Effects of Moisture Stress on Acid-Soluble Phosphorus Compounds in Trifolium Subterraneum [1, 2]

A. M. Wilson [3] and R. C. Huffaker

Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture, and Department of Agronomy, University of California, Davis, California

Little is known about phosphorus metabolism in plants suffering from moisture stress. Several metabolic processes, which are completely dependent upon phosphorylated intermediates, are noticeably affected by moisture stress conditions. Photosynthetic rate decreases markedly in severely stressed plants (13). Rate of fixation of CO_2 in the dark decreases (12). Above a diffusion pressure deficit (DPD) of about 5 atmospheres, incorporation of glucose into cell-wall polysaccharides decreases with an increase in DPD (10). Such observations indicated the importance of determining the effects of moisture stress on phosphorylated intermediates involved in these processes.

In the present study, subterranean clover plants (*Triofolium subterraneum* L. cv. Mt. Barker) were subjected to a brief period of soil moisture depletion. Moisture stress caused decreases in a number of organic phosphorus compounds. Increases in concentrations of organic phosphorus compounds during a 24-hour period after irrigation indicated a partial recovery from the effects of moisture stress.

Materials and Methods

Growth of Plants. Subterranean clover was planted in 3 kg of Auburn fine silty loam fertilized with 20 mg phosphorus and 100 mg nitrogen per kilogram of oven-dry soil. Phosphorus was applied as H_3PO_4 and nitrogen as NH_4NO_3. Phosphorus was labeled with 200 μc of P^{32} per flat. Approximately 125 seedlings emerged within 7 days in each of the 18- by 26-cm plastic flats. Plants were grown in a controlled environment with a light intensity of 1000 ft-c, temperature of 20°, and daylength of 15 hours. Relative humidity was not controlled, but varied from 60 to 80 % during the day and from 90 to 95 % during the night. Plants were irrigated daily during the preliminary growing period.

Moisture-Stress Treatments and Procedure at Harvest. Beginning 3 weeks after emergence, water was withheld in sequence so that all plants would have been exposed to the desired period of moisture depletion on the day of harvest. After exposure to stress and 24 hours before harvest, one treatment was irrigated so that recovery from moisture stress could be measured. All plants were harvested on the same day. Plants were harvested within 6 to 8 hours after the beginning of the light period to avoid diurnal fluctuations in levels of phosphorus compounds.

Relative turgidity was determined essentially as described by Weatherly (15), except that whole leaves rather than discs were used and correction was not made for loss in dry weight (about 8 %) during floating.

Three plants from each flat were harvested and dried at 100° for 24 hours to determine percent moisture. The remaining plants were clipped, quickly frozen in liquid nitrogen, and stored in liquid nitrogen until extraction. Plant materials from 4 flats in one experiment and from 3 flats in a second experiment were combined for a single analysis.

Soil moisture was determined gravimetrically at harvest. Percentage moisture at harvest and records of daily water loss were used to calculate changes in soil moisture throughout the drying cycle. Disturbed soil samples were used in preparing the soil moisture characteristic curve (11).

Extraction of Acid-Soluble Phosphorus Compounds. Plant material (about 10 g dry wt) was homogenized twice in 120 ml of 0.6 N $HC10_4$ at 0°. Homogenizing a third time removed little additional phophorous. The cell debris was removed by centrifuging at 35,000 × *g* for 10 minutes, and the supernatant solution was stored at − 15° after neutralizing with 4 N KOH and removing the $KC10_4$ precipitate.

Anion-Exchange Chromatography. A variable-gradient device similar to the one described by Peterson and Sober (9) and a modification of the formic acid solvent system described by Hurlbert et al. (7) were used to separate phosphorus compounds. The concentration gradients of formic acid and ammonium formate are given in figure 1.

The neutralized extract in a volume of 250 to 300 ml was adsorbed to the anion-exchange column (Dowex-1 X-8, 200 to 400 mesh, 1 × 40 cm) at 0°. Column separation was carried out at room

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[3] Plant Physiologist, Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture. (Present address: Department of Forestry and Range Management, Washington State University, Pullman, Washington.)

temperature.

Radioassay of Phosphorus Compounds. Portions of the fractions collected were pipetted into concentric-ringed stainless steel planchets and dried on a hot plate. P^{32}-labeled compounds were measured with a gas flow Geiger-Müller counter. Total phosphorus in 3 fractions of the inorganic phosphate peak was determined colorimetrically, and the specific activity of phosphorus for each column was calculated. Radioactivity measurements for all fractions were then converted to μmoles of phosphorus per 10 g dry weight of plant material. The specific activity of samples counted one week after harvest was 4000 cpm per μmole P.

Paper Chromatography. Fractions from individual peaks were pooled and passed through a cation-exchange column (Dowex-50, H form) to remove ammonium ions from the effluent. In this step a large part of the pigments in the effluent were adsorbed to the cation-exchange resin. Samples were lyophilized to dryness and taken up in a small volume of water for paper chromatography.

The chromatography procedures described by Bieleski and Young (6) were used in the identification of unknowns. Chromatograms were developed descendingly at room temperature in n-propyl acetate: 90 % formic acid: water 11: 5: 3 v/v or in n-propanol: ammonium hydroxide of 0.90 specific gravity: water 6: 3: 1 v/v. Phosphorus compounds were detected with a perchloric-molybdic acid spray (2), and aldoses were detected with aniline phthalate (8).

Chemical Analyses. Chemical determinations were made as follows: Total P and P_i, modification of the Fiske-Subbarow method (4); hexose, modification of the anthrone method (3); fructose, modification of the cysteine-carbazole method (3); ribose, modification of the orcinol method (1); P-glycerate, 4,5-dihydroxy-2, 7-naphthalenedisulfonic acid (chromotropic acid) method (5). Because of interfering substances in the effluent from the column, phosphorylated compounds were purified on a second column before sugar, P-glycerate, and UV absorbance measurements were made.

Results

Identification of Phosphorus Compounds. Twelve detectable peaks were separated with the variable gradient of formic acid and ammonium formate shown in figure 1. Peaks are numbered with Roman numerals.

Fractions in peak IV contained equal amounts of phosphate and hexose (anthrone determination). Glucose-6-P and fructose-P and perhaps small amounts of other sugar phosphates were present in peak IV. Fructose-P, identified on the basis of its spectrum in the cysteine-carbazole reaction, was detected in the trailing fractions of peak IV. Fructose-P accounted for about 14 % of the phosphorus in peak IV. All fractions of peak IV except those containing fructose-P were combined and glucose-6-P

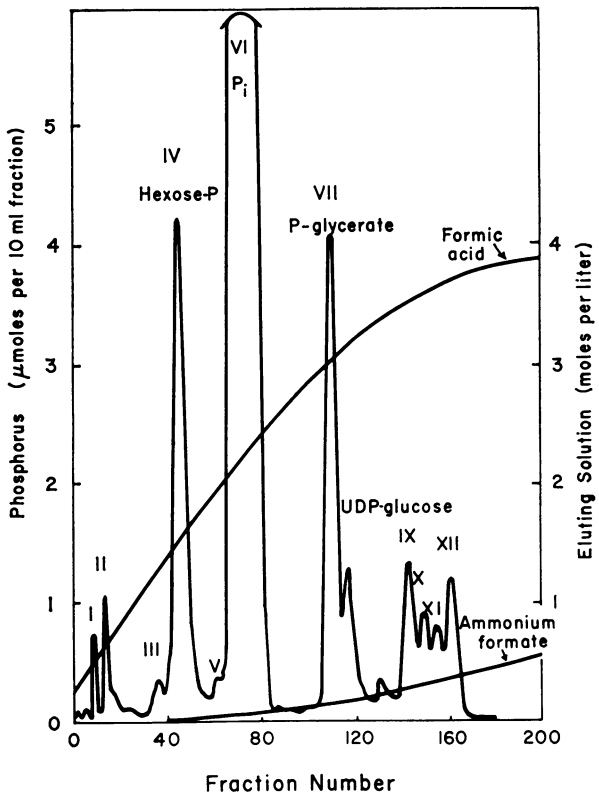


FIG. 1. Separation of acid-soluble phosphorus compounds on an anion-exchange column (Dowex-1 X-8, 200 to 400 mesh, 1 × 40 cm). The indicated gradient was obtained using a 5-chamber variable gradient device (9). Formic acid concentrations in chambers 1 to 5 were initially 0.25, 2.0, 4.0, 4.0, and 3.0 N, respectively. In addition chambers 4 and 5 contained 0.6 M and 0.8 M ammonium formate, respectively. Initial chamber volume was 500 ml. Flow rate was 40 ml per hour. The phosphorus compounds were extracted from 10 g (dry wt) of plant material.

was determined in the glucose-6-P dehydrogenase assay using a purified enzyme obtained from a commercial source. Glucose-6-P accounted for 86 % of the phosphorus in these fractions. Fractions containing fructose-P were not used in the assay because the enzyme preparation slowly isomerized fructose-P.

Fractions in peak VI contained over 90 % P_i as determined by phosphate analyses before and after acid hydrolysis.

P-glycerate from subterranean clover and authentic P-glycerate gave identical spectra in the chromotropic acid reaction (5). P-glycerate accounted for all of the phosphate in peak VII.

Fractions in peak VIII contained the expected ratio of glucose (anthrone), phosphate, ribose (orcinol), and uridine (absorbance at 260 mμ). After hydrolysis (1 N HCl at 100° for 7 min), the sugar moiety was identified as glucose by cochromatography with authentic glucose. The compound was

Table I. *Travel Characteristics of Phosphorus Compounds on Paper Chromatograms*

Compound	Rp*, **	
	Acidic solvent	Basic solvent
Glucose-6-P		
Unknown	65	111
Known	65	(111)
Mixture***	66	...
Fructose-6-P		
Unknown	56	137
Known	56	(143)
Mixture	57	...
3-phosphoglycerate		
Unknown	88	115
Known	82	(115)
Mixture***	88	...
UDP-glucose		
Unknown VIII	17	...
Known	15	...

* $R_p = \frac{\text{Travel of compound}}{\text{Travel of } P_i} \times 100.$

** Values in parentheses are from Bieleski and Young (6).

*** The mixture of known and unknown traveled as a discrete spot.

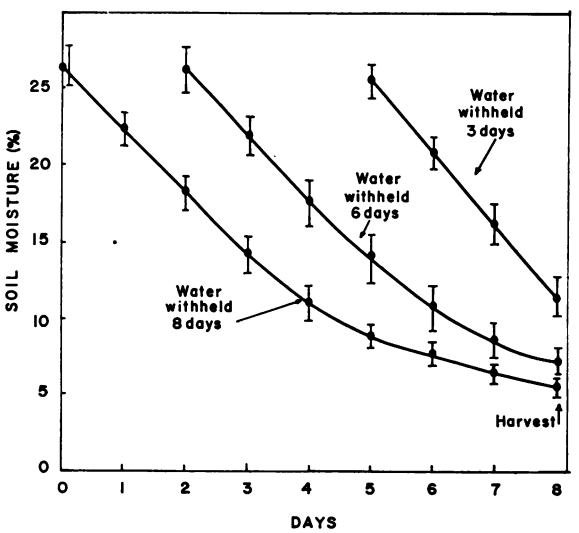
identified as a uridine nucleotide on the basis of its characteristic UV spectra at pH 7 and 11.

Compounds which were not identified did not absorb UV radiation at wavelengths typical of nucleotides. Although traces of sugar diphosphates might have been present in peaks IX to XII, none were detected in the anthrone (except in peak IX), cysteine-carbazole, and orcinol tests. A small part of peak IX reacted in the anthrone test (0.14 μmole per μmole of P using glucose as the standard). ATP added to an extract of P^{32}-labeled plant material was eluted with peak XI; however, paper chromatography of peak XI indicated that although a small part of the phosphate traveled with ATP, the major component of peak XI was not ATP.

Travel of the identified compounds from subterranean clover with authentic compounds on anion-exchange columns and paper chromatograms corroborated these identifications (table I).

Change in Concentration of Phosphorus Compounds during Moisture Depletion. To measure the effect of moisture stress on concentrations of acid-soluble phosphorus compounds, we withheld water from subterranean clover plants for 0, 3, 6, or 8 days. At harvest, plants not irrigated for 3 days showed no evidence of water deficit; plants not irrigated for 6 days were visibly wilted; and plants not irrigated for 8 days had remained visibly wilted for 2 days. The 2 replications were run consecutively. The change in soil moisture per cent during the drying cycle is illustrated in figure 2.

Percentage soil moisture, soil moisture tension, relative turgidity of the unifoliate leaf, and moisture content of the unifoliate leaf at harvest are given in table II. Because of small differences in rate of water depletion among flats, plant material used for a given treatment represented a range of relative turgidity values.



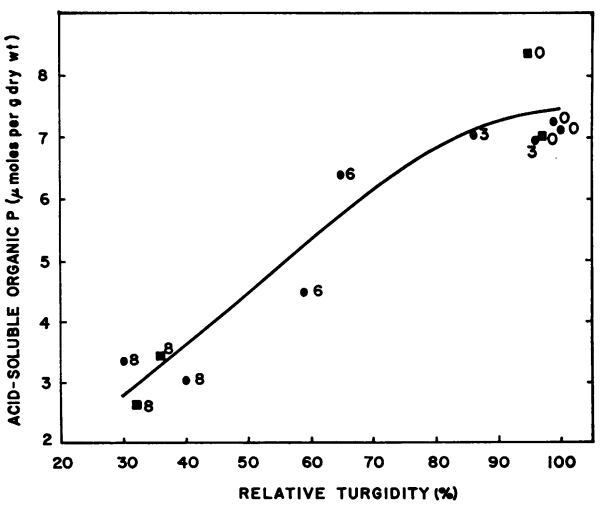


FIG. 2 (*left*). Change in soil moisture percent during the drying cycle. The percentage moisture at harvest was determined gravimetrically; other points were calculated from records of daily water loss during the drying cycle. Each point represents the average moisture percent of soil from 8 flats (4 in each replication). Standard deviations are indicated by vertical lines at each point.

FIG. 4 (*right*). Decrease in total acid-soluble organic phosphorus with decreasing relative turgidity. Each point is the organic phosphate from a single column corrected for phosphate represented by the base line. Relative turgidity is the average of flats combined for analysis. Conditions at harvest are given in table II (circles) and table IV (squares). The number by each point indicates days water was withheld.

Table II. *Percent Soil Moisture, Soil Moisture Tension, Relative Turgidity of Unifoliate Leaf, and Percent Moisture in Unifoliate Leaf at Harvest*

These values are means and standard deviations of 8 samples taken from 8 separate flats.

Days H_2O withheld	Soil Moisture %	Soil Moisture tension atm	Relative turgidity %	Moisture in unifoliate leaf %
0	23.9 ± 0.7	0.36	100 ± 1	85.8 ± 1.8
3	11.5 ± 1.2	3.5	91 ± 12	84.2 ± 1.3
6	7.2 ± 0.8	>15	62 ± 11	72.6 ± 3.7
8	5.7 ± 0.5	>15	34 ± 12	62.4 ± 6.6

The single drying cycle did not have a measurable effect on dry weight of top growth. The average for the 4 treatments was 2.8 g per flat.

The elution chromatograms for plants not irrigated for 0, 6, and 8 days are shown in figure 3. While the position of peaks varied only a few fractions among columns, differences in the separation of closely associated peaks may be noted. In some instances peak V was clearly distinguishable from P_i. UDP-glucose sometimes appeared as a shoulder on the P-glycerate peak.

Total acid-soluble organic phosphorus remained relatively constant in plants whose relative turgidity was from 80 to 100 %, but decreased in plants whose relative turgidity was from 50 to 75 % (fig 4). In severely wilted plants (relative turgidity 20 to 45 %) the concentration of acid-soluble organic phosphorus decreased to less than half that in control plants.

The changes in amount of phosphorus in individual peaks or in closely associated peaks are summarized in table III. Phosphorus represented by the base line was subtracted from the total phosphorus in each peak. Hexose-P decreased from approximately 22 μmoles per 10 g dry weight in plants receiving

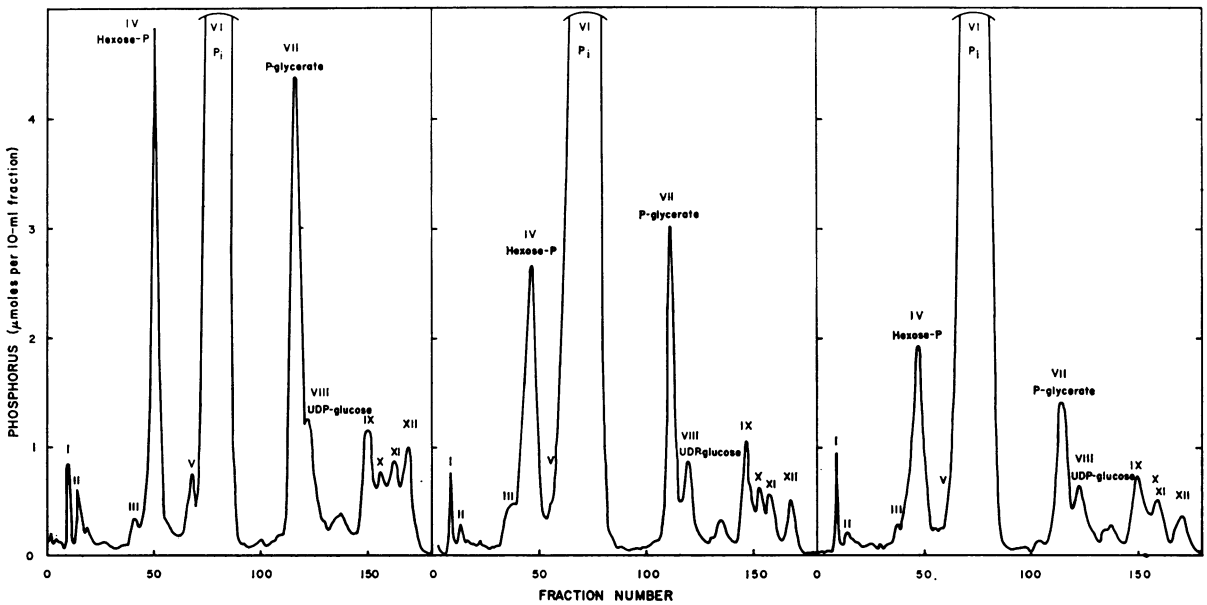


FIG. 3. Separation of phosphorus compounds extracted from 10 g of plant material (dry wt). Phosphate compounds were extracted from plants irrigated daily (left), not irrigated for 6 days (center), and not irrigated for 8 days (right). Soil moisture depletion during the drying cycle is given in figure 2. Conditions at harvest are given in table II.

Table III. *Effect of Water Deficit on Concentration of Acid-Soluble Phosphorus Compounds in Subterranean Clover*

Days H_2O withheld**	Phosphorus peak*						
	I	III & Hexose-P	V & P_i	P-glycerate & UDP-glucose	IX	X & XI	XII
0	0.92 ± 0.06	22.4 ± 0.8	314 ± 27	28.2 ± 1.5	6.2 ± 0.2	7.0 ± 0.2	5.5 ± 0.3
3	0.69 ± 0.03	24.0 ± 3.4	280 ± 34	25.6 ± 0.4	5.4 ± 0.0	6.8 ± 0.4	6.1 ± 1.9
6	0.78 ± 0.04	19.6 ± 0.8	294 ± 32	19.0 ± 3.5	4.6 ± 0.5	5.7 ± 1.9	3.6 ± 2.0
8	1.10 ± 0.10	12.4 ± 0.4	254 ± 16	10.8 ± 0.6	3.2 ± 0.8	2.0 ± 0.5	1.1 ± 0.5

* Micromoles phosphorus per 10 g dry wt. Values are means and average deviations of 2 replications.
** Conditions at harvest are summarized in table II.

adequate moisture to 12 μmoles in severely stressed plants. Correction was not made for the small amount of phosphate in peak III associated with hexose-P. The combined P-glycerate and UDP-glucose peak decreased to 11 μmoles phosphorus per 10 g dry weight, approximately one-third that in control plants. In elution chromatograms where UDP-glucose separated well from P-glycerate the concentration of UDP-glucose was approximately 2.5 μmoles per 10 g dry weight in control plants as compared with 1.6 μmoles in severely stressed plants. A decrease in amount of unknowns IX, X, XI, and XII with increasing water deficit was also noted.

Moisture stress had no effect on unknown I. Reproducible results were not obtained for unknown II. The levels of inorganic phosphate were variable, but no change due to moisture stress was detected.

Recovery of Plants after Irrigation. Plants which had depleted soil moisture for 8 days were irrigated 24 hours before harvest. For comparison, a second group of plants were allowed to deplete soil moisture 8 days, but were not irrigated before harvest. The control plants were irrigated daily. The 2 replications were run concurrently. Plant material from 3 flats was used for a single analysis.

After 8 days of moisture depletion the relative turgidity of the first 2 sets of plants was similar (table IV). The relative turgidity of plants after recovery from moisture stress was equal to that of control plants.

Results summarized in table V indicate that changes in concentration of phosphorus compounds due to severe moisture stress were comparable with those reported in table III. The level of P_i was not affected by moisture stress. The amounts of hexose-P and phosphorus in the combined P-glycerate and UDP-glucose peak doubled during the 24-hour recovery period. A small increase in amount of unknown IX and a larger increase in unknowns X and XI were observed. The amount of phosphorus in peak XII did not change measurably during the recovery period.

Discussion

The primary effects of moisture stress may be many steps removed from the metabolic disturbance observed in this study. Evidently changes in metabolism occur which lower the steady state levels of phosphorylated intermediates. A disruption of phosphorylation processes (18), increase in phosphatase activity (16, 17), increase in respiration (18), stomatal closure (14), and inactivation of enzyme systems involved in the synthesis of phosphorylated intermediates are possible explanations for the lower steady state concentrations. These areas need further exploring before their relative importance can be established.

With the decrease in concentrations of organic phosphorus compounds, a corresponding increase in P_i perhaps would be expected. Additions to the P_i pool resulting from hydrolysis of organic compounds might have been partly offset by decreased uptake of phosphate from the soil during water deficit. The fact that the concentration of P_i did not change during the drying cycle suggests that P_i was not limiting to phosphorylating reactions.

The observation that little change occurred in the concentration of phosphorylated intermediates until

Table IV. *Percent Soil Moisture, Soil Moisture Tension, Relative Turgidity of the Second Trifoliate Leaf, and Percent Moisture in the Second Trifoliate Leat at Harvest.*

These values are means and standard deviations of 6 samples taken from 6 separate flats.

Days H_2O withheld	Soil moisture %	Soil Moisture tension atm	Relative turgidity %	Moisture in second trifoliate leaf %
8	5.1 ± 0.2	>15	35 ± 6	61.0 ± 3.8
8 Before irrig.*	...	...	32 ± 6	63.1 ± 1.7
At harvest	23.4 ± 0.9	0.40	95 ± 2	82.2 ± 0.9
0	22.9 ± 1.9	0.44	96 ± 3	80.7 ± 1.9

* Measured before irrigation.

Table V. *Increase in Concentration of Acid-soluble Phosphorus Compounds After Irrigation*

Days H_2O withheld**	Phosphorus peak*						
	I	III & Hexose-P	V & P_i	P-glycerate & UDP-glucose	IX	X & XI	XII
8	1.34 ± 0.18	11.1 ± 1.2	254 ± 17	9.7 ± 0.4	3.3 ± 0.3	1.6 ± 0.2	1.4 ± 0.1
8 + H_2O***	1.34 ± 0.00	21.4 ± 0.9	224 ± 2	21.4 ± 0.1	4.8 ± 0.1	5.6 ± 0.1	2.0 ± 0.2
0	0.88 ± 0.16	25.0 ± 1.7	240 ± 1	28.6 ± 3.6	6.4 ± 0.2	7.4 ± 0.7	5.6 ± 0.3

* Micromoles phosphorus per 10 g dry wt. Values are means and average deviations of two replications.
** Conditions at harvest are given in table IV.
*** Plants which had not been irrigated for 8 days were watered 24 hours before harvest.

near wilting does not mean that moderate moisture stress was without effect. The synthesis and utilization of intermediates could be affected equally, while the steady state concentrations would remain the same. Similarly, increases to near normal during the 24-hour recovery do not indicate that synthesis and utilization of intermediates had returned to normal.

A low or high concentration of an intermediate does not necessarily indicate that the reaction in which it is a substrate will proceed at a low or high rate. Available information concerning reaction controlling mechanisms in cells, enzyme activities, and substrate concentrations is inadequate to predict what substrate concentrations would be limiting to the rate of a reaction. Nevertheless, it is interesting to conjecture that during drought the depression of polysaccharide synthesis (10), for example, is partially due to low levels of UDP-glucose. Similarly, the rates of other reactions could be affected by changing concentrations of phosphorylated intermediates during drought and recovery.

Intermittent rainfall during growth of subterranean clover on arid rangelands results in periods of severe water deficit followed, at times, by adequate moisture. Under such conditions changes in levels of phosphorus compounds may be comparable with those observed in these experiments.

Summary

Concentrations of acid-soluble phosphorus compounds in month-old *Trifolium subterraneum* L. plants were measured at several stages of soil moisture depletion and after a 24-hour recovery from moisture stress. Phosphorylated compounds were extracted with 0.6 N $HC10_4$ at 0° and separated on anion-exchange columns. Concentrations of hexose phosphate, phosphoglycerate, uridine diphosphate glucose, and several unidentified phosphorus compounds decreased in plants whose relative turgidity was 50 to 75 %. In severely wilted plants (relative turgidity 20 to 45 %), the concentration of most phosphorylated compounds decreased to less than half that in plants with a relative turgidity near 100 %. The concentration of inorganic phosphate, however, was not affected by moisture stress.

Plants which had depleted soil moisture until severe water deficits developed were irrigated 24 hours before harvest. Levels of most organic phosphorus compounds which had decreased as a result of moisture stress showed a marked increase during the 24-hour recovery period.

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