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Levels of Phosphate Esters in Spirodela

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Abstract. The duckweed *Spirodela oligorrhiza* was grown in sterile nutrient solutions that contained 1 mM phosphate-^{32}P at various specific activities. In solutions with activities higher than 2 μc per μmole per ml, plant growth was inhibited after a time, and the physical appearance of the plants was affected. The critical level of radiation, at which growth was first affected, corresponded to 5 kilorads.

Plants were grown for 9 days (5 generations) in a culture solution containing phosphate at 0.5 μc per μmole per ml (radiation load approx 0.5 kilorads) so that all phosphorus-containing materials in the tissue became uniformly labeled. The various radioactive compounds were extracted, chromatographed, identified, and their radioactivity was measured. From this radioactivity plus the specific activity of the supplied phosphate, the amount of each compound was calculated. The data constitute a complete balance-sheet for phosphorus in a plant tissue. The identity of 98 % of the phosphorus in the tissue was determined. Inorganic phosphate (32,700 mμmoles/g fr wt) was the predominant phosphorus-containing compound; RNA (5100 mμmoles P/g fr wt) was the main organic phosphate; phosphatidyl choline (1600 mμmoles/g fr wt) was the main phospholipid, and glucose-6-phosphate (500 mμmoles/g fr wt) the main acid-soluble phosphate ester. Amounts of other phosphorus compounds are given.

Despite the central role of phosphorus in cellular metabolism, there is little information about the amounts of the various phosphate esters which may be present in plant tissues. Experimental difficulties exist because numerous phosphorus-containing compounds are present, in low concentrations. Sensitive and specific enzyme methods can be used to estimate each ester in turn (1, 2, 35); but the procedure is not suitable for obtaining a balance-sheet. Procedures for separating the various esters chromatographically and estimating each ester chemically (12, 39, 45) have been limited by a lack of sensitivity in the chemical estimation procedures. A greatly increased sensitivity can be obtained by using radiochemical procedures; either through neutron activation (4) or through using plant material which has been grown in $^{32}P_i$ long enough for equilibrium labeling to be reached. The latter procedure is not suitable for large plants which are growing in soil. However, the duckweed *Spirodela oligorrhiza* (Kurz.) Hegelm., a minute floating water plant, grows vegetatively in axenic culture on a completely defined medium, doubles its weight every 2 days, and is ideally suited to such a study.

In this study, *Spirodela* was grown for several "generations" in $^{32}P_i$, to provide a uniformly labeled plant tissue which was analyzed for its phosphorus-containing constituents. To avoid the possibility of radiation damage confusing the results, the radiation sensitivity of *Spirodela* was first determined.

Materials and Methods

Culture of Spirodela. *Spirodela oligorrhiza,* obtained from Thimann (42), was normally grown within 50 ml conical flasks in 20 ml medium which contained 4 mM $(NH_4)_2SO_4$, 2 mM $CaSO_4$, 2 mM $MgSO_4$, 1 mM K_2SO_4, 1 mM KH_2PO_4, 1 % glucose w/v, Fe-EDTA at 3 parts per million, B and Mn at 0.5 parts per million, Zn at 0.05 parts per million, and Cu and Mo at 0.02 parts per million (15).

Medium was dispensed and autoclaved, then inoculated with 3 or 5 three-frond units, that is, 6 to 10 mg tissue (a 3-frond unit comprises a mature mother frond subtending an old daughter frond on one side and a younger one on the other). Sterile $CaCO_3$, about 50 mg, was added to each flask at the time of inoculation to buffer the culture medium during growth of the tissue. Plants were grown at 24° in continuous "daylight" fluorescent light, approximately 250 ft-c, and harvested or subcultured after 8 to 14 days.

Sensitivity of Spirodela *to Beta Radiation.* In these experiments, to conserve tracer, only 10 ml culture medium per flask was used. Standard medium minus P_i was made up; then 0.2 M KH_2PO_4-(^{32}P) solutions of various specific activities were prepared by serial dilution with unlabeled 0.2 M KH_2PO_4, and the appropriate amount was added to its portion of the culture medium. Levels of radioactivity employed were 0, 0.6, 2.0, 6, and 20 μc per

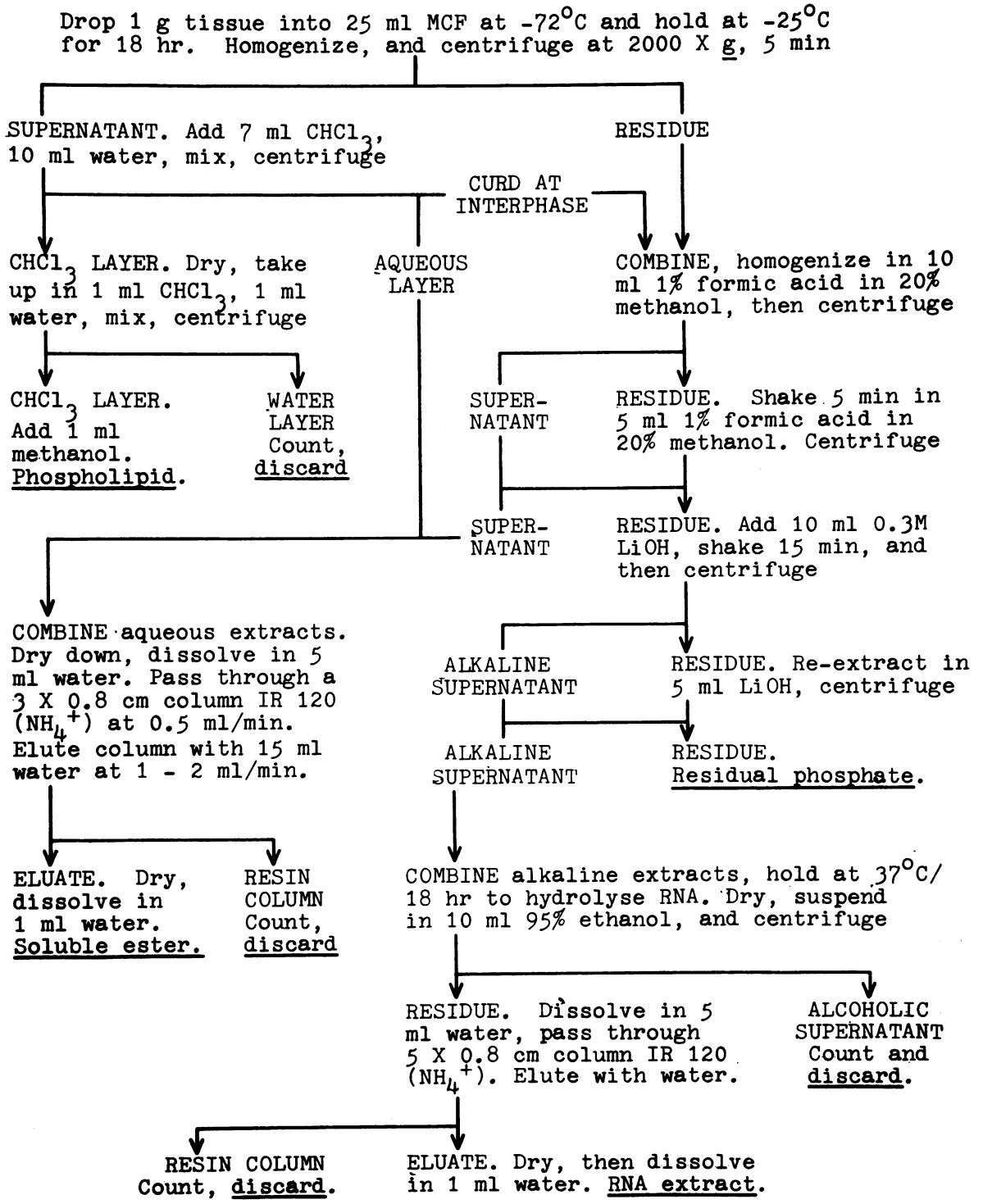

FIG. 1. Flow sheet for extraction of phosphate-containing fractions from *Spirodela* tissue. MCF, methanol: chloroform:formic acid:water, 12:5:1:2 v/v.

μmole P_i per ml (Expt 1) and 0, 1.2, 4, and 12 μc per μmole P_i per ml (Expt 2). At each sampling time, 2 flasks were harvested from each treatment and emptied into a Buchner funnel: the plants were rinsed in a stream of water from a wash bottle, then floated in 3 changes of 50 ml water during 3 minutes so as to remove any P_i contained in the apparent free space, then blotted and weighed. A weighed portion of each sample was measured for radioactivity, in order to determine the amount of phosphorus taken up.

Growth of Uniformly-labeled Tissue. Fronds were inoculated into medium containing 0.5 μc ^{32}P per μmole P_i per ml, and grown for 9 days, during which time the tissue increased in weight 20 to 30 times. Tissue, 4 g, was harvested, washed, killed, and extracted.

Extraction of Phosphorus Compounds From Tissue. The killing procedure employs methanol: chloroform:formic acid:water, 12:5:1:2 v/v (MCF) at $-25°$ for 18 hours, to minimize phosphatase action (9). Tissue was macerated in either a Virtis homogenizer or (small samples) a Duall conical glass homogenizer. Extracts were dried at 35° under vacuum in either a rotary film evaporator or (small samples) a Buchler "Evapomix". The extraction procedure (fig 1) for phospholipids and phosphate esters was essentially that of Bieleski and Young (14). The resulting residue was treated with 0.3 M LiOH to extract RNA; LiOH was removed from the resulting extract by ethanol extraction. Divalent cations were then removed from the ester extract, and any remaining LiOH from the RNA hydrolysate as NH_4OH, by passing each extract through IR 120 (NH_4^+). Significant amounts of nucleotide were not retained on the columns provided that the resin was large-beaded, in the (NH_4^+) form, and of minimum column length. ^{32}P-labeled material was lost when KOH hydrolysis/$HClO_4$ neutralization was used for obtaining the RNA extract (14, 44).

Separation of Components of Extracts. P_i was determined by precipitating it from the ester extract (40) and then measuring its radioactivity. The amount of phosphorus in each fraction or compound could be obtained, from the measured radioactivity, and the known specific activity of the P_i originally supplied to the tissue.

Phospholipids were separated by 2-dimensional thin-layer chromatography on 20 × 20 cm, 250 μ thick, mixed silica gel/cellulose layers [20 g silica, Merck H, + 8 g cellulose, MN 300, + 100 ml water, blended and spread in the usual manner (13)]. Plates were activated at 115° for 1 hour, cooled, and immediately spotted with 10 to 100 μl phospholipid extract, then chromatographed in solvent 1 (chloroform:methanol:water 65:25:4 v/v) (26), a small beaker of chloroform being included in the tank to maintain chloroform in the vapor phase and thereby reduce tailing. The plate was dried thoroughly then chromatographed in solvent 2, methyl isobutyl ketone:acetic acid:water 8:5:1 v/v. Chromatograms were autoradiographed and spots were removed from the chromatogram, for counting, by a cellulose acetate peel technique (13). Duplicate chromatograms were sprayed with suitable detection reagents (26), and known phospholipids were used as carrier compounds. Phosphatidyl choline plus phosphatidyl ethanolamine, from egg yolk, was given by Dr. R. Weenink, and phosphatidyl ethanolamine plus phosphatidyl serine was prepared from brain (27). Phosphatidic acid was obtained by the action of phospholipase D, prepared from brussels sprouts (18), on phosphatidyl choline. Phosphatidyl inositol and phosphatidyl serine came from Koch-Light Laboratory Incorporated.

Phosphate esters and P_i contained in the soluble ester extract were separated by 2-dimensional paper chromatography, 50 to 200 μl/origin, with or without added authentic carrier compounds, in solvent 3, *n*-propanol:ammonia:water:EDTA, 6:3:1:0.01 v/v/v/w then solvent 4, *n*-propyl acetate:formic acid:water 11:5:3 v/v (14). Carrier compounds were detected by standard procedures (14, 20). Generally, when a carrier was added to the extract, trace compounds normally chromatographing in the same position were excluded by it. In autoradiographs, these compounds appeared as a rim at the edge of the carrier. The 2 areas were eluted individually, and then separated along with other carrier compounds by electrophoresis at pH 3.6 (10, 14) or by chromatography on paper buffered with pH 8, 0.25 M borate + 0.1 % EDTA in an ethanol (95 %):borate buffer, 72:28 v/v solvent (solvent 5) (14).

Further information was obtained by 2 dimensional paper chromatography of the original extract in solvent 4 and then (after neutralizing the dried paper in NH_3 then spraying it with buffer) in solvent 5 so as to separate the sugar phosphates, and 2'- + 3'- from 5'-nucleotides (14). Two-dimensional thin-layer chromatography and thin-layer electrophoresis (10) were also used in confirming results. Other chromatographic systems were tested, but they did not yield any additional information.

Ribonucleotides contained in the RNA extract were separated by the procedures described above, except that electrophoresis was used to obtain the primary separation. A phosphorus-containing material that always remained at the origin was partly eluted by 1 N KOH, and was identified as DNA. The amount present in the original extract was estimated chemically (16).

Attempts to render soluble the small amount of phosphorus-containing material retained in the residue fraction were not successful.

Results

Effect of Radiation Intensity on Plant Growth. The rate of growth of *Spirodela* depended on the specific activity of the $^{32}P_i$ supplied in the culture

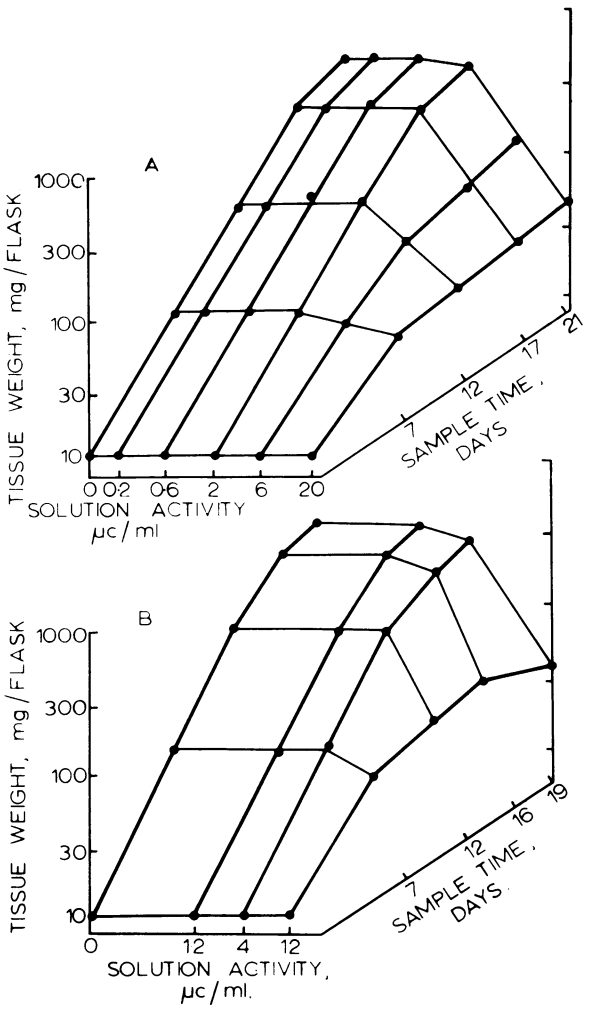

FIG. 2. Effect of specific activity of phosphorus in culture medium on growth of *Spirodela* with time.

medium, and on the duration of exposure (fig 2). Control plants grew logarithmically for 14 days, until limited by the availability of nutrients. Plants in 0.2, 0.6, and 1.2 μc/μmole grew at the same rate as control plants. Plants in 2.0 and 4.0 μc/μmole showed a slight decrease in growth after 21 and 16 days respectively. Growth in 6 μc/μmole was markedly reduced after 12 days; and in 12 and 20 μc/μmole, was virtually halted after 9 and 6 days respectively. New fronds became small, curled inwards and down, lost their buoyancy and died.

The phosphorus content of the tissue was related to these growth effects. Normally-growing plants contained about 40 μmole P/g fresh weight until the P_i of the medium became exhausted, at day 14. Plants grown in 0.2 to 1.2 μc/μmole behaved in a similar way. Plants grown in 6 to 20 μc/μmole however, increased their phosphorus content with time, from about 40 to 60 to 75 μmole P/g fresh weight. Apparently, radiation intensities which halted growth did not halt P_i accumulation, so that the phosphorus content of the radiation-damaged tissue rose. This would tend to heighten the radiation damage and produce a more marked endpoint to tissue growth.

Proportion of Total Phosphorus in Various Extracted Fractions. The extraction procedure divided the tissue phosphorus into 4 main fractions, plus 4 minor ones that were discarded (resin columns, water wash of phospholipids, alcohol wash of RNA extract). The amount and percentage of phosphorus present in each fraction is given in table I. At the end of extraction, over 99 % of the tissue phosphorus had been recovered within phospholipid, soluble ester and RNA extract fractions.

Components of Phospholipid Fraction. A consistent pattern was obtained with all phospholipid extracts of *Spirodela* (fig 3). There were at least 12 spots separable by chromatography, but 3 of these contained about 80 % of the total lipid phosphorus. One co-chromatographed with authentic phosphatidyl choline, was hydrolyzed by phospholipase D, and reacted with Dragendorff reagent. One co-chromatographed with authentic phosphatidyl ethanolamine and reacted with ninhydrin. The third chromatographed in the position expected for phosphatidyl glycerol (26), reacted with periodate/Schiffs reagent (4) and alkaline $AgNO_3$ (43), and upon deacylation gave a product that chromatographed in water-saturated phenol at an R_F characteristic of the deacylation product of phosphatidyl glycerol (4, 26). They are identified as phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl glycerol respectively.

Three other compounds were identified on the basis of their co-chromatography with authentic compounds and their reactions with spray reagents: phosphatidyl inositol (blue fluorescence with Rhodamine B, weak reaction with alkaline $AgNO_3$), phosphatidyl serine (very weak ninhydrin reaction) and phosphatidic acid. The material which remained near the origin was probably a complex phospholipid, for it gave positive ninhydrin and Dragendorff reactions, and a red fluorescence with Rhodamine B. The tissue content of the various phospholipids is given in table II.

Components of the Soluble Ester Fraction. The phosphate ester pattern obtained after chromatography of the soluble ester extract from *Spirodela* (fig 4) closely resembled patterns obtained for a variety of other plant tissues; for example, potato tuber (12), apple phloem, celery and *Brassica* vascular bundles (11) and cultured tobacco pith cells (3). During this earlier work the major compound present in each chromatogram spot was identified, and the present chromatogram (fig 4) has been annotated accordingly; but rechromatography with carrier compounds has now shown that some spots contained other compounds as well. The following spots were essentially pure (abbreviations, see fig 4, 5): P_i, PC, PE, PGA, αGP, UDP. The fol-

Table I. *Amount and Proportion of Phosphorus in Extracted Fractions from* Spirodela *Tissue*

Fraction	P, μmoles/ g fr wt		P, % of total P	
	Expt A	Expt B	Expt A	Expt B
Phospholipid	3.84	3.05	8.2	6.7
Soluble esters - inorganic phosphate	32.80	32.60	70.3	71.9
- phosphate esters	2.18	2.28	4.7	5.0
RNA extract - inorganic phosphate	0.18	0.17	0.4	0.4
- esterified phosphate	7.24	6.67	15.5	14.7
Residual phosphate	0.14	0.13	0 3	0.3
Water wash, phospholipid extract	0.00	0.00	0.0	0.0
Alcohol wash, dried RNA extract	0.04	0.06	0.1	0.1
Resin column, soluble ester extract	0.23	0.43	0.5	0.9
Resin column, RNA extract	0.00	0.01	0.0	0.0

Table II. *Amount and Proportion of Phospholipids in Phospholipid Extract from* Spirodela *Tissue*

Compound	P, mμmoles/ g fr wt		P, % of P-lipid	
	Expt A	Expt B	Expt A	Expt B
Phosphatidyl choline	1735	1460	44.8	47.8
Phosphatidyl ethanolamine	820	725	21.2	23.7
Phosphatidyl glycerol	510	420	13.2	13.7
Phosphatidyl inositol	260	180	6.7	5.9
Phosphatidyl serine	75	70	1.9	2.3
Phosphatidic acid	35	60	0.8	1.9
Origin	195	80	5.0	2.6
Other compounds	240	65	6.2	2.0

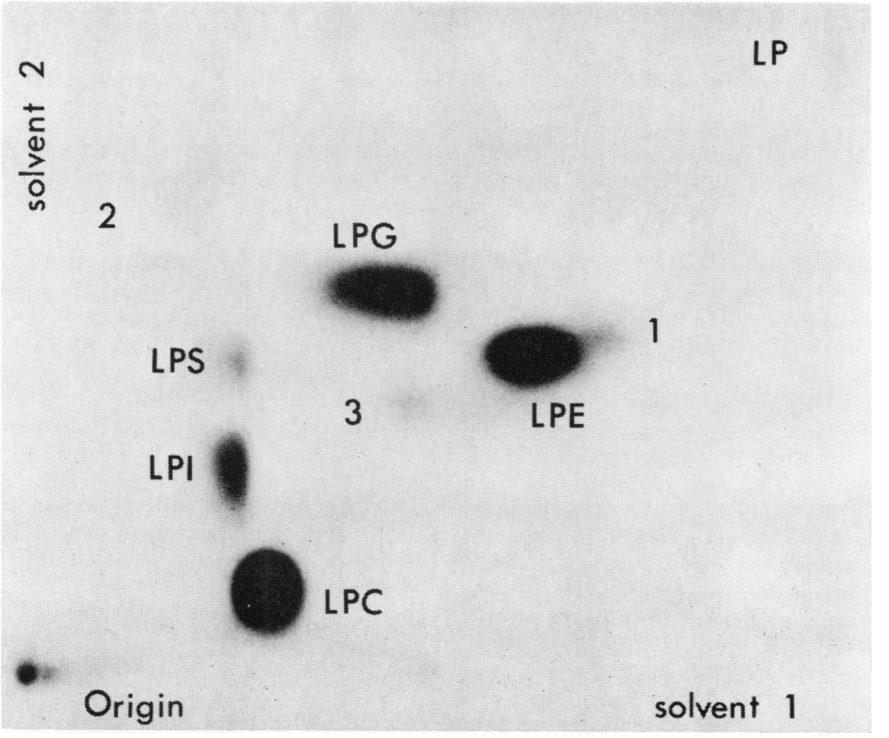

FIG. 3. Autoradiograph of ^{32}P-phospholipids present in phospholipid extract and separated by 2-dimensional thin-layer chromatography. Abbreviations: LPC, phosphatidyl choline; LPE, phosphatidyl ethanolamine; LPG, phosphatidyl glycerol; LPI, phosphatidyl inositol; LPS, phosphatidyl serine; LP, phosphatidic acid; 1,2,3, unknown compounds that appear consistently in these positions with all extracts.

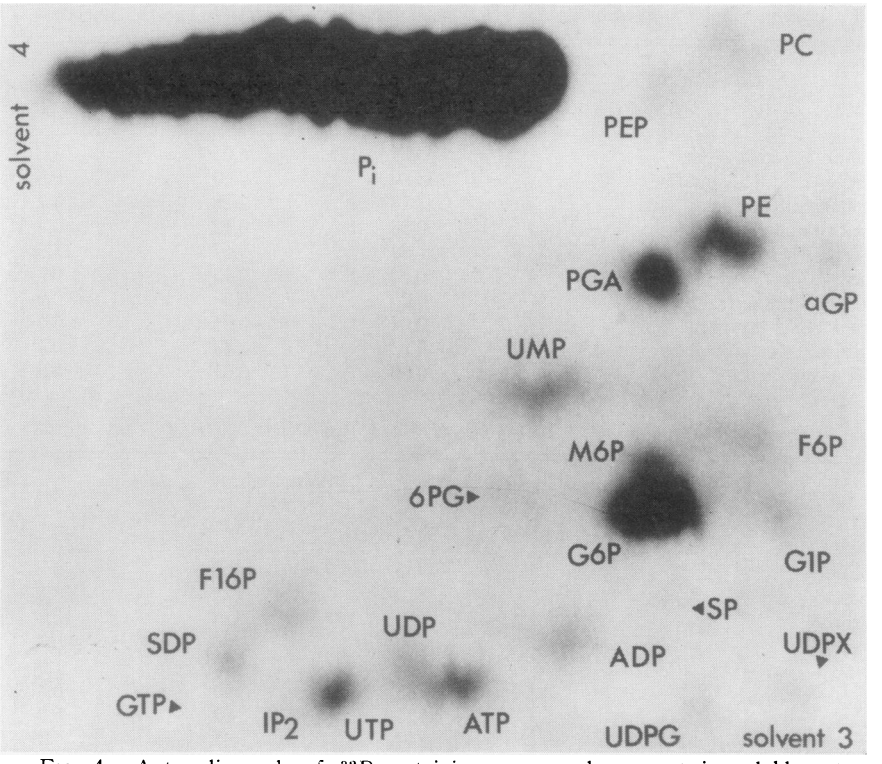

FIG. 4. Autoradiograph of ^{32}P-containing compounds present in soluble ester extract and separated by 2-dimensional paper chromatography. Abbreviations: AMP, ADP, and ATP, adenosine 5′-mono-, di-, and triphosphate; CTP, CDP, and CMP, cytosine 5′-mono-, di-, and triphosphate; GMP, GDP, and GTP, guanosine 5′-mono-, di-, and triphosphate; UMP, UDP, and UTP, uridine 5′-mono-, di-, and triphosphate; UDPG and UDPX, UDPglucose and an unidentified uridine diphospho compound; G1P, glucose-1-P; G6P, M6P, and F6P, glucose-, mannose-, and fructose-6-phosphates; SP, sucrose phosphate; SDP, sugar diphosphates; F1,6P, fructose-1,6-diP; PC, phosphoryl choline; PE, phosphoryl ethanolamine; 6PG, 6-phosphogluconate; PGA, 3- (and 2-) phosphoglycerate; PEP, P-enolpyruvate; αGP, α-glycerophosphate; IP_2, inositol diphosphate.

lowing spots contained 1 to 8 % of 1 or 2 other compounds (in brackets): PEP (PC or P_i); F6P (UMP + M6P); M6P (G6P); G6P (M6P); G1P (G6P); ADP (CDP). The following spots contained less than 90 % of the activity in the nominated compound: UMP (10–30 % F6P); 6PG (10–40 % G6P); UTP and IP_2 (incompletely separated from each other); ATP (CTP + 10 % unidentified nucleotide); F1,6P (10 % of a possible nucleotide + 5–30 % other sugar diphosphates); GDP (50–85 % sugar diphosphates). The following compounds were shown to be virtually absent: AMP, CMP, GMP, A3P, C3P, G3P, U3P, βGP, GTP, PP_i, IP_6, and P-serine. CTP could not be completely separated from the ATP; but it was less than 20 % of the ATP. CDP was present as 8 to 14 % of the ADP spot.

Several compounds require comment. The UDPglucose spot upon electrophoresis showed P_i, UMP and a hexose phosphate as impurities. These would have come from the labile UDPglucose during drying of the chromatogram and elution of the spot. Other evidence (10) suggested that over half of the UDPglucose present in the original extract had already broken down during paper chromatography in solvent 3. Carrier UDPglucose was largely broken down into a trail of UMP during paper chromatography in solvent 3, and ^{32}P-UMP on the chromatograms showed a similar trailing pattern. Thin-layer chromatograms showed relatively higher levels for UDPglucose (and UDPX) than did paper chromatograms. The evidence all suggested that 65 to 80 % of the UDPglucose broke down to UMP + G1P during paper chromatography.

UDPX was a compound that chromatographed close to UDPglucose. On electrophoresis it gave products similar to those given by UDPglucose, as revealed by radioactive areas coincident with UMP, UDPglucose, and hexose phosphate. It is probably a UDP-sugar.

SP chromatographed where sucrose phosphate would be expected, though it could also have been

a false spot resulting from trailing of G6P. Its electrophoretic mobility appeared to be slightly lower than that of G6P, however. IP_2 was a single compound with the chromatographic and electrophoretic mobility of inositol diphosphate, but this identification is provisional only. A fifth compound, not shown in figure 4, chromatographed in the region where breakdown products of NAD are found. It migrated as a single compound during electrophoresis. Attempts to identify it more positively were not successful. The region marked SDP contained at least 4 unidentified compounds which had electrophoretic mobilities ranging from 94 to 110 (relative to P_i = 100) — values typical of sugar diphosphates (14).

Chromatography of the original soluble ester extract in solvent 4–solvent 5 allowed accurate figures for radioactivities present in free nucleotide 3′- (and 2′-) phosphates to be obtained. The hexose monophosphates were separated more cleanly and their identity was confirmed. It became apparent that a small amount of galactose-6-P was present, and though it did not separate completely from the G6P, it appeared to comprise from 4 to 10 % of the G6P. Glyceraldehyde-3-P was also separated as a compact spot in this system.

These data have been used to estimate the amount and proportion of each ester detected in the soluble ester extract (table III).

Components of the RNA Extract. Electrophoresis of the hydrolyzed RNA extract yielded 5 strongly radioactive bands, and about 10 weakly radioactive ones. All 5 main bands were weakly UV-absorbing: 1 was at the origin and the remaining 4, with rela-

Table III. *Amount and Proportion of Phosphate Esters in Soluble Ester Extract From* Spirodela *Tissue*

The following compounds, if present in the soluble ester extract, were there at less than 2 mμmoles/g fr wt tissue: PP_i, methyl phosphate, β-glycerophosphate, inositol hexaphosphate, adenosine 3′-P, cytosine 3′-P, guanosine 3′-P, and uridine 3′-P.

Compound	P, mμmoles/g fr wt		P, % total ester P	
	Expt A	Expt B	Expt A	Expt B
Inorganic phosphate	32800	32600	1460	1485
Glucose-6-P	515	480	22.9	21.9
Mannose-6-P	125	110	5.6	5.0
Fructose-6-P	100	100	4.4	4.6
Glucose-1-P	55	40	2.4	1.8
Galactose-6-P	*ca* 40	In G6P	1.8	...
Triose-P	15	In F6P	0.7	...
Pentose-P	10	In F6P	0.4	...
Fructose-1,6-diP	40	45	1.8	2.0
Inositol diphosphate ?	55	65	2.4	3.0
Sugar diphosphates ?	100	105	4.4	4.8
Sucrose phosphate ?	45	20	2.0	0.9
Phosphoglyceric acid(s)	250	205	11.1	9.3
Phosphoenolpyruvate	38	36	1.7	1.6
6-Phosphogluconate	19	17	0.8	0.8
α-Glycerophosphate	15	20	0.7	0.9
Phosphoryl ethanolamine	135	205	6.0	9.3
Phosphoryl choline	55	95	2.4	4.3
ATP	135	95	6.0	4.3
GTP	12	5	0.5	0.2
UTP	45	60	2.0	2.7
ADP	65	70	2.9	3.2
CDP	7	8	0.3	0.4
GDP	7	7	0.3	0.3
UDP	55	80	2.4	3.6
UDPglucose	65	40	2.9	1.8
Uridine diphospho-X	40	35	1.8	1.6
Hydrolyzed UDPglucose[1]	105	115	4.7	5.2
AMP	7	10	0.3	0.5
CMP	2	<2	0.1	0.0
GMP	2	<2	0.1	0.0
UMP	50	75	2.2	3.4
Nucleotide 3′-monophosphates	7	In UMP etc.	0.3	...
Nicotine adeninedinucleotide ?	20	30	0.9	1.4
Other compounds	15	25	0.7	1.1

[1] The amount of UDPglucose (and UDPX) hydrolyzed during chromatography was estimated from the activity present in the trailing portion of the UMP and glucose-1-P spots and added to the total. It is likely to be an underestimate.

tive electrophoretic mobilities 11, 23, 47, and 61, coincided with carrier C3P, A3P, G3P, and U3P respectively. The identity of each was confirmed by eluting it and chromatographing it in solvent 5, which clearly separates the 3′- (and 2′-) ribonucleotides from most other phosphate esters. Only traces of other compounds were found in the ribonucleotide bands.

Some of the weaker bands separated by electrophoresis were also found to coincide with carrier compounds, for example those at mobility 83 (αGP), 92 (ATP), 100 (P_i), 122 (UTP), 146 (PP_i), and 168 (IP_6). These were further studied by chromatographing the original RNA extract in solvent 3 and then solvent 4 (fig 5). Radioactive areas that coincided with A3P, C3P, U3P, G3P, αGP, P_i, AMP, ADP, ATP, UMP, UDP, UTP, GDP, GTP, PP_i, IP_3, and IP_6, were found. All were eluted and further separated by electrophoresis with additional carrier compounds. The original chromatogram spots G3P, IP_6, P_i, ATP, and αGP, were all essentially pure, though βGP may have been present in the αGP spot. The A3P-C3P-U3P area contained 3 % of its radioactivity in 2 unidentified compounds. The remaining spots contained secondary compounds as well as the nominated one: UMP (20–30 % G3P); ADP (5–30 % unidentified compounds); UDP (0–25 % UTP); UTP (0–40 % UDP); PP_i (0–15 % UDP + 20–30 % unidentified); GDP (70–80 % unidentified); GTP (35–45 % IP_3, 10–25 % GDP, 10 % unidentified). The area X contained a compound with zero electrophoretic mobility, which was probably derived from hydrolysis of residual phospholipid. The area Y contained about 65 % of its activity in C3P and U3P, and the rest in unidentified compounds.

Several of the unidentified compounds absorbed UV light, suggesting that they were either minor ribonucleotides, or alkali-resistant dinucleotides, derived from the RNA. About 10 % of the ^{32}P-activity in the extract remained at the origin during either chromatography or electrophoresis. It absorbed UV light, and gave a positive Dische reaction, indicating the presence of DNA. Part of this material was eluted from the origin with 1 N KOH, precipitated with dilute H_2SO_4, and redissolved in 2 drops 1 N KOH: 40 % of the origin activity was recovered. DNA in this extract, estimated colorimetrically (16), accounted for 85 % of the radioactivity. A DNA estimation performed on the original RNA extract, before chromatography, gave a value (595 mμmoles DNA-phosphorus/g fr wt in Expt B) rather higher than the total origin-bound phosphorus. It is concluded that the phosphorus-

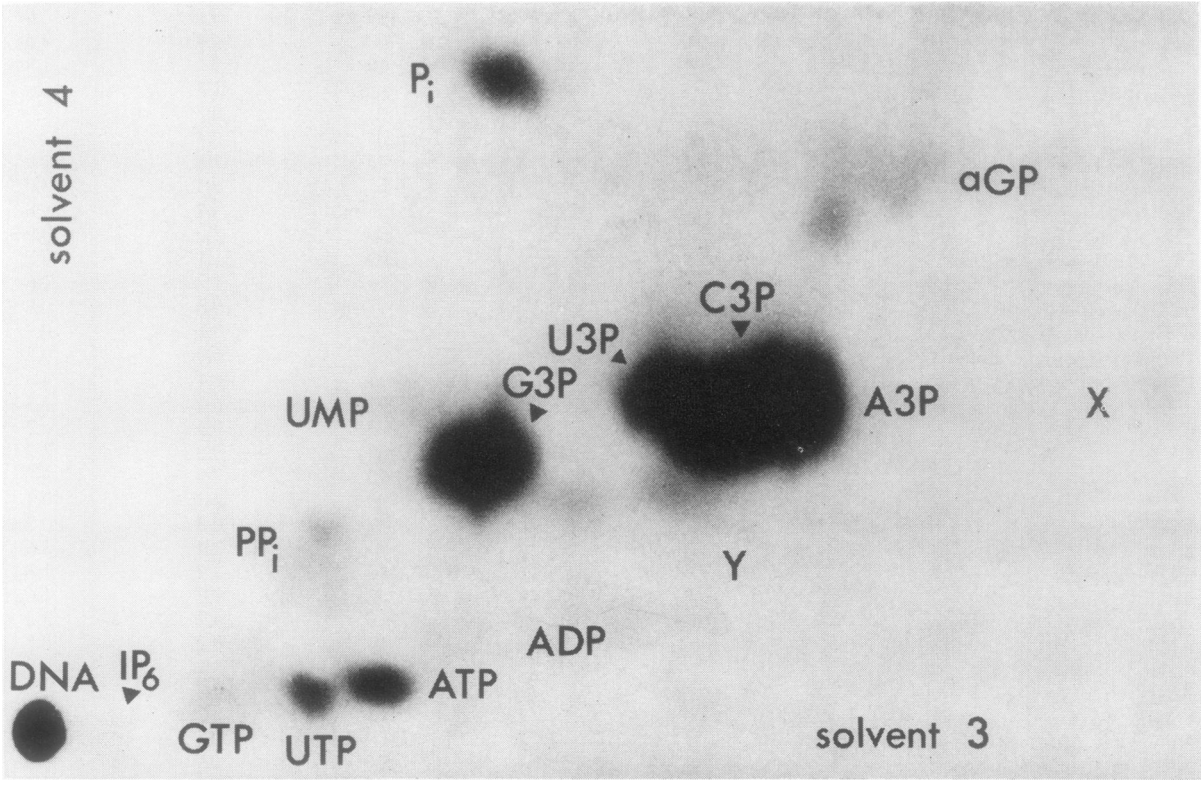

FIG. 5. Autoradiograph of ^{32}P-containing compounds present in RNA extract and separated by 2-dimensional paper chromatography. Abbreviations: as for figure 4, and; A3P, adenosine 2′- and 3′-phosphates (mixed); C3P, cytosine 2′- and 3′-phosphates; G3P, guanosine 2′ and 3′-phosphates; U3P, uridine 2′- and 3′-phosphates; IP_3 and IP_6, inositol tri- and hexaphosphate (IP_6 is phytic acid); X and Y, unknown compounds.

Table IV. *Amount and Proportion of Phosphorus-containing Compounds in the RNA Extract From* Spirodela *Tissue*

Compound	P, mμmoles/g fr wt		P, % of RNA extract	
	Expt A	Expt B	Expt A	Expt B
RNA ribonucleotides	5230	4800	71.6	70.5
Possible RNA nucleotides[1]	90	70	1.2	1.0
Possible RNA dinucleotides[1]	60	25	0.8	0.4
DNA (origin)	680	565	9.3	8.3
ATP	165	180	2.3	2.6
UTP	85	70	1.1	1.0
GTP	30	25	0.4	0.4
ADP	65	55	0.9	0.8
UDP	30	50	0.4	0.7
GDP	15	30	0.2	0.4
AMP	5	10	0.1	0.1
UMP	60	40	0.8	0.6
α-Glycerophosphate	180	330	2.4	4.8
Inorganic phosphate	180	170	2.4	2.5
Inorganic pyrophosphate	85	100	1.1	1.5
Inositol hexaphosphate	70	35	0.9	0.5
Inositol triphosphate	40	30	0.5	0.4
Other compounds	230	225	3.3	3.3

[1] Unknown compounds, interpreted as minor ribonucleotides or alkali-resistant dinucleotides arising from hydrolysis of RNA, see text.

containing material bound to the origin is mostly DNA.

These data have been used to estimate the amount and proportion of each phosphorus-containing compound present in the RNA extract (table IV).

Discussion

Radiation Sensitivity. The higher the specific activity of the ^{32}P supplied to the tissue, the sooner an inhibition of growth resulted. The critical level of radiation at which growth first became affected was calculated. In its geometry, the system is equivalent to a flat plate of tissue 0.10 mm thick, resting on an infinitely thick layer of solution. There were 2 components to the radiation received by the tissue, from the ^{32}P in the tissue and from the $^{32}P_i$ in the solution. Consider the situation at the critical point when inhibition of growth had just become apparent in the sample. The solution contained (activity at t = 0) 6 μc/ml; the tissue content was 270 μc/g fresh weight; inhibition was apparent after t = 9 days; and ^{32}P has a mean beta energy $\overline{E}$ = 0.69 Mev and a half-life of 14 3 days. The apparent absorption coefficient for ^{32}P beta particles in tissue is 9.2 cm^2/g (23). These data can be used to calculate that the dose due to the solution was 760 rads, to the tissue itself was 4400 rads, total 5200 rads. A similar critical dose is obtained for tissues growing at other radiation intensities. The value obtained is likely to be an overestimate because the tissue takes about 2 days to reach its final radioactivity, and the damage takes a finite time to appear (33). It is similar to that obtained for the effect of ^{32}P on barley (38), but higher than for the effect of x-rays on *Lemna* (33) or on *Spirodela* (Bollard, unpublished data).

Separation Procedure. The first extraction step gave a chloroform-methanol extract which was then converted into a 2-phase mixture; centrifuging left a thin curd between the two. The chloroform phase contained virtually all the phospholipid and no other phosphorus compounds; the aqueous phase contained much of the acid-soluble material; and the curd contained about 13 % of the tissue RNA and a trace of phospholipid. Solubility of s-RNA in lipid solvents under acid conditions, has been reported (21), suggesting that the curd represents the s-RNA fraction of the tissue. In these experiments it was returned to the residue, to be later hydrolyzed along with the rest of the RNA.

The acid-soluble phosphate esters plus P_i were extracted mainly in the aqueous phase of the chloroform-methanol extract (31,800 mμmoles P/g fr wt). Two following extractions of the tissue with 1 % formic acid in 20 % methanol removed 3100 mμmoles and 200 mμmoles P/g tissue respectively. Despite this, about 1100 mμmoles P/g tissue of acid-soluble ester material was apparently retained in the residue (see later). Material retained on the column was mostly finely particulate RNA: it was not recovered.

Almost all of the phosphate in the residue was extracted with alkali. Much was in the form of RNA and DNA (5200 and 600 mμmoles P/g tissue). However a surprisingly large amount (1070 mμmoles/g tissue) was present as simple phosphate esters. The α-glycerophosphate would have arisen from alkaline hydrolysis of phospholipids that remained in the tissue after chloroform-methanol extraction (4). P_i probably arose from hydrolysis of

other compounds, and inositol triphosphate may have come from hydrolysis of inositol hexaphosphate. Inositol hexaphosphate and PP_i are known to form acid-insoluble salts with some polyvalent cations, explaining why they were not recovered earlier in the acid extracts. The surprise lay in the amount of nucleoside di- and triphosphate (over 40 % of the total) which was recovered in the alkaline extract (compare tables III, IV). Fortunately these nucleotides, unlike RNA, are stable to alkaline hydrolysis (less than 5 % of ATP, UTP or GTP was broken down in tests under the conditions used here); and so a tissue total of each nucleotide can be obtained. The UMP probably came from hydrolysis of adsorbed UDPG.

All these nucleotides should have been completely extracted, along with the other simple esters, in the acid extracts: yet they were firmly held, as they could not be completely removed by further extractions with 1 % formic acid in 20 % methanol, 5 % formic acid, or 5 % trichloroacetic acid. Probably the nucleotide was not chemically bound (*e.g.* in the form of s-RNA end groups) as the amount was far too great, but rather bound adsorptively onto the RNA (34). It sounds a cautionary note to those making quantitative nucleotide studies by using acid extraction procedures on plant tissues.

Tissue Content of Phosphorus Compounds. In general, the concentrations and relative proportions of the various phosphorus compounds present in *Spirodela* are in line with the rather fragmentary results for other plant tissues. The amount of RNA in *Spirodela,* 5200 mμmoles RNA P/g fresh weight, is similar to that in beetroot, old and young pea leaves [790, 4500, and 22,500 mμmoles/g respectively (39)], and in apple leaf [approx 1800 mμmoles/g (30)]. The amount of DNA, 690 mμmoles DNA-phosphorus/g fresh weight, is similar to that in beetroot, old and young pea leaves [77, 330, and 1000 mμmoles/g (39)] and in young tobacco leaf [550 mμmoles/g (28)].

There appears to be very little information on the actual amount of phospholipid present in plant tissues (46); though several studies have been made on their identity and their proportions. Phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl glycerol were the major ones found in potato tuber (26), oat coleoptiles (47), bean, beet, and spinach leaves (24, 46), and apple fruit (29). They are also the major phospholipids present in *Spirodela.* The amount of each (1600 mμmoles phosphatidyl choline/g fresh weight, 770 mμmoles phosphatidyl ethanolamine/g and 470 mμmoles phosphatidyl glycerol/g is high when compared with the other phosphorus-containing compounds of the tissue.

A number of attempts has been made to measure the concentrations of the various hexose phosphates present in plant tissues, particularly potato. The earliest attempts, based on barium fractionation techniques, were unsatisfactory (see 2, 37). Subsequent enzyme and chromatographic techniques have yielded results which are much more convincing, and in agreement with one another. Typical values for hexose phosphate, in mμmoles/g fresh weight, include 77 and 173 [beet petiole parenchyma and bundles, (32)], 80 to 100 [green apple, tomato, and banana fruits, (1)], 100 [potato, (12)], 100 to 300 [potato, (1, 2)], and 300 [carrot root, (19)]. Compared to these, the present value, 770 mμmoles hexose phosphate/g fresh weight *Spirodela,* is high. The ratio G6P:F6P, 3:1 for beet, 5:1 for green banana and strawberry leaf, and 8:1 for green fruits compares with 5:1 for *Spirodela.* Values for the concentration of PGA, 12 to 25 mμmoles/g fresh weight; PEP, 8 to 25 mμmoles/g; dihydroxyacetone-P, 0.2 to 6 mμmoles/g; and F1,6P, 0.3 to 6 mμmoles/g have also been reported (1, 2, 12). Again, the corresponding values found for *Spirodela* tissue are of the order of 5 times higher. However, *Spirodela* is metabolically very active, and is essentially equivalent to a young leaf, whereas most of the tissues discussed above are storage tissues. Values found for strawberry leaf, 200 to 250 mμmoles hexose phosphate/g fresh weight (1, 2) and clover leaf, 320 mμmoles hexose phosphate/g and 280 mμmoles PGA/g (45) are more similar to the *Spirodela* values.

There is rather more published information on the identity and concentrations of nucleotides present in plant tissues; and once again, concentrations are higher in *Spirodela* than in most other tissues. In the various studies the nucleotide pattern obtained has to some extent been a function of the method used as well as the tissue studied. One or two generalizations can be made. Uridine nucleotide is approximately equal in amount to adenosine nucleotide: guanosine nucleotide is less than 0.2, and cytosine nucleotide less than 0.1 this amount (6, 12, 25, 32). When extracting and separating conditions have been mild, avoiding strongly acid or alkaline solutions, UDPG is a major uridine-containing nucleotide (6, 32, 36). Uridine diphosphogalactose may also be present (5).

The ratio nucleoside triphosphate:nucleoside diphosphate:nucleoside monophosphate has been of major interest in many studies (37). When uridine nucleotides are studied as well as adenosine nucleotides, it is generally found that the ATP:ADP and ADP:AMP ratios are higher than the corresponding UTP:UDP and UDP:UMP ratios (8, 17, 32). In the present study, for example, ATP:ADP:AMP and UTP:UDP:UMP, in mμmoles/g fresh weight, were 100:65:15 and 45:55:110 respectively. The adenosine nucleotide ratio itself can vary: ATP:ADP:AMP rises from 70:200:450 to 140:100:130 during germination of pea seeds (7); ATP:ADP can rise (130:90 to 320:80 in cantaloupe) or fall (120:0 to 15:90 in banana) during onset of the climacteric of a fruit (37). However, when 2 tissues are being compared, it must be remembered that ATP:ADP can also depend heavily on the tissue extraction procedure used. It has already

been shown that phosphatase activity can persist during some extraction procedures, leading to gross changes in the ATP:ADP ratio (9, 10). Results in the present paper further show that it can be very difficult to recover nucleotides quantitatively from plant tissues by simple acid extraction procedures. It is of note that high ATP:ADP:AMP ratios, typically 50:20:10, have always been obtained in this laboratory, using a variety of green and non-green tissues, whenever phosphatase action has been prevented and nucleotide recovery has been nearly quantitative.

Certain compounds that would be expected to be present in *Spirodela* were not identified by the procedures used. All 4 coenzymes have been found in leaf tissue in measurable amounts: NAD, 50 mμmoles/g fresh weight; NADP, 15 mμmoles/g; NADH 1.5 mμmoles/g and NADPH, 8 mμmoles/g (22), and have also been demonstrated in cultured tobacco cells in concentrations approximately 0.15 the above (41). They would have been broken down under the extracting and chromatographing conditions employed here. ADP glucose has been found in developing rice grains [30 mμmoles/g fresh weight, (31)], but if present was probably largely broken down, like UDPglucose, in the alkaline solvent. Other UDP sugars could also have been present (32).

Some of the data presented in this paper merely confirm those obtained for other tissues; others appear to be the first records of the amounts of those esters. However, the main object of this paper has been to present all values for one tissue at one time; and to measure not only the amounts of the identifiable compounds, but also the amounts and nature of the unidentifiable ones. In this way it has been possible to set up a balance sheet for phosphorus in this plant, in which about 98 % of the tissue phosphorus has been accounted for in known compounds. The study should be of value, not only in establishing the amount of each identified compound, but also in fixing limits on the amounts of other compounds that might be present. Occasional compounds, for example phytic acid, will undoubtedly be present in widely differring amounts in different tissues. It has been the experience of this laboratory that the relative proportions of most of the various soluble phosphate esters are remarkably consistent from tissue to tissue and time to time, and in this respect, the present values are typical.

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