operation by the design and size of the plant and by the rate of production. To ascertain how much ammonia would be absorbed under granulation conditions but with low gas flows, ammonia at levels of 4.0 and 4.9 pounds per unit were added over varying lengths of time. The results (Table XII) show that longer times gave higher efficiencies, but at the high rate of addition, a maximum absorption value just over 4 pounds per unit occurs in the apparatus using Product 1. The operating conditions not listed in the table were maintained constant, and the water level used was 100 ml. per 1000 grams of triple superphosphate.

Effect of Particle Size

A portion of run-of-pile triple superphosphate (Product 1) was separated into its constituent particle size fractions, and the fractions were ammoniated at two levels of ammonia addition with constant amounts of water added. The efficiency results appear in Table XIII.

The medium-sized particles exhibited high ammonia absorption, whereas the larger particles did not. This lower absorption of the large particles can be caused by the short reaction time employed (3 minutes). The time necessary for diffusion of gases into the interstices of the particle, over the relatively short reaction time employed may explain the lower absorption into these larger particles. Should the chemical reaction of ammonia with monocalcium phosphate cause complete blocking of inkbottle pores, suggested by Caro and Freeman (*2*) as existing in triple superphosphate, one would expect reduced absorption. Also, there is a greater probability of pores that are blocked off from any exterior surface in large particles which would reduce contact of solid with gas. The low efficiency of the −100 mesh size was due to the rapid granulation which occurred, giving subsequent isolation of a large portion of the material from further possibility of contact with ammonia gas. It is evident that the particle size distribution present in the granulation is more important to ammoniation efficiency than the particle sizes charged in the dry state.

Literature Cited

(1) Brook, A. L., *Fertiliser Soc. Engl. Proc.* **47** (1957).
(2) Caro, J. H., Freeman, H. P., J. AGR. FOOD CHEM. **9,** 182 (1961).
(3) Giddings, S. A., DeLapp, D. F., *Ibid.*, **10,** 65 (1962).
(4) Haines, H. W., Jr., Lange, F., *Ind. Eng. Chem.* **48,** 996 (1956).
(5) Hardesty, J. O., Ross, W. H., *Ibid.*, **29,** 1283 (1937).
(6) *Ibid.*, **30,** 668 (1938).
(7) Highett, T. P., Slack, A. V., J. AGR. FOOD CHEM. **5,** 814 (1957).
(8) Kumagai, R., Rapp, H. F., Hardesty, J. O., *Ibid.*, **2,** 25 (1954).
(9) Newitt, D. M., Papadopoulos, A. L., *Fertiliser Soc. Engl. Proc.* **55,** (1959).
(10) Sauchelli, V., *Agr. Chem.* **1956,** p. 34.
(11) White, L. M., Hardesty, J. O., Ross, W. H., *Ind. Eng. Chem.* **27,** 562 (1935).

Received for review July 23, 1962. Accepted October 23, 1962.

PHOSPHORUS STATUS OF CROPS

Phosphorus Fractions in High and Low Phosphate Plants

CHARLES E. BARR and ALBERT ULRICH

Plant Department of Soils and Nutrition, University of California, Berkeley, Calif.

Lima bean plants were grown in nutrient solutions at low and high levels of phosphate addition. Comparable samples of leaflet-blades and petioles were collected for fresh-freezing and for oven-drying. The fresh-frozen samples were analyzed for orthophosphate, organic phosphate soluble in trichloroacetic acid, and acid-insoluble phosphate. Dried materials were analyzed for acetic acid–soluble phosphate and insoluble phosphate. The results indicate that much of the organic phosphate is broken down as a consequence of the drying process; nevertheless, the use of dried samples for assessing the phosphorus status of the plant would seem to be as adequate as the use of fresh materials, under ordinary conditions. For fresh-frozen material, the differences in phosphate supply produce little variation in acid-insoluble organic phosphate; intermediate variation in acid-soluble organic phosphate; and a very large variation in orthophosphate, with the orthophosphate reflecting the external phosphate most closely.

CHEMICAL ANALYSIS of plant parts as a means of evaluating the nutritional status of field crops has generally involved oven-dried materials. Although the advantages of dried over fresh materials have been well appreciated (*17*), it is also true that during the process of drying a considerable number of chemical transformations involving some of the essential elements will occur, resulting in a loss of some information. As the first step in an attempt to discover which phosphorus compound or fraction present in fresh material is best correlated with the adequacy of the phosphate supply to the plant, the amounts of phosphorus in the three conventional phosphate fractions (orthophosphate, acid-soluble organic phosphate, and acid-insoluble phosphate) were determined on fresh-frozen parts of high- and low phosphate lima bean plants. For comparison, analyses of oven-dried plant parts were also run.

Methods and Materials

Growing of Plants. Lima bean seeds (*Phaseolus limensis*, var. Fordhook concentrated), which had been dusted with Arasan, dieldrin, and Delsan, were planted in vermiculite on November 7, 1958, and watered daily with distilled water. On November 20, healthy seedlings were selected and set out (cotyledons attached), five per 20-liter container, in nutrient solutions at four phosphate concentrations. All solutions at the time of transplanting contained the following concentrations of mineral nutrients: 3 mmoles KNO_3, 1 mmole $MgSO_4$, 0.5 mmole NaCl, 0.5 mmole

K_2SO_4, 2.5 mmoles $Ca(NO_3)_2$, 0.25 mmole Na_2SiO_3, 0.25 p.p.m. B (as H_3BO_3), 0.25 p.p.m. Mn, 0.025 p.p.m. Zn, 0.01 p.p.m. Cu, 0.005 p.p.m. Mo (as MoO_3), and 2.5 p.p.m. Fe (as EDTA). Phosphate as KH_2PO_4 was provided at four levels: three containers at zero P level, two at the 1/128 P level (1/64 mmole), three at the 1P level (2 mmoles), and two at the 12 P level (24 mmoles). Adjustment of the pH of the zero P and 1/128 P solutions to 5.6 or 5.7 was made with sulfuric acid; pH of the 1 P solutions was left at 6.3 and that of the 12 P solutions at 5.2, their original values. About halfway through the 9-week growth period a second dose of nutrients (excluding KH_2PO_4) was added, equal in amount to that provided in the original solutions. Plants were harvested January 23, 1959.

Sampling. Samples were collected according to leaf position for each of the five plants of a container and placed together. The leaf nearest the shoot apex having leaflet-blades greater than 1 inch in length is designated as leaf No. 1. All shoots having at least two leaves of the required size were sampled; smaller shoots were combined to form a residual sample.

In order to have comparable samples for parallel analyses, i.e., on fresh-frozen and on oven-dried materials, one lateral leaflet-blade of a trifoliolate leaf was taken for freezing, and the paired leaflet-blade was taken for oven-drying. Petioles of a given leaf position were arbitrarily separated into two approximately equal lots, one for freezing and the other for drying. Rachises and petiolules were discarded. Fibrous roots were blotted with cheese cloth, cut up, and divided into two lots.

Samples to be frozen were weighed, put into plastic bags, and then placed between sheets of dry ice. The frozen samples were stored at −19° C. until analyzed. The samples to be dried were put into paper bags and placed in a forced-draft oven at 80° C. for 24 hours. After drying, the samples were weighed, ground in a Wiley Mill or, for smaller samples, in a "Wig-L-Bug" electric mortar (Spex Industries, Inc., Queens Village, N.Y.), and stored in stoppered plastic vials.

Analyses. Fresh-Frozen Samples. Frozen samples, ranging in weight from 0.5 to 12 grams, were extracted with 75 to 200 ml. of 2% TCA (trichloroacetic acid) depending on the amount of phosphate anticipated. Pulverized dry ice was added to the measured volume of 2% TCA in a Waring Blendor until a slushy consistency was obtained. The frozen plant material was added to the TCA slush and blended for 2.5 minutes, after which the resulting slurry (all ice now melted) was filtered (Whatman No. 12) into a 500-ml. Erlenmeyer flask at 0° to 5° C. When filtration was virtually complete, the filtrate flask was stoppered and kept at 3° C. The residue in the filter paper was then washed with 2% TCA at 0 to 3° C. until orthophosphate was no longer detectable (usually three washings; discarded), and kept at 3° C. until analyzed for insoluble P.

Within 48 hours of the extraction, orthophosphate was determined directly on an aliquot of the filtered extract by a method identical with that given by Johnson and Ulrich (*12*) with the exception that the treatment with H_2O_2 over steam was omitted to avoid the possibility of hydrolysis or other degradation of the organic phosphate present. The work of Weil-Malherbe and Green (*18*) suggests that the breakdown of labile phosphates, such as ATP, would be negligible under the conditions of the analysis. Creatine phosphate and acetyl phosphate are considerably more labile than ATP (*18*), but the presence of these substances in higher plants has not been reported, and presumably they are lacking or are present only in very small amounts. The highest concentration of TCA present in any of the analysis mixtures (0.2%) did not affect the amount of orthophosphate detected, agreeing with the results of Dellamonica *et al.* (*5*). These authors also reported that silicate in the presence of TCA is also detected, i.e., gives a blue color like phosphate. Since silicate had been used in the nutrient solutions, a silicate series was run with two levels of TCA, 0.025% and 0.25%, covering the range of the present experiment. No effect of silicate was detected in the phosphate determinations. Presumably the difference is attributable to differences in the analytical methods used. Dellamonica *et al.* used the method of Fiske and Subbarow (*8*) with either ferrous sulfate or aminonaphtholsulfonic acid as the reducing agent, whereas stannous chloride served here.

For determination of total acid-soluble phosphorus, a 25- or 50-ml. aliquot of the TCA extract plus 1.0 ml. of 50% $Mg(NO_3)_2.6H_2O$ were brought to dryness in a 70-ml. evaporating dish and placed in a cold muffle furnace set at 600° C. for 5 hours. The ash was brought into solution with 5 ml. of 7% HCl on the steam bath, brought to dryness, and taken up in 25 ml. of 2% acetic acid. Phosphate was determined on an aliquot as above. Acid-soluble organic P is assumed to be the difference between total acid-soluble P and orthophosphate P. Among the compounds expected in this fraction are sugar phosphates, various nucleotides, phosphoglyceric acid, thiamin pyrophosphate, and phosphoryl choline (*15*). However, also possibly included in this fraction would be inorganic pyrophosphate and metaphosphate (cyclic polyphosphate); these have been found in cottonseed meal (*10*) and in *Euglena* (*2*). Although previously the failure to detect inorganic polyphosphates in higher plants seemed to indicate that these substances were limited to lower organisms (*9*, *13*), their presence in spinach leaves was recently reported by Miyachi (*16*). Possibly they occur in this fraction, as well as in the insoluble fraction.

For determination of TCA-insoluble P, the washed residue and filter paper were placed in a large evaporating dish, wetted down with 3.0 ml. of the $Mg(NO_3)_2$ solution, and brought to near dryness on the steam bath. Samples were charred by adding 10 to 15 ml. of 95% ethanol, igniting, and allowing the materials to burn out, after which they were ashed as above. The ash was brought into solution with 10 ml. of 7% HCl, brought to dryness on the steam bath, and taken up in 50 to 75 ml. of 2% acetic acid. An aliquot was analyzed for phosphate as above. This fraction is assumed to contain phosphate from nucleic acids, phosphoproteins, phospholipides (*1*), and possibly inorganic polyphosphates (*16*).

Oven-Dried Samples. Oven-dried materials were analyzed for phosphate soluble in 2% acetic acid by the method given by Johnson and Ulrich (*12*). A 25- to 100-mg. sample was used depending on the amount of phosphate anticipated. A preliminary experiment indicated that organic phosphate may constitute only a few per cent of the total phosphate in this extract, but most or all of it is decomposed to orthophosphate during the peroxide treatment.

Total phosphate analysis of oven-dried material was likewise carried out on a 25- to 100-mg. sample, which was wetted down in a 50-ml. evaporating dish with 1.0 ml. of 10% $Mg(NO_3)_2.6H_2O$ (w./v.) in 95% ethanol and ignited. After charring, ashing was carried out as above.

In preliminary experiments on non-deficient lima bean plants, the total phosphate in blades was 6 to 8% greater for fresh material than for dried, but no such difference was found for petioles. Whatever the source of this discrepancy for blades, it presumably is also present in this experiment, but direct comparison cannot be made since dry-weight measurements were not made with sufficient accuracy. Agreement between frozen and dried petioles cannot be so good as that for blades because of the arbitrary manner necessary for dividing the petioles into two lots for analysis.

Preliminary experiments also indicated that no more orthophosphate is extracted from fresh material with 5% TCA than with 2% TCA, and that no measurable breakdown of organic phosphate into orthophosphate occurred when fresh materials were subjected to

Table I. Comparison of Growth of Lima Bean Plants in Relation to Phosphorus Supply

Phosphate Added[a]	Fresh Weight, Grams			Dry Weight, Grams		
	Tops	Roots	Total	Tops	Roots	Total
Zero P	119	44	163	25.5	4.5	30.0
	125	40	165	28.0	4.4	32.4
1/128 P	130	43	173	28.4	4.2	32.6
	137	52	189	29.7	5.1	34.8
	145	44	189	31.4	4.4	35.8
Total	656	223	879	143.0	22.6	165.6
1 P	404	73	477	58.7	4.6	63.3
	467	76	543	80.7	6.8	87.5
	426	77	503	72.4	6.3	78.7
12 P	363	78	441	63.7	6.9	70.6
	392	75	467	60.2	6.9	67.1
Total	2052	379	2431	335.7	31.5	367.2

[a] P equals 2.0 mmoles of PO_4 per liter of nutrient solution.

freezing. Although TCA extracts were always analyzed for orthophosphate within 48 hours of the time of extraction, extracts kept as long as 3 weeks at 3° C. showed no measurable increase in orthophosphate during this time.

Dried materials extracted with 2% TCA yielded the same amounts of phosphate as those extracted with 2% acetic acid.

Experimental Results

Fresh-Frozen Samples. As indicated in Table I, yields for the 1/128 P plants were only about 12% greater than those for the zero P plants, and since no significant differences were found between them in any of the phosphorus fractions, the two are grouped together as low-P plants. Similarly, the 1 P and 12 P plants are grouped together as high-P plants; there is a 10% reduction in yield in the 12 P plants as compared to the 1 P, which may be partly attributable to the fact that salts crept up the roots of the 12 P plants and deposited on the cotton plugs surrounding the hypocotyls.

The low-P plants were about a third the size of the high-P plants; the former were spindly, sparsely branched, and had small, dull-green leaves. The high-P plants were bushy and had dark green leaves. Both developed flowers, but those on the low-P plants were very small. No fruit developed on either the low- or high-P plants.

If the results of the phosphorus analyses in Figure 1 for the fresh material are examined in terms of the ratio of phosphorus concentration in the high-P plants to that in the low, values ranging from 1.5 to 150 are obtained depending on the phosphate fraction and on the nature and position of the plant part. Orthophosphate ratios are highest, thus most closely reflecting the external supply of phosphate; soluble organic P is intermediate; and insoluble P is the most conservative fraction. The level of insoluble phosphate in the petioles is almost independent of the external supply, but orthophosphate accumulates greatly under high-phosphate provision especially in the older petioles; presumably the lower parts of the stem behave similarly under these conditions. The older blades of high-P plants, however, maintain about the same level of orthophosphate as do the younger ones. Among the plant parts, the roots accumulate not only the highest concentration of orthophosphate but also the highest concentration of soluble organic phosphate when the supply is abundant. When the supply is low, the roots are the lowest among the plant parts in all three fractions.

The ratio of insoluble P in the blades of the high-P plants to that in the low is about three, and this ratio is maintained for each leaf position even though the absolute amounts of insoluble P are dependent on the age of the leaf. This constancy of the ratio is perhaps indicative of a maximum and a minimum phosphorus to protoplasm ratio for high-P and low-P plants or of some process working independently of the nutrient status of the plant.

Oven-Dried Samples. For each of the total phosphorus values of the fresh-frozen samples, there is a parallel result for the dried samples. The ratio of the two varies to some extent because of the variation in dry weight to fresh weight ratio among the different plant parts and among plant parts in different stages of development. In addition, larger discrepancies are to be expected and are present for the petioles because of the unequal division of the petioles as previously discussed. In a few cases, especially for leaf 4, only two or three petioles were available per leaf position for the five plants of a given container. With samples so meager as these, some divergence in the results can be expected. Thus, for example, in petiole 4 of the high-P plants, it is quite probable that the 1380 p.p.m. value for fresh material is too low, and 12,200 p.p.m. for dried material is too high.

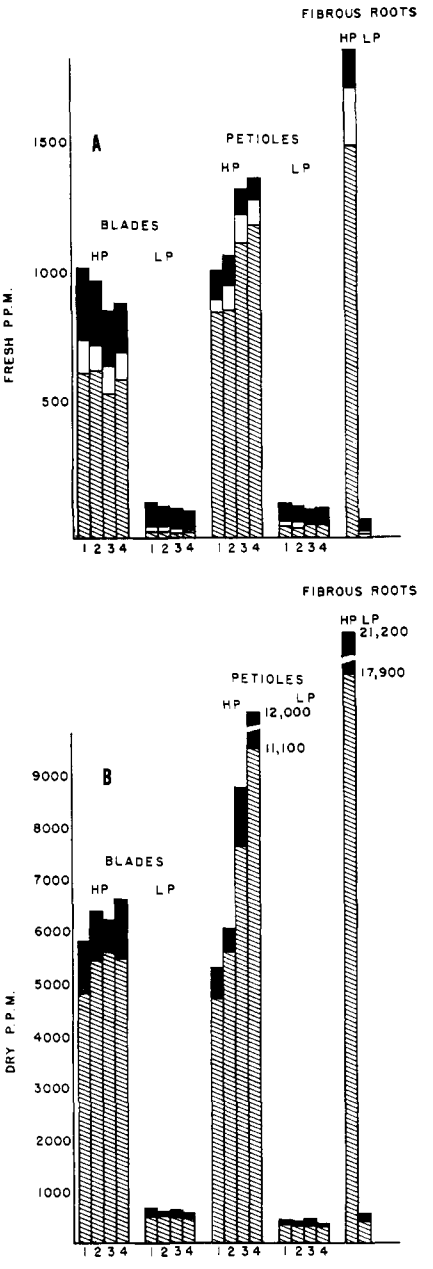

Figure 1. Comparison of phosphorus fractions in fresh frozen (A) and dried (B) plant material of lima bean plants provided with ample (HP) and deficient (LP) amounts of phosphorus

The comparison is made for blades and petioles of leaves 1, 2, 3, and 4 (downward) and for fibrous roots. ■ Insoluble phosphorus; □ soluble organic phosphorus; ▨ orthophosphorus

The proportion of total phosphorus accounted for by soluble phosphate is much larger for the dried material than it is for the fresh (Figure 1). This indicates that a considerable breakdown of insoluble phosphate to the soluble form occurs during drying. For low-P blades, about 70% of the insoluble P is decomposed; for the petioles it is about 58%. Calculations for the high-P plants cannot be made with much accuracy since insoluble P constitutes only a small fraction of the total P.

As previously mentioned, the acetic acid extract of dried materials contains some organic phosphate compounds, and most or all of these are decomposed during the peroxide treatment. A portion of these are perhaps derived from insoluble organic phosphates partially decomposed and thus solubilized during the drying process.

Discussion

Since the two groups of plants in this experiment represent the extremes of phosphate provision, it is impossible to make detailed generalizations for the whole range of phosphate levels. However, it is evident that orthophosphate is the fraction in fresh and dried material which gives the best indication of the magnitude of the phosphorus reserves within the plant, and the use of acetic acid extracts of dried material would seem to be generally adequate for an evaluation of the phosphorus status of the plant even though most of the soluble organic phosphate and part of the insoluble phosphorus compounds are decomposed and solubilized during the drying process. Whether petioles or blades are used does not appear to be critical for the dried material. Petioles tend to provide a better indication of the external supply of phosphate, but there is more variation among the petioles at different leaf positions than there is among the blades; also, petioles of a given leaf position tend to be more variable than the blades. Much of this greater variability of petioles over blades may, however, be due to the small number of petioles in the sample and the arbitrary manner used in separating the petioles into two groups for analysis.

The variation of orthophosphate P in the plant can also be expressed as a per cent of the total P. For fresh materials of high-P plants, the orthophosphate fraction comprises about 65% of the total P in the blades and about 85% in the petioles. In the low-P plants, the orthophosphate fraction in the blades drops to 16% of the total P and the petioles to 25%. Drying the plant material causes the acid-soluble fraction (measured as orthophosphate) in the blades of the high-P plants to rise from about 65% to approximately 86% of the total P, and in the petioles from 85 to 90% of the total P. In the low-P plants, the effects of drying are much more pronounced; the acid-soluble P rises from 16% to nearly 80% of the total P in the blades and from 25% to approximately 75% of the total P in the petioles. These increases in acid-soluble phosphate upon drying are associated with a nearly complete loss of soluble organic P and a commensurate loss of insoluble P.

Possible use of the organic fractions of fresh materials for diagnostic purposes would be warranted in cases in which phosphorus deficiency symptoms occur in the presence of a supposedly adequate supply of orthophosphate within the plant. Biddulph (*3*) has set up experimental conditions in which iron phosphate is precipitated within the tracheary elements of the plant. Here an acetic acid extraction of dried material might bring some of the iron phosphate into solution, and the phosphorus deficiencies within the plant would not be revealed. But an analysis of one of the organic P fractions together with an orthophosphate determination on fresh material would throw some light on the deficiency problem.

Somewhat surprising was the threefold increase of insoluble P in the blades of the high-P over that of the low-P plants (Figure 1). Exactly how much of the phosphorus in this fraction is actively involved in photosynthetic reactions is not known. The recently reported detection of inorganic polyphosphates in spinach leaves (*16*) suggests that they may accumulate in the high-phosphate lima bean plants, serving as a storage form of high energy phosphate bonds, as they do in yeast (*4*). In a review article, Henderson and Le Page (*11*) make the suggestion that most of the ATP and ADP in plants may be bound to some particular component of the cell.

The work of Eaton (*6, 7*) shows that in phosphorus-deficient plants no diminution of carbohydrates is manifested, and at times an accumulation even occurs. This would indicate that in phosphorus-deficient plants the basic synthesis of carbohydrates goes on at a rate that is more than adequate, i.e., in relation to other processes involved in growth. This points to the role that phosphorus may play in mediating the transfer of energy required in a great many synthetic reactions, a role which was first postulated by Lipmann (*14*) and which is now well substantiated. On this basis, the authors suggest that the photosynthetic apparatus tends to monopolize phosphorus when it is scarce, and that other synthetic reactions essential to growth are correspondingly subjected to greater stress.

Acknowledgment

The authors wish to thank Wade L. Berry for his help in culturing and harvesting the plants.

Literature Cited

(1) Albaum, H. G., *Ann. Rev. Plant Physiol.* **3,** 35 (1952).
(2) Albaum, H. G., Schatz, A., Hutner, S. H., Hirshfield, A., *Arch. Biochem.* **29,** 210 (1950).
(3) Biddulph, O., in "Mineral Nutrition of Plants," Emil Truog, Ed., pp. 261–78, University of Wisconsin Press, Madison, Wis., 1951.
(4) Bukhovich, E., Belozerskii, A. N., *Dokl. Akad. Nauk SSSR Biochem. Sect.* **124,** 1147 (1959), *English transl.* **124,** 37 (1959).
(5) Dellamonica, E. S., Bingham, E. W., Zittle, C. A., *Anal. Chem.* **30,** 1986 (1958).
(6) Eaton, S. V., *Botan. Gaz.* **110,** 449 (1949).
(7) *Ibid.*, **111,** 426 (1950).
(8) Fiske, C. H., Subbarow, Y., *J. Biol. Chem.* **66,** 375 (1925).
(9) Hackett, D. P., *Ann. Rev. Plan Physiol.* **10,** 113 (1959).
(10) Hardin, E. B., *S. Carolina Agr. Expt. Sta. Bull.* **8,** 10 (1892).
(11) Henderson, F. J., Le Page, G. A., *Chem. Rev.* **58,** 645 (1958).
(12) Johnson, C. M., Ulrich, A., *Calif. Agr. Expt. Sta. Bull.* **766,** 25 (1959).
(13) Kuhl, A., *Ergeb. Biol.* **23,** 144 (1960).
(14) Lipmann, F., *Advan. Enzymol.* **1,** 99 (1941).
(15) Miettinen, J. K., Savioja, T., *Acta Chem. Scand.* **13,** 1693 (1959).
(16) Miyachi, S., *J. Biochem. Tokyo* **50,** 367 (1961).
(17) Ulrich, A., *Ann. Rev. Plant Physiol.* **3,** 207 (1952).
(18) Weil-Malherbe, H., Green, R. H., *Biochem. J.* **49,** 286 (1951).

Received for review June 29, 1962. Accepted October 26, 1962.