation of samples of corticotropin-B for this work is gratefully acknowledged.

(11) S. Johnsson and B. Högsberg, *Nature*, **169**, 286 (1952).
(12) I. I. Geschwind, W. D. Reinhardt and C. H. Li, *ibid.*, **169**, 1061 (1952).
(13) H. B. F. Dixon, S. Moore, M. P. Stack-Dunne and F. G. Young, *ibid.*, **168**, 1044 (1951).
(14) E. B. Astwood, M. S. Raben, R. W. Payne and A. B. Grady, THIS JOURNAL, **73**, 2969 (1951).
(15) C. H. Li, H. M. Evans and M. E. Simpson, *J. Biol. Chem.*, **149**, 413 (1943).
(16) G. Sayers, A. White and C. N. H. Long, *ibid.*, **149**, 425 (1943).
(17) W. W. Umbreit, K. H. Burris and S. F. Stauffer, "Manometric Techniques and Related Methods for the Study of Tissue Metabolism," Burgess Publishing Co., Minneapolis, Minn., 1945, p. 103.

RAHWAY, NEW JERSEY

[CONTRIBUTION FROM THE RADIATION LABORATORY AND DEPARTMENT OF CHEMISTRY, UNIVERSITY OF CALIFORNIA]

Phosphorus and Photosynthesis. I. Differences in the Light and Dark Incorporation of Radiophosphate[1]

BY M. GOODMAN, D. F. BRADLEY AND M. CALVIN

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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.

Since the majority of significant photosynthetic intermediates are phosphorylated,[2-5] 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[6-11] that have heretofore been employed in such studies have not per-

(1) The work described in this paper was sponsored by the U. S Atomic Energy Commission.
(2) M. Calvin and A. A. Benson, *Science*, **107**, 476 (1948).
(3) A. A. Benson, J. A. Bassham, M. Calvin, T. C. Goodale, V. A. Haas and W. Stepka, THIS JOURNAL, **72**, 1710 (1950).
(4) J. G. Buchanan, *et al.*, "Phosphorus Metabolism," Vol. II, Johns Hopkins Press, Baltimore, Md., 1952.
(5) E. W. Fager, J. L. Rosenberg and H. Gaffron, *Federation Proc.*, **9**, 535 (1950).
(6) M. D. Kamen and S. Spiegelman, *Cold Spring Harbor Symposia on Quantitative Biology*, **13**, 151 (1948).
(7) R. L. Emerson, J. F. Stauffer and W. W. Umbreit, *Amer. J. Bot.*, **31**, 107 (1944).
(8) W. W. Umbreit, R. H. Burris and J. F. Stauffer, "Manometric Techniques," Burgess Publishing Co., Minneapolis, Minn., 1945, Chap. XV.
(9) S. Aronoff and M. Calvin, *Plant Physiology*, **23**, 351 (1948).
(10) H. Gest and M. D. Kamen, *J. Biol. Chem.*, **176**, 299 (1948).
(11) W. Simonis and K. H. Grube, *Z. Nat.*, **7b**, 194 (1952).

mitted much chemical characterization of the fractions. In addition, some of the procedures used, *i.e.*, strong acid extractions,[6-8,10,11] produce irreversible chemical changes in the materials.

We have, therefore, initiated a series of investigations of short-time radiophosphate assimilation using paper chromatography to separate the intermediates, followed by radioautography in conjunction with cochromatography 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 in a 1% by volume suspension in distilled water.[15] The experiments were carried out in a water-jacketed circular illumination vessel with plane sides 1 cm. apart.[16]

In the light experiments,[16] the vessel was illuminated by two General Electric photospots No. RSP2 placed 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 infrared 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°. In the 30-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 30 minutes with a stream of air bubbling through the suspension. In all dark experiments, the illumination was stopped after 15 minutes and the suspension kept in the dark with air bubbling through for an additional 15 minutes to stop all residual photosynthetic activity.[13] At the end of the adaptation period, the bubbler was removed and radiophosphate was added rapidly. The amount of carrier-free P^{32} added as KH_2PO_4 varied from 0.1 to 0.75 millicurie 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 suspending 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. Eighty per cent. 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 *in vacuo* at room temperature to 2.0 ml. Aliquots of these concentrates were placed on Whatman 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. Percentages based on total organic P^{32}-activity appearing on the chromatograms 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 before killing 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 and celite were counted on aluminum plates.[18] In all counting a thin mica-windowed, helium-filled, Scott Geiger–Mueller tube was used.[18]

In the one-hour experiments, the algae were exposed for 60 minutes in the light with a relatively small amount of radiophosphate (approximately 0.25 millicurie) at which time a sample was taken. The light was then turned off and after an additional 15 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 g. of picric acid, 80 ml. of *t*-butanol, 20 ml. of 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. of 95% ethanol and 3 vol. of molar ammonium acetate solvent.[20] Samples were also subjected to seven-minute hydrolysis in *N* HCl at 100° 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 treated with a commercial phosphatase preparation (General Biochemicals "Phosphatase") 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} mole of carrier-free radiophosphate was added to 500 microgr ms o authentic samples of PGA, G-6-P, F-6-P, AMP, ATP, phos phoglycol and glycerol-phosphate, in a total volume of 0.5 ml. The initial *p*H 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.

(12) O. Lindberg and L. Ernster, *Exper. Cell Research*, **3**, 209 (1952).

(13) A. A. Benson, M. Calvin, V. A. Haas, S. Aronoff, A. G. Hall, J. A. Bassham and J. W. Weigl, "Photosynthesis in Plants," Iowa State Coll. Press, Ames, Iowa, 1949, Chap. 19, pp. 381–401.

(14) L. Jacobson, *Plant Physiol.*, **26**, 411 (1951).

(15) S. Kawaguchi, A. A. Benson, P. Hayes and M. Calvin, THIS JOURNAL, **74**, 4477 (1952).

(16) M. Calvin, "The Harvey Lectures," Series XLVI, 218 (1950–1951), Charles C Thomas.

(17) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).

(18) B. Axelrod and R. S. Bandurski, *J. Biol. Chem.*, **193**, 405 (1951).

(19) The authors are indebted to Drs. L. F. Leloir and H. M. Kalckar for specimens of UDPG, to Dr. J. T. Park for a sample of UDP and to Dr. W. E. Cohn for a sample of UMP.

(20) A. C. Paladini and L. F. Leloir, *Biochem. J.*, **51**, 426 (1952).

(21) C. E. Carter, THIS JOURNAL, **72**, 1466 (1950).

(22) A. F. Lukovnikov, V. P. Medvedev, M. V. Nieman, An. N. Nesmeyanov and I. S. Shaverdina, *Doklady Akad. Nauk S.S.S.R.*, **70**, 43 (1950) [*C. A.*, **44**, 4315i (1950)].

(23) D. E. Hull, THIS JOURNAL, **63**, 1269 (1941).

Results

Compounds Isolated.—The radioactivity on the chromatograms (Fig. 1) 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 only in small amounts.

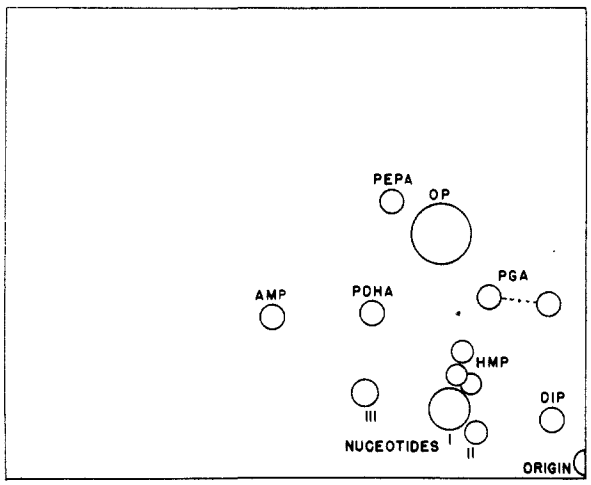

Fig. 1.—Relative distribution of activity on chromatograms of extracts from *Scenedesmus* exposed to $H_2P^{32}O_4^-$. Abbreviations used are as follows: OP, ortho phosphate; PGA, 3-phosphoglyceric acid; HMP, hexose monophosphate area; DIP, sugar diphosphates; nucleotide I, predominantly adenosine triphosphate; nucleotide II, predominantly uridine diphosphate glucose; nucleotide III, adenosine diphosphate; AMP, adenosine monophosphate; PDHA, phosphodihydroxy acetone; PEPA, phosphoenolpyruvate.

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 radioactive 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 phenol direction and completely 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, phospholipides, 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 30 seconds all of the intermediates observed in one-hour exposures are labeled, 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 30-second experiments, time did not permit this

(24) J. M. Gulland and E. O. Walsh, *J. Chem. Soc.*, 169 (1945).

(25) A. W. D. Avison and J. B. Hawkins, *Quart. Rev.*, **5**, 171 (1951).

(26) J. Sacks and C. H. Altschuler, *Amer. J. Physiol.*, **137**, 750 (1942).

(27) O. Kandler, *Z. Naturforech*, **5b**, 423 (1950).

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 Figs. 2 and 3. Although these results are consistent with the 30-second and one-hour experiments, the latter have not been included in the figures because of the difference in experimental procedures.

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

TABLE II

FRACTIONATION OF HMP AREA

	% of HMP activity						
Compound	Dark 1 min.	Dark 10 min.	Dark 25 min.	Light 1 min.	Light 10 min.	Light 25 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)[a]

[a] Mannose-6-P only.

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]

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 75 minutes as in 60 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.

(28) B. L. Strehler, "Phosphorus Metabolism," Vol. 2, Johns Hopkins Press, Baltimore, Md., 1952.

(29) The values given were obtained by enzymatic equilibration among G-6-P, F-6-P and M-6-P by M. W. Slein, *J. Biol. Chem.*, **186**, 753 (1950); *cf.* K. Lohmann, *Biochem. Z.*, **262**, 137 (1933).

(30) M. Goodman, Thesis, Ph.D., University of California, 1952.

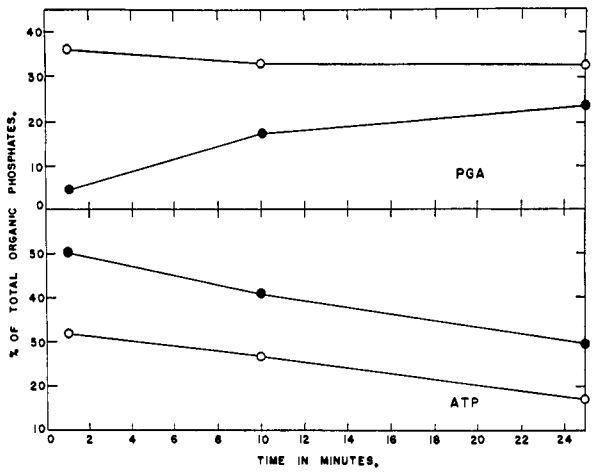

Fig. 2.—PGA, % of 3-phosphoglyceric acid; ATP, % of adenosine triphosphate as function of time: O, in light; ●, in dark, ordinate is the % of total activity in soluble organic compounds; data are from series II.

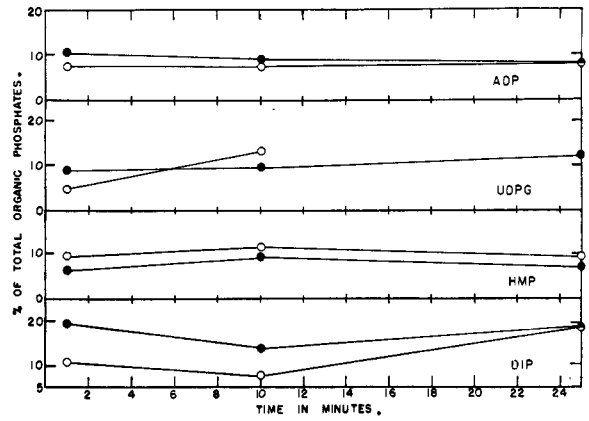

Fig. 3.—ADP, % of adenosine diphosphate; UDPG, % of uridine diphosphate glucose; HMP, % of hexose monophosphates; DIP, % of sugar diphosphates as function of time. Ordinate is the % of total activity in soluble organic compounds. Data are from series II.

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 vari-

(31) F. Lipmann and L. C. Tutle, *J. Biol. Chem.*, **159**, 21 (1945).

(32) E. R. Stadtman and H. A. Barker, *ibid.*, **180**, 1095 (1949).

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

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 rate 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.[7,8,33] 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 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 regeneration of ATP from the AMP and ortho phosphate would yield ADP labeled in the terminal phosphate, presumably through the action of myokinase.

It is of interest to relate the results with the known elements of respiration and photosynthesis.[4] Figure 4 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

(33) G. A. LePage and W. W. Umbreit, *J. Biol. Chem.*, **147**, 263 (1943).

(34) J. T. Park, "Phosphorus Metabolism," Vol. 1, Johns Hopkins Press, Baltimore, Md., 1951, p. 93.

(35) R. Caputto, L. F. Leloir, C. E. Cardini and A. C. Palladini, *J. Biol. Chem.*, **184**, 333 (1950).

(36) A. Kornberg, "Phosphorus Metabolism," Vol. 1, Johns Hopkins Press, Baltimore, Md., 1951, p. 392.

(37) F. Lipmann, M. E. Jones, S. Black and R. M. Flynn, THIS JOURNAL, **74**, 2384 (1952).

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 percentage 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 saturating 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,[27,28,38-40] leading to the production of PGA. This would account for the rapid labeling of PGA in the light.

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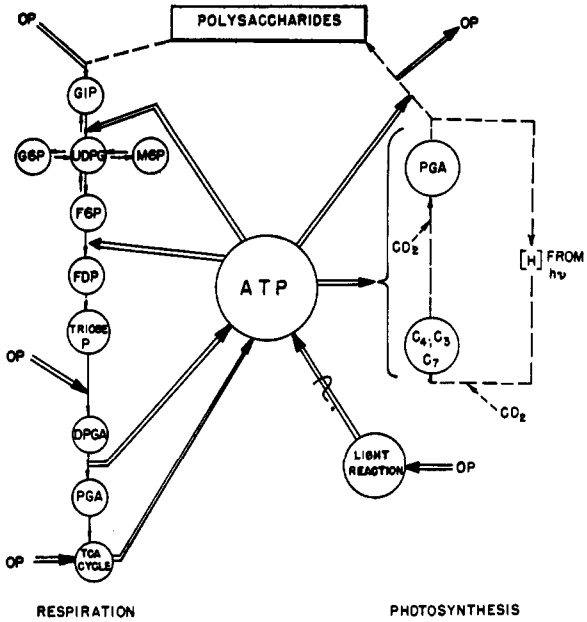

Fig. 4.—Schematic representation of the relationship between ortho phosphate and certain organic phosphates Double lines indicate phosphate transfer; dotted lines, a change in the carbon skeleton; single lines, other types of transformation. "TCA" denotes the tricarboxylic acid cycle and "Light Reaction" indicates methods of converting ortho phosphate to high energy phosphate in the form of ATP by means dependent upon photochemical reactions and not involving the reduction of CO_2 followed by reoxidation of the products.

(38) E. C. Wassink, J. F. G. M. Wintermans and J. E. Tjia, *Proc. Kon. Akad. Wetenschap*, **54c**, 41 (1951).
(39) W. Lindeman, *ibid.*, **54c**, 287 (1951).
(40) E. C. Wassink, J. E. Tjia and J. F. G. M. Wintermans, *ibid.*, **52**, 412 (1949).

[CONTRIBUTION FROM THE NATIONAL INSTITUTES OF HEALTH]

The Use of Various Aminomalonates in the Synthesis of α-Substituted Tryptophans

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Dibenzyl formaminomalonate (IV), carbobenzyloxyaminomalonate (V) and formaminopiperidinomethylmalonate (II) were prepared and tested in condensation reactions with 2-phenyl- and 2-carbethoxy-3-diethylaminoindoles (VIII and IX). XIII, the condensation product of V with IX, on catalytic debenzylation lost all three benzyl groups and gave 2-carbethoxytryptophan (XIX) in good yield. 2-Phenyltryptophan (XXI) and its ethyl ester (XXII) were prepared by four different routes. XV, the condensation product of IX with diethyl nitromalonate (VI), yielded, *via* XVII, 2-carbethoxytryptophan ethyl ester (XX) or the 4,5,6,7-tetrahydro-2-carbethoxytryptophan ethyl ester (XVIII) depending on the type of reduction.

The difficulties of obtaining the presumable primary breakdown product of tryptophan[1] would be expected to be smaller in a tryptophan derivative bearing a substituent in the α-position. In such a compound the well-known readiness with which β-hydroxy(or hydroperoxy)-indolenines undergo internal (or external) addition reactions, *e.g.*, with the alanine side chain, should be lessened or suppressed. 2-Phenyltryptophan (XXI) was synthesized in the hope of using the activating influence of the phenyl group for autoxidation experiments,[2] while 2-carbethoxytryptophan (XIX) was thought to be a suitable starting material for a β-hydroxyindolenine which could then lose the carbethoxy group with the same ease of saponification and decarboxylation as indoleninecarboxylic acid or α-picolinic acid.

2-Phenyltryptophan (XXI).—This amino acid and its ethyl ester (XXII) were obtained by the following four different routes: (i) The Mannich base VIII from 2-phenylindole (VII),[3] formaldehyde and diethylamine was condensed in the usual

(1) A. Ek, H. Kissman, J. B. Patrick and B. Witkop, *Experientia*, **8**, 36 (1952).
(2) B. Witkop and J. B. Patrick, THIS JOURNAL, **74**, 3855 (1952).
(3) H. Kissman, D. K. Farnsworth and B. Witkop, *ibid.*, **74**, 3948 (1952).