Analysis of Phosphate Esters in Plant Material

EXTRACTION AND PURIFICATION

By F. A. ISHERWOOD AND F. C. BARRETT

Food Research Institute, Low Temperature Research Station, Cambridge

(Received 8 March 1967)

1. A critical study was made of the quantitative extraction of nucleotide and sugar phosphates from plant tissue by either boiling aqueous ethanol or cold trichloroacetic acid. The effect of the extraction technique on the inactivation of the enzymes in the plant tissue and the possibility of adsorption of the phosphate esters on the cell wall were especially considered. 2. In the recommended method the plant tissue was frozen in liquid nitrogen, ground to a powder and then blended with cold aqueous trichloroacetic acid containing 8-hydroxyquinoline to prevent adsorption. 3. The extract contained large amounts of trichloroacetic acid, cations, chloride, sugars, amino acids, hydroxy organic acids, phytic acid, orthophosphoric acid and high-molecular-weight material including some phosphorus-containing compounds. All of these were removed as they were liable to interfere with the chromatographic or enzymic assay of the individual nucleotide or sugar phosphates. 4. The procedure was as follows: the last traces of trichloroacetic acid were extracted with ether after the solution had been passed through a column of Dowex AG 50 in the hydrogen form to remove all cations. High-molecular-weight compounds were removed by ultrafiltration and low-molecular-weight solutes by a two-stage chromatography on cellulose columns with organic solvents. In the first stage, sugars, amino acids, chloride and phytic acid were separated by using a basic solvent (propan-1-ol-water-aqueous ammonia) and, in the second stage, the organic acids and orthophosphoric acid were separated by using an acidic solvent (di-isopropyl ether-formic acid-2-methylpropan-2-ol-water). The final solution of nucleotide and sugar phosphates was substantially free from other solutes and was suitable for the detection of individual phosphate esters by either chromatography or enzymic assay. 5. The recovery of D-glucose 6-phosphate or adenosine 5'-triphosphate added to a trichloroacetic acid extract simulating that from peas and potatoes, and isolated according to the standard procedures, was better than 95%. Estimation of naturally occurring D-glucose 6-phosphate and adenosine 5'-triphosphate in the initial extract of peas and potatoes and in the final purified extract also indicated a recovery of about 95%. A similar estimation of uridine diphosphate glucose in potatoes showed that little or no breakdown occurred.

Experience indicates that the direct application of the classical procedures for the determination of phosphate esters, such as fractionation of the barium salts by precipitation with alcohol, or column (Khim & Conn, 1953) or paper (Rappaport & Chen, 1960) chromatography applied to unpurified extracts from higher plants, is unsatisfactory because many of the non-phosphorylated substances present interfere seriously with the clear-cut separation of the phosphate esters. It seems that at least a preliminary partial fractionation is necessary before the application of these methods and, in view of the complexity of the mixture of phosphate esters likely to be present, the complete removal of other substances is desirable.

In the present paper the whole extraction procedure has been considered in detail as a necessary preliminary to the final analysis either by column or paper chromatography, or if individual esters only are being studied, by means of specific enzymes (Slater, 1953; Newsholme & Randle, 1961; Fawaz & Fawaz, 1962). Interest has centred on the low-molecular-weight water-soluble phosphate esters; the high-molecular-weight compounds (mol.wt. over 30000) such as nucleic acids and polysaccharides, which are also present in the crude extract, have been excluded by filtration through viscose film. Special attention has been given to the denaturation of the enzymes in the biological tissue before the extraction of the phosphate esters since

the small amounts present under normal physiological conditions and their close association with highly active enzyme systems makes it difficult to control the complex reactions that occur during the killing of the tissue, especially if a heat treatment is used (Isherwood & Niavis, 1956). The speed with which the various esters are interconverted is shown by the work of Bielecki & Laties (1963) on the uptake of [^{32}P]phosphate by potato slices. They showed that many of the phosphate esters were labelled in about the same time and that the time to reach half-saturation was of the order of a minute. The fact that many of the esters were labelled at the same apparent rate means that the rate of interconversion of these phosphate esters must be extremely high as compared with the rate of diffusion of orthophosphate into the slices, otherwise the esters would have been labelled at different rates. Apart from the enzymes which are concerned with the interconversion of the various phosphate esters, there are present in plants very active phosphates which will readily hydrolyse sugar phosphates to free sugars (Benson, 1955).

The inactivation of the enzymes is only part of the problem involved in the extraction of the phosphate esters. The adsorption of the esters on the cell-wall material has also been especially studied since esters present in small amount might be almost completely lost. This adsorption has been linked with the presence of naturally occurring alkaline-earth and heavy-metal cations which form insoluble compounds with the phosphate esters. It is of interest therefore to compare the amounts of some of these with figures for the amounts of sugar phosphates in the same tissue. The whole potato contains Ca^{2+} , 8–22, Mg^{2+} , 16–34, Fe^{2+} , 0.4–2.6, Al^{3+} , 0.7–2.2 mg./100 g. fresh wt. (Talbert & Smith, 1959), and it is noticeable that the amounts of Ca^{2+} and Mg^{2+} , 2–5 mm and 6–14 mm respectively, are much larger than the concentrations of 3-phosphoglycerate or fructose 1,6-diphosphate, 0.3 mm and 0.04 μM (Barker, Isherwood, Jakes, Solomos & Younis, 1962). It seems clear that if Ca^{2+} and Mg^{2+} do cause adsorption there is a considerable excess present. Adsorption is also important in chromatography and in this connexion the experience of Hanes & Isherwood (1949) is especially valuable. They found that various inorganic impurities in the filter paper profoundly affected the character of chromatograms of phosphate esters either in basic or acidic solvents. Elimination of the inorganic ions either by washing the paper or by chelation with suitable reagents prevented the adsorption, which suggested that purified cellulose itself was not adsorbing the phosphate esters. By analogy it may be supposed that the polysaccharide material from plant tissue would behave similarly. Various chelating agents have therefore been added to the

extracting fluid in an attempt to eliminate adsorption, and of these the sodium or ammonium salt of EDTA in neutral extracts and 8-hydroxyquinoline in acid extracts have proved to be valuable.

EXPERIMENTAL

Evaporation procedures

All operations were so designed as to avoid as far as possible breakdown of labile phosphate esters. Separations involving column techniques or lengthy manipulations such as ultrafiltration were carried out in a room at 0°. Aqueous solutions containing large quantities of organic solvents were first reduced in volume until most of the organic solvent had been removed and then the mainly aqueous solution was concentrated by freeze-drying in an all-glass freeze-drier (Quickfit and Quartz Ltd., Stone, Staffs.). The round-bottomed flasks used had wide necks with flat flanges so that the dry residues or syrups remaining could be quantitatively mixed with cellulose powder for subsequent chromatographic separations. The organic solvent was removed either in a rotary film evaporator (Craig, 1950) at 12 mm. Hg pressure and a bath temperature not exceeding 30°, or, for larger quantities of solution, in a climbing film evaporator (Bartholomew, 1949). The latter was slightly modified from the original design; hot water at 70–75° was used as the heating fluid instead of live steam, and this was circulated through the jacket of the evaporator by means of a small centrifugal pump (Stuart Pump, Stuart Turner Ltd., Henley-on-Thames). The maximum vapour-liquid temperature at the top of the evaporator was 22° at a pressure of about 20 mm. Hg and the volume was reduced about two-thirds in each evaporation cycle (most of the organic solvent was removed after three cycles). The period during which the solution was heated was so short that no breakdown took place. In general concentration of these aqueous organic solutions either in the rotary film evaporator or in the climbing film evaporator was carried out so to avoid subjecting the whole bulk of the solution to the heat treatment.

Chemicals

D-Fructose 1,6-diphosphate (sodium salt), ATP (sodium salt), 3-phosphoglycerate (barium salt), D-ribose 5-phosphate (sodium salt), D-glucose 1-phosphate (potassium salt), D-glucose 6-phosphate (sodium salt) and uridine 5'-diphosphate glucose (sodium salt) were purchased from L. Light and Co. Ltd., Colnbrook, Bucks. The other chemicals and organic solvents were of analytical reagent grade.

The propan-1-ol was further purified before use by refluxing for several hours with a small quantity of solid NaOH (10 g. to 1 l.) and distilling (Isherwood & Hanes, 1953). To remove any alkali which might have been carried over in the spray during the distillation, the purified propan-1-ol was made slightly acid with conc. H_2SO_4 and redistilled. Since large quantities of purified propan-1-ol were required, as much as possible was recovered from the waste solvent from the cellulose column. This mixture of propan-1-ol, water and NH_3 (80–90% propan-1-ol) was treated with excess of anhydrous K_2CO_3 for several hours to remove water and the propan-1-ol layer separated. Conc. H_2SO_4 was added until just acid and the $(\text{NH}_4)_2\text{SO}_4$ and K_2SO_4 were allowed to

crystallize. The filtrate was distilled, giving a purified propan-1-ol containing less than 5% of water which was quite suitable for the preparation of chromatographic solvents.

Pure ammonium phytate was prepared from crude sodium phytate (Boatwell, 1917) by a procedure based essentially on that described later in this paper for the trichloroacetic acid extract of plant tissues (cf. 'Removal of chloride, amino acids and phytin'). The ammonium phytate on the cellulose column was eluted with 60% propan-1-ol-water and the solution evaporated to dryness. This sample was chromatographically homogeneous (Anderson, 1955).

Analytical procedures

Determination of phosphorus. Routine measurements were made by the method of Allen (1940). If the extract contained much formic acid, a small amount of oxalic acid appeared to be formed during the incineration and this interfered with the development of the molybdenum blue colour; the colour only slowly reached its maximum intensity and showed a purplish tint at one stage. To avoid the formation of oxalic acid, as much formic acid as possible was removed by a preliminary distillation and the incineration with perchloric acid continued for several hours.

Determination of trichloroacetic acid in biological extracts. The small amounts remaining after such extracts had been repeatedly shaken with ether were measured by the method recommended by the General Medical Council (1953).

Enzymic determination of D-glucose 6-phosphate, adenosine 5'-triphosphate and uridine diphosphate glucose. D-Glucose 6-phosphate was measured by the method described by Barker, Jakes, Solomos, Younis & Isherwood (1964). ATP was measured as D-glucose 6-phosphate by causing it to react with glucose in the presence of the hexokinase enzyme, and uridine diphosphate glucose by oxidation with NAD in the presence of the appropriate dehydrogenase (Bergmeyer, 1963). Both these methods were sensitive to the presence of interfering substances and especially traces of trichloroacetic acid and formic acid. If present these must be removed. The best criterion for a satisfactory assay is that the progress curve of the enzyme reaction in the extract follows the same pattern as for pure substrates under the same standard conditions.

Killing and extraction of plant tissues

Three different procedures were tested: extraction with cold trichloroacetic acid, with boiling ethanol and with boiling water. Each procedure is described below but since preliminary tests showed that the use of cold trichloroacetic acid gave the most complete extraction of the phosphate esters this is described first in the form finally developed for the isolation of the phosphate esters in a state of purity. The other two procedures were only used in the preliminary experiments.

Plant material. Stored and new potatoes (variety King Edward) were used. Strawberry leaves (variety Royal Sovereign) were collected from the field immediately before analysis. Peas (Laxton Superb, Onward) were germinated as follows: vermiculite (40g.) was mixed with water (100ml.) and allowed to stand overnight. Peas (100g.) were sown in the damp vermiculite and kept at 15° for 5 days to germinate. Peas germinated in this manner were free from mould

infection and closer to those sown under natural conditions.

Extraction with cold trichloroacetic acid. The procedure was similar whether potatoes, strawberry leaves or soaked peas were used. The plant material (usually sliced) was frozen in liquid N₂, ground to a powder and then added to the aqueous trichloroacetic acid. In the few cases where it has been possible to determine individual phosphate esters (Barker *et al.* 1964) the results appear to be similar whether the material is first frozen in liquid N₂ and then added to aqueous trichloroacetic acid or merely added directly to the cooled aqueous trichloroacetic acid. In a typical small-scale experiment, the potatoes (two or three, each about 10g. in weight) were sliced with a razor and each slice (1–2mm. thick) was dropped immediately into liquid N₂ in a wide-mouthed Dewar vessel and left for a few minutes. The process of slicing the potatoes into the liquid N₂ can be done rapidly after a little practice (strawberry leaves can be dropped into the liquid N₂ without any treatment). The frozen slices were then transferred to an aluminium saucepan and crushed to a fine powder with a flat stainless-steel pestle, the powder and slices being kept under liquid N₂ all the time. The finely ground frozen powder (20g.) was added gradually to aq. 10% (w/v) trichloroacetic acid (20ml.) containing 0.15% (w/v) 8-hydroxyquinoline in the vortex tube of a high-speed blender (Measuring and Scientific Equipment Ltd.; with a more powerful motor, 14000 rev./min., 0.25 h.p., so that the performance does not drop appreciably under load). Addition was made so that the mixture in the vortex tube remained just above freezing point. When all the powder had been added, the vortex tube was surrounded with a mixture of crushed ice and water and blending continued for 15 min. Ice-cold water (10ml.) was used to wash adhering powder from the beaker in which the sample had been weighed into the vortex tube. If the potato slices or soaked peas were added directly into the aqueous trichloroacetic acid, the vortex tube would be surrounded with a mixture of crushed ice and alcohol at the start of the blending. The extract was then centrifuged at 18000g for 15 min. in a room at –3°. The clear supernatant was decanted and the precipitate extracted with ice-cold aq. 5% (w/v) trichloroacetic acid (20ml.) containing 0.15% (w/v) 8-hydroxyquinoline by blending for 5 min. and then centrifuging as before. The whole process was repeated and the supernatants were combined (pH 1.3).

Removal of trichloroacetic acid and cations. The extract (100ml.) was immediately shaken with ether (50ml., six times) to remove as much trichloroacetic acid as possible (pH rises to 2.6) and then passed through a short column of Dowex AG 50 in the H⁺ form (8–10% cross-linked, 100–200 mesh; A.R. grade, BioRad Laboratories, Richmond, Calif., U.S.A.; 25 m-equiv. of resin or 15ml. of resin bed is usually sufficient) to remove all cations. The resin bed was washed with ice-cold water (10ml., three times) and the filtrates were combined. Estimation by flame photometry of Ca²⁺ and K⁺ still remaining indicated less than 1 p.p.m. present. The solution was extracted with ether (50ml., six times) to remove the trichloroacetic acid (pH rises to 3.1) and then neutralized with dilute NH₃ soln. to between pH 7 and 8 (vol. 120ml.).

Removal of high-molecular-weight material (including polysaccharide in solution). This was accomplished by ultrafiltration through a viscose film which did not allow solutes with a molecular weight greater than 30000 to pass. For this the ultrafilter LKB 6300A (LKB Produkter AB,

Stockholm 12, Sweden) was found suitable. As filtration proceeded, the rate of flow of liquid through the membrane dropped and to prepare a sample (100 ml.) free from polysaccharides within a reasonable time (10–12 hr.) several filters were used in conjunction. The pH of this sample was adjusted if necessary to about 8 by the addition of dilute NH_3 soln. and then the solution was concentrated to a thin syrup (0.5 ml.).

Removal of chloride, sugars, amino acids and phytin. The extract contained considerable amounts of both reducing and non-reducing sugars, amino acids, phytin and, with potato, chloride. These were separated from the nucleotide and sugar phosphates by chromatography on a cellulose column by using mixtures of propan-1-ol and water as developing solvents. The solvent used for the preparation of the column and for the removal of the sugars and amino acids was propan-1-ol-water-aq. NH_3 (sp.gr. 0.88)–0.2M-EDTA (90:4:9.5:0.1:0.1, by vol., d_4^{25} 0.83). To prepare the column, cellulose powder (Chromedia CF1, Whatman, W. and R. Balston Ltd., 30g.) was passed through a nylon sieve, made into a slurry with solvent and then poured into a suitable glass tube (2 cm. diam. with a sintered-glass plate, porosity 0, at the bottom). As soon as the cellulose had packed down, the column was washed with more solvent (0.5 l.) to remove all soluble impurities and equilibrate the cellulose powder against the solvent (length of column 48 cm.).

To the extract containing the phosphate esters (0.5 ml.) was added dry cellulose powder (3g.) and the whole thoroughly mixed. We have found that the extract must be dried separately from the cellulose as a mixture of the two seems to react in some way during the drying process, which prevents the phosphate esters being eluted subsequently on the column. The powder was made into a slurry by the addition of solvent (3–4 ml.) and then poured on to the top of the column. When the top of the column was dry, more solvent (3–4 ml.) was added and allowed to drain in. This was repeated and then the column developed with the same solvent until the chloride, sugars and amino acids had been washed out (1.5–2.0 l.). No phosphate esters were eluted and in fact the column could be washed with even more of the solvent without eluting any (the flow rate was about 40 ml./hr.).

To elute the nucleotides and the sugar phosphates, the solvent was changed to one with more water (72.5:27.3:0.1:0.1, by vol., d_4^{25} 0.867) and the development continued. The eluate was collected in fractions (200 ml.) and each fraction concentrated separately. The progress of the elution was followed by estimation of P in each fraction until all the nucleotide and sugar phosphates and P_i had been eluted (1.5 l.). Washing the column with a further quantity of solvent (0.5 l.) did not remove any more P-containing material. The phytic acid remained behind on the column and was not eluted. It can be obtained by further development of the column with a solvent (1 l.) containing more water (60:39.8:0.1:0.1, by vol., d_4^{25} 0.894). Examination of the phytic acid on a paper chromatogram (Anderson, 1955) showed that it was mainly inositol hexaphosphate with a trace of a faster-running component, which could be the tetraphosphate.

Separation of organic acids and orthophosphoric acid from nucleotides and the sugar phosphates. The extract containing the nucleotides and the sugar phosphates was rechromatographed on a cellulose column with an acidic solvent

for development. The malic acid and citric acid and P_i which contaminated this fraction were rapidly eluted, leaving the phosphate esters behind on the column. These were easily recovered by subsequently washing the column with an aqueous alcoholic solvent. The procedure was as follows: the solvent was isopropyl ether-formic acid (90%, w/v)–2-methylpropan-2-ol-water (18:6:5:5, by vol.; top layer), which had been allowed to stand for 24 hr. at 0° to equilibrate. To prepare the column, cellulose powder (40g.) was thoroughly mixed with the bottom layer of the solvent mixture (7.5 ml.) and then made into a slurry with solvent. This was poured into a suitable glass tube (length 65 cm., diam. 3.5 cm.) and allowed to settle. As soon as the cellulose had packed down the column was washed with more solvent (1.5 l.) to remove soluble impurities and when the excess of solvent had soaked in was ready for the addition of the eluate from the previous column. The syrup (2 ml.) containing the phosphate esters was thoroughly mixed with dry cellulose powder (10g.), made into a slurry with solvent (a short treatment in a blender helps to give a uniform suspension), and then poured on to the top of the column. When the excess of solvent had disappeared the organic acids and P_i were washed through by developing with more solvent (2 l.).

With the plant materials examined (potatoes, peas and strawberry leaves) all the P_i was removed without any combined phosphorus being present. The formic acid was now removed from the column by washing with solvent (500 ml.) free from formic acid. This was prepared in the same way as the standard solvent except that the formic acid component was omitted. A small amount of combined phosphorus was sometimes washed through at this stage (possibly glycerophosphoric acid) and if this occurred the fraction was concentrated separately from the main bulk of the phosphate esters because of its content of formic acid.

The phosphate esters on the column were eluted by changing the solvent to methanol-water (1:1, v/v; 1.0 l.). The eluate was neutralized with NH_3 soln. and concentrated

Killing and extraction with hot ethanol. The ground frozen potato powder (see above; 10g.) was slowly added to boiling aq. 60% (v/v) ethanol (150 ml. containing 0.5% of EDTA) under reflux so that the mixture continued to boil. A small amount of aq. NaOH soln. (the precise volume is determined in a preliminary experiment) was added so that the pH of the final aqueous concentrate was not less than 6. After the last addition, the mixture was boiled for 10 min. and then filtered. The residue was suspended in aq. 60% ethanol (100 ml., with EDTA as before), homogenized in a high-speed blender for 10 min. and then refluxed for 15 min. It was filtered hot and the extraction repeated once more. The combined supernatants (500 ml.) were concentrated to about 7 ml.

Killing and extraction with boiling water. The ground frozen potato powder (see above, 10g.) was added to vigorously boiling water (100 ml. containing 0.5% EDTA) together with sufficient NaOH to adjust the final pH to not less than 6, and the mixture refluxed for 15 min. It was then centrifuged and the residue re-extracted with boiling water (50 ml.) as before. The combined supernatants (150 ml.) were frozen and left overnight. They were then allowed to thaw at room temperature. The starch and other polysaccharides, which had been dispersed in the boiling water, separated as a fluffy fibrous mass which was easily removed by centrifuging, leaving a clear solution. This was concentrated to about 7 ml.

Paper chromatography

The apparatus and procedure were similar to those described by Hanes & Isherwood (1949). The preferred method for splitting the P_i from acid-resistant phosphate esters was by heavy-metal-catalysed photolysis under ultraviolet light. The air-dried chromatogram was sprayed lightly and evenly with 1% (w/v) CuSO_4 in water and allowed to dry at room temperature (Co and Mn were also effective). It was irradiated for 30 min. (Bandurski & Axelrod, 1951), under a portable ultraviolet lamp (Chromatolite; Hanovia, Slough) from which the Woods glass filter had been removed, lightly sprayed with the molybdate reagent (Hanes & Isherwood, 1949), allowed to dry and finally sprayed with 0.2M SnCl_2 in $N\text{-HCl}$ [10g. of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ dissolved in 25 ml. of HCl ($d_{1.18}$) and 2 ml. of this diluted to 20 ml. by the addition of water just before it was required]. The position of the P_i was revealed as an intensely blue spot on a white background (sensitivity $0.01 \mu\text{g./cm.}^2$).

The method worked smoothly when the paper chromatogram had been developed with a basic alcoholic solvent but was unreliable if the paper was contaminated with phenol or formic acid. It was essential to remove these by a preliminary steaming treatment before spraying with the copper solution.

RESULTS

Extraction of phosphorus-containing compounds from potato

A preliminary survey was made of various methods for the extraction of phosphorus-containing compounds from potato, the object being (1) to inactivate the enzymes as rapidly as possible, (2) to bring the phosphorus compounds into solution quantitatively, (3) to cause the minimum amount of breakdown of the phosphorus compounds and, if possible, (4) to introduce no extraneous solutes into the final extract. The investigation started with the use of boiling alcohols since these have been employed extensively in the past for the extraction of phosphate esters (Benson, 1955) and have the advantage that no foreign salts are introduced into the extract. In some of the experiments the alcohol contained mineral acids (perchloric acid), which could be removed subsequently as insoluble salts (potassium perchlorate) and thus leave the extracts free from acid. Apart from boiling alcohols extraction with aqueous acids was also investigated. The results of the preliminary experiments showed that the amount of combined phosphorus in the extracts varied widely with different conditions of extraction, the overall conclusion being that several factors contributed. The most important of these was the complex nature of the mixture of phosphorus-containing compounds in the cell, many of which have high molecular weights and are attached to protein. Since our main interest was in the low-molecular-weight compounds and these form only a small part of the total phosphorus-containing

compounds, it was clear that the figure for the combined phosphorus in the extract was not a true index of the extraction. Another factor which contributed to the variability of the results was adsorption of combined phosphorus on the polysaccharide cell-wall material and the possible influence of calcium and magnesium and heavy metals. After this study attention was concentrated on two types of extractant, boiling aqueous ethanol and aqueous trichloroacetic acid. The others were eliminated because they either introduced extraneous salts into the extract or there was doubt about the efficiency with which they inactivated enzymes in the tissue. These later experiments were concerned with the recovery of low-molecular-weight phosphate esters added to potato tissue and also with the extraction of the naturally occurring low-molecular-weight phosphate esters.

Extraction with boiling ethanol-water mixtures containing ethylenediaminetetra-acetic acid. The results of experiments in which known amounts of D-glucose 1-phosphate or fructose 1,6-diphosphate were added to samples of frozen potato powder (the amount of naturally occurring phosphate esters in this is very low) and the whole extracted with boiling aq. 80% ethanol either alone or with the addition of EDTA to chelate the Ca^{2+} and Mg^{2+} (pH about 7) indicated that the addition of EDTA increased the recovery of D-glucose 1-phosphate in the ethanolic extract almost to the theoretical figure but had a smaller effect with fructose 1,6-diphosphate. This may be due to the low solubility of EDTA in aq. 80% ethanol and possibly its low reactivity towards Ca^{2+} and Mg^{2+} in the presence of small amounts of water.

A similar series of experiments in which the phosphate esters were added to the residue after extraction of frozen potato with boiling aq. 80% ethanol are shown in Table 1.

The figures in Table 1 show that extraction with boiling aq. 80% ethanol either with or without the addition of EDTA did not remove all the added phosphate ester. The addition of EDTA appeared to prevent the extraction of the phosphate ester to some extent and this may be due to a salting-out effect. Extraction with boiling water gave higher recoveries, and with the addition of EDTA the recovery of added phosphate ester was practically quantitative. It seems clear that EDTA can only be fully effective in the presence of sufficient water to swell the cell wall. The amount of phosphorus in the residue reflects the amount of phosphate ester adsorbed.

Extraction with aqueous trichloroacetic acid. Apart from boiling alcohol, aq. trichloroacetic acid is the most common reagent in use for the extraction of phosphate esters from plant tissue.

Table 1. *Extraction of phosphate esters from potato cell wall*

Extraction of added glucose 1-phosphate (3.55 mg. of P) and fructose 1,6-diphosphate (2.8 mg. of P) from alcohol-extracted potato powder (2 g. dry wt. equivalent to 10 g. of frozen potato powder) with boiling aq. 80% ethanol or boiling water. EDTA (0.5%, w/v) was added to the extracting fluid where indicated. Figures in parentheses are recoveries (% of added phosphate ester), suitable allowance being made for the blank.

Extracting fluid	Phosphate ester added	Total phosphorus (mg.)	
		Extract	Residue
Aq. 80% ethanol	None	0.1	1.1
Aq. 80% ethanol	Glucose-1-phosphate	2.5 (66)	2.1
Aq. 80% ethanol	Fructose 1,6-diphosphate	1.7 (56)	2.3
Aq. 80% ethanol-EDTA	None	0.0	1.1
Aq. 80% ethanol-EDTA	Glucose 1-phosphate	1.8 (50)	3.2
Aq. 80% ethanol-EDTA	Fructose 1,6-diphosphate	0.5 (16)	3.9
Water	None	0.6	0.5
Water	Glucose 1-phosphate	3.5 (80)*	0.7
Water	Fructose 1,6-diphosphate	3.1 (88)	0.6
Water+ EDTA	None	0.7	0.2
Water+ EDTA	Glucose 1-phosphate	4.3 (100)*	0.2
Water+ EDTA	Fructose 1,6-diphosphate	3.4 (97)	0.2

*Confirmed by determination of P_i (as P) liberated by acid hydrolysis in extract (7 min. at 100° in N acid).

Table 2. *Extraction of frozen potato powder with cold trichloroacetic acid and removal of trichloroacetic acid by solvent extraction*

Glucose 1-phosphate (37 mg. of P) and fructose 1,6-diphosphate (1.7 mg. of P in Expt. 2, otherwise 1.55 mg. of P) were added to frozen potato powder (10 g.) and the whole was extracted with 10% (w/v) trichloroacetic acid (15 ml.). 8-Hydroxyquinoline (0.15%) was added where indicated. Figures in parentheses are recoveries (% of added phosphate ester), suitable allowance being made for the blank.

Expt. no.	Additions to extracting fluid	Phosphate ester added	Total phosphorus in extract (mg.)		Comment
1	—	Glucose-1-phosphate	38.4	38.0*	Not solvent-extracted
			38.4	37.6*	Extracted with ether
2	—	{ None	1.9		
		{ Fructose 1,6-diphosphate	3.2 (85)		
3	8-Hydroxyquinoline	{ None	1.8		8-Hydroxyquinoline not added to
		{ Fructose 1,6-diphosphate	3.2 (90)		ether for extraction but only to
					extract
4	—	{ None	2.05		P in residue 1.6
		{ Fructose 1,6-diphosphate	3.15 (72)		P in residue 2.0
5	8-Hydroxyquinoline	{ None	2.1		P in residue 1.76
		{ Fructose 1,6-diphosphate	3.62 (98)		P in residue 1.6

* P_i (as P) liberated by acid hydrolysis (7 min. at 100° in N acid).

A similar series of experiments to those described above for boiling ethanol-water mixtures was carried out with aq. trichloroacetic acid as extractant, except that the final extracts were shaken out with ether to remove the trichloroacetic acid. The results of these experiments are given in Table 2. The effect of adding a small amount of 8-hydroxyquinoline to the aq. trichloroacetic acid was examined since it is known that phosphate esters

are adsorbed even under acid conditions if traces of copper, iron or lead are present (Hanes & Isherwood, 1949).

The results of Expt. 1 shows that the solvent extraction of the excess of trichloroacetic acid from the solution does not cause any loss of added glucose 1-phosphate. The figures for the total amount of phosphorus and acid-hydrolysable phosphorus are almost identical whether the

solution was extracted with solvent or not. The results of Expts. 2 and 4 show that fructose 1,6-diphosphate is not quantitatively removed and analysis of the residue (4) indicates that it is adsorbed on the insoluble material. Addition of 8-hydroxyquinoline to the aq. trichloroacetic acid prevented this adsorption and in Expt. 5 the recovery of fructose 1,6-diphosphate was theoretical. 8-Hydroxyquinoline was added to the ether used for extraction of the trichloroacetic acid as certain of the metal chelates are extracted best between pH 2 and 4 (Martell & Calvin, 1952). The recovery of fructose 1,6-diphosphate (3) was not quantitative when 8-hydroxyquinoline was omitted from the ether.

Extraction by trichloroacetic acid of the naturally occurring phosphate esters. The experiments described above were designed to test the quantitative recovery of phosphate esters which had been added to potato tissue and in these experiments the temperature was kept at 0° and all operations were carried out as quickly as possible to prevent breakdown. These conditions are ideal for the extraction of soluble phosphate esters but with those occurring naturally the possibility had also to be considered that some might be bound to cell wall or to protein by the kind of labile link suggested by Wildman, Campbell & Bonner (1949). If this were so, more drastic conditions than those described above might increase the amount extracted. The following experiments were devised to investigate this possibility. The results are given in Table 3.

Comparison of the results of Expt. 1 with those of Expts. 2, 3, 4 and 5 indicates that even mild treatment of the residue will liberate extra combined phosphorus into the supernatant. Fat solvents (acetone and ether) have the same effect as 5% (w/v) trichloroacetic acid either at 20° for 24 hr. or at -20° for 8 days. Analysis of the phosphorus compounds in the supernatants in the various experiments indicates that it is the combined phosphorus fraction which is liberated, for the orthophosphate is not changed significantly. A short dialysis of the supernatants in Visking tubing against distilled water (Expts. 6 and 7) removed most of the orthophosphate but caused very little loss of combined phosphorus. In the material extracted from the residue by hot acid treatment (Expt. 7) there was practically no change. Low-molecular-weight phosphorus compounds would have been removed by dialysis and it is clear that the extra combined phosphorus extracted from the residue by the additional treatment with trichloroacetic acid is almost entirely high-molecular-weight material such as nucleic acid. Hot acid liberated more combined phosphorus than any other treatment and it is possible that two types of compounds are concerned: those liberated by prolonged treatment with cold 5% trichloroacetic acid and fat solvents, and those which require a mild hydrolysis to free them from protein and cell-wall material. The main conclusion from these experiments was that, though the amount of combined phosphorus in the extract can vary considerably, the amount of

Table 3. *Effect of different conditions on extraction of phosphorus compounds by trichloroacetic acid from potato*

Frozen potato powder (10 g.) was blended with 10% (w/v) trichloroacetic acid (15 ml. plus 0.15%, w/v, 8-hydroxyquinoline) at 0° for 15 min.

Treatment	Phosphorus (mg.)				Expt. no.
	Super-natant	Dialysis residue	Residue	Total	
Homogenate centrifuged immediately at 0°	1.6 { 1.25* 0.33† }	—	1.4	3.0	1
Homogenate heated at 100° for 2 min.	2.6 { 1.27* 1.30† }	—	0.6	3.2	2
Homogenate held at 20° for 24 hr.	2.12 { 1.27* 0.85 }	—	1.1	3.2	3
Homogenate held at -20° for 8 days	2.0 { 1.27* 0.75† }	—	1.2	3.2	4
As Expt. 1 but residue treated with acetone and ether and extracted with water. Supernatants combined	2.0	—	1.1	3.1	5
Supernatant from Expt. 1 was dialysed against water	1.6	0.4 { 0.09* 0.31† }	—	—	6
Residue from Expt. 1 heated with aq. trichloroacetic acid at 100° for 2 min. Supernatant dialysed against water	1.08	1.06	—	—	7

* P₁ (as P).

† Combined phosphorus (as P).

low-molecular-weight material (equivalent to 0.02–0.04mg. of phosphorus) does not change significantly. It will only be possible to determine if small amounts are liberated when a much more sensitive method of phosphorus analysis is available.

Preferred method. Extraction with cold aq. trichloroacetic acid containing 0.15% 8-hydroxyquinoline gives a quantitative recovery of phosphate esters added to potato tissue and removes all the naturally occurring low-molecular-weight phosphorus compounds. This method of extraction is free from the criticism that enzymes in the tissue are stimulated to higher activity before killing, and provided that the operations are carried out as quickly as possible is unlikely to cause breakdown of labile phosphate esters. Extraction with cold aq. trichloroacetic acid has therefore been used as the basis for the isolation of phosphate esters from plant tissue. The details of the procedure developed to remove all interfering materials from the initial extract are set out in the Experimental section and the reasons for the adoption of each step are discussed below.

Extraction with boiling ethanol containing EDTA was not considered further because this method of extraction did not quantitatively remove added fructose 1,6-diphosphate and moreover was open to the criticism that the enzymes in the tissue were stimulated by the heat treatment to higher activity before being destroyed.

Analysis of extracts from potatoes, peas and strawberry leaves

The results of the application of the preferred method to the isolation of phosphate esters from potatoes, peas and strawberry leaves are given in Table 4. The complete procedure was also checked by using a synthetic extract (equivalent to 100g. of peas) and treating it exactly as if it were a plant extract. The extracts from potatoes (430ml. from 165g.) and soaked peas (510ml. from 439g.) were ultrafiltered. The residue from the filtration was viscous and contained polysaccharides which could be precipitated by the addition of ethanol (15ml., the residue from peas, contained 5% of the total phosphorus in the original extract). The extract from strawberry leaves (380ml. from 120g. of leaves) was not ultrafiltered since it contained only a small amount of mucilaginous material. The size of the first column was adjusted on the basis of the soluble solids content of the extract (see the Discussion section) and for all but strawberry leaves was 300g. with 2l. effluent fractions. For strawberry leaves a 120g. column was used with 800ml. effluent fractions (this is to scale).

Certain aspects of the results described in Table 4 deserve comment. The figure for the combined phosphorus is the difference between total phosphorus and P_i (as phosphorus) and since it is small in relation to these two quantities is likely to lack

Table 4. *Analysis of column effluents*

For details of chromatography see text.

Constituent analysed	Fractions in which detected	Amount recovered (mg./100g. fresh wt.)			
		Control	Potatoes	Peas	Strawberry leaves
Column 1					
Chloride	1–3	—	24	29	—
Amino acids	2–5	—	—	—	—
Sugar, reducing, or glucose	1–8	500	700	93	51
non-reducing as sucrose	2–9	—	100	2190	143
Combined P (as P)	11–17	8	1.7	11	5.3
P_i (as P)	11–17	21	27.3	25	19.6
Organic acids (as malic)	11–17	300	300	100	—
Phytic acid (as P)	19–21	—	6	151	—
Column 2					
P_i (as P)	1–4*	21	27.3	25	18.4
Organic acids	1–3	—	—	—	—
Combined P (as P)	5, 6	7.8	2.0	11	5.25
Recovery of phosphate esters (% from original extract)					
D-Glucose 6-phosphate	5, 6	95	98	99	See text
ATP	5, 6	95	87	95	See text
UDP-glucose	5, 6	—	80	—	—

* Each fraction was 500 ml.

precision. Any discrepancies in the recorded figures for combined phosphorus in the Table are due to this cause.

With strawberry leaves, washing the second column with the formic acid-free di-isopropyl ether solvent removed a small amount of combined phosphorus (0.18 mg.). After removal of the solvent this was added to the rest of the phosphate esters.

Analysis of the original extracts by enzymic methods was only possible for potatoes and peas. Strawberry leaves contained large amounts of polyphenols, which inhibited the enzymes. Even with potatoes and peas the enzymic analyses were difficult because the reactions did not reach a constant end point within the standard time. With potato the reaction after the initial rapid reaction was over changed in a linear fashion with time. For the actual estimation the course of the reaction was plotted against time and then the curve extrapolated to zero time and this figure used for the final calculation. The recovery figures for the phosphate esters indicated that negligible breakdown occurred and it is significant that the amount of P_i (as phosphorus) which reflects any breakdown of phosphate esters remained unchanged throughout the procedure. No recovery figures are given for strawberry leaves but the final purified extract was analysed without difficulty. It contained D-glucose 6-phosphate and ATP, 13.4 and 2.44 μ moles/100 g. fresh wt. respectively. The figure for D-glucose 6-phosphate is similar to that obtained on strawberry-leaf extracts cleared with activated charcoal (Barker *et al.* 1962). No figures for ATP are available for comparison since charcoal adsorbs nucleotides as well as polyphenols.

DISCUSSION

The two most commonly used methods for killing and the extraction of phosphate esters from plant material are treatment either with hot ethanol or with cold trichloroacetic acid. Either of these methods if properly carried out on the finely divided plant material ensures that the enzymes are denatured, though the criticisms raised in an earlier paper on α -oxo acids (Isherwood & Niavis, 1956) against any heat-inactivation method apply to the hot-ethanol method. There is always a suspicion that before the enzymes are denatured they are stimulated by the increase in temperature to a far higher activity, especially during the period when the cell is being disorganized. The cold-extraction method avoids this. A detailed analysis of the factors affecting the rate of killing is given in the discussion to the paper on the α -oxo acids and most of them apply with equal force to the estimation of the phosphate esters.

However, even with a cold-extraction method it is essential to prevent enzymic activity during the

brief period when the cells are being disrupted during extraction. That this is a real danger is shown by some experiments described by Heslop (1964). He showed that, with frozen house-fly thoracic tissues in which the cells had been disorganized by freezing, the ATP and arginine phosphate were stable for 1 hr. when the storage temperature was below -3° , but that at -2° and above there was extensive breakdown. No such breakdown occurred in unfrozen thoraces dissected from chilled flies at 0° , presumably because the cells were not disorganized. The changes occur because at -2° some parts of the frozen tissue are beginning to thaw. Freezing in liquid nitrogen is a convenient method of preserving tissue before extraction and so long as the temperature is sufficiently low no changes occur, but it is clear that when this tissue is extracted the enzymes must be inactivated immediately the frozen tissue begins to thaw in the presence of the extractant. In the present method the finely divided plant material at -196° was blended rapidly with chilled aq. trichloroacetic acid (-3°) in such a way that inactivation of the enzymes occurred in the shortest possible time. It is highly significant that with this method the amount of AMP as compared with ADP and ATP in extracts from strawberry leaves was very low, indicating that negligible breakdown had occurred (cf. Minard & Davis, 1962; Heslop, 1964) during the extraction procedure.

Apart from the question as to whether a hot or a cold killing method is desirable, it seems clear from the results of the experiments in Table 1 that adsorption of the phosphate esters occurs in ethanol-water mixtures even when a calcium- and magnesium-chelating agent is added; only when no ethanol is present is the chelating agent effective and the recovery of added phosphate esters quantitative. This fact alone would indicate that ethanolic solvents were not satisfactory extractants either hot or cold. With extraction with cold aq. trichloroacetic acid, some adsorption of the phosphate esters occurs (Table 2) but this is entirely eliminated by the addition of 8-hydroxyquinoline. The nature of the heavy metal causing the adsorption is uncertain but the presence of iron is suspected because the addition of 8-hydroxyquinoline caused a darkening in the colour of the ether extract when the trichloroacetic acid was removed.

It was from a consideration of the advantage of using a cold killing method and the fact that adsorption could be easily eliminated by addition of 8-hydroxyquinoline that led us to adopt extraction with aq. trichloroacetic acid as the basis of the recommended procedure in the present paper. Force was given to this decision by the fact that it was possible to remove all added trichloroacetic acid by solvent extraction, and thus produce a solution of

the phosphate esters free from extraneous substances.

By comparison, other methods which have been proposed for the cold extraction of phosphate esters from plant tissues were considered less satisfactory. Bielecki & Young (1963) suggested either a mixture of chloroform, methanol and water or 0.2M-formic acid in 20% (v/v) ethanol-water for the extraction but offered no evidence as to the efficiency of these extractants for the inactivation of the enzymes other than a radioautograph showing the presence of ATP, ADP and AMP in the final extract. It seems likely that many of the enzymes would survive for an appreciable time under the conditions of the extraction. In addition adsorption of the phosphate esters on the cell-wall material would occur from the aq. ethanolic medium in much the same manner as observed with the boiling aq. ethanol solvents described in Table 1. Perchloric acid (3N) has been employed by several workers (Lowry, Passonneau, Hasselberger & Schultz, 1964; Polakis & Bartley, 1966) and we found that it gave a clean extraction of both P_i and combined phosphorus compounds. However, attempts to remove the perchloric acid as the potassium salt were only partly successful since potassium perchlorate is appreciably soluble in water (0.75%, w/v, at 0°). Addition of several volumes of ethanol precipitated the potassium perchlorate but caused losses of phosphorus-containing material on the precipitate (in some cases 30% of the P_i and 15% of the combined phosphorus were lost). Even so the removal of the potassium perchlorate was far from complete because of its solubility in the aq. ethanol (0.6% in 75% ethanol-water at 20°).

A detailed study of the recommended method as applied to various plant materials showed that cold aq. trichloroacetic acid extracted a wide range of phosphorus compounds and the completeness of the extraction of the total phosphorus depended largely on the tissue. Germinated peas yielded up to 70% of their phosphorus whereas frozen potato flour gave only 50%. Modifications of the extraction procedure, namely heating at 100° for 2 min., treatment with acetone and storage at -20° for 8 days, all increased the total amount of phosphorus extracted. With frozen potato flour this extra material was almost entirely high-molecular-weight substances such as starch phosphate, phytic acid and nucleic acid and therefore was not of interest in the present method, which is primarily concerned with low-molecular-weight compounds. The amount in the residue in peas was smaller (30%) and heating with aq. trichloroacetic acid removed 10%. This material was mainly of low molecular weight and may have been present in the residue as a result of incomplete extraction. It is significant that further extraction of the original residue with cold

aq. trichloroacetic acid removes an appreciable amount of soluble phosphorus-containing material. The question as to whether there are any bound low-molecular-weight phosphates in the pea remains open, but, if so, they appear to be liberated by repeated treatment with cold aq. trichloroacetic acid.

The extract, after separation of the cell debris, usually contained an appreciable amount of soluble polysaccharide material as well as the cations present in the plant tissue. These were removed before the extract was fractionated by cellulose chromatography. The polysaccharides increase the viscosity of the solution and in addition tend to block chromatographic columns, particularly in the presence of ethanolic solvents in which the polysaccharides are insoluble. The cations prevent the complete extraction of the trichloroacetic acid and those which form insoluble compounds with phosphate esters tend to cause losses of phosphate esters through adsorption on the cellulose. EDTA is an effective 'complexing' reagent in aqueous solution for calcium and magnesium but is much less efficient in the presence of alcohols, and the trace of EDTA in the solvent used for the main chromatographic separation of the phosphates was added as a safeguard against accidental contamination. In addition the precision of the chromatographic separations is improved if no other cations are present except ammonium. Even the presence of variable amounts of sodium and potassium affects the reproducibility of the analysis. The procedure was therefore to remove as soon as possible the bulk of the trichloroacetic acid by ether extraction to prevent hydrolysis of labile phosphate esters, all cations by treatment with a cation-exchange resin, the trichloroacetic acid liberated from salt formation by a further extraction with ether and finally to neutralize the solution with ammonia. The solution was then ultrafiltered through a viscose membrane to remove polysaccharides. The ultrafiltration is not always necessary and may be omitted if the amount of polysaccharide is small. It was necessary with peas and potatoes but less so with strawberry leaves. Phosphorus analyses on the filtrate and on the residual solution indicated that with peas most of the phosphorus-containing compounds passed through the membrane readily since the two solutions had a similar composition (vol. 510 ml., total phosphorus 504 mg., and vol. 30 ml., total phosphorus 27 mg., respectively). However, addition of ethanol to the residual solution not only precipitated the polysaccharides but also brought down over half the phosphorus (16 mg.). This meant that in all probability some high-molecular-weight phosphate esters were present since the P_i in the solution was unaffected.

The extract was now fractionated by chromatography on a cellulose column. This separated the nucleotides and sugar phosphates from the chloride, sugars, amino acids and phytic acid. It was essential to remove these compounds because they were present in relatively large amounts and, unless removed, interfered with the isolation of individual phosphate esters. Phytic acid also contains a large amount of esterified phosphorus (28%) and, since the amount was usually several times that of the combined sugar phosphates, it tended to obscure the interpretation of the phosphorus analyses. The size of the column depended upon the amount of the sugars present since it was important not to concentrate the extract so that the residual syrup became too thick to be easily absorbed by the cellulose powder. In general it was better to use a slightly larger column than appeared necessary even though the quantities of solvent required were correspondingly larger. In practice the amount of solvent used was not fixed but was adjusted so that the sugars were completely eluted. An excess did no harm because the phosphate esters on the column were not affected (90% propan-1-ol-water-ammonia). Similarly the phosphate esters could be eluted by using an excess of the appropriate solvent (72.5% propan-1-ol-water-ammonia) without affecting the phytic acid on the column. However, this clear-cut distinction between the fractions depended on the amount of water in the solvent being precisely defined and for this the most convenient and sensitive method was measurement of the density by hydrometer. The density of propan-1-ol-water mixtures ranges from 0.807 to 1.00 in a nearly linear relationship with the percentage of water in the mixture, and, since it is simple to measure the density to 0.001 by hydrometer, the water content can be estimated to 0.2%.

The fraction from the first column containing the phosphate esters also contained all the malic acid and citric acid as well as the P_i . These compounds were present in concentrations greater than that of the combined nucleotides and sugar phosphates (P_i is of the same order and the organic acids are 10–20 times the concentration) and when present in these amounts hinder the subsequent separation of individual nucleotides or sugar phosphates either by ion-exchange or paper chromatography. They were removed by chromatography on a fresh cellulose column with an acidic solvent.

The size of the column was theoretically regulated by the amount of malic acid and citric acid present but since the amounts of material involved were small, a column using 40 g. of cellulose powder was conveniently adopted as standard. The amount of solvent used for development was critical since the column procedure was designed to give the quickest possible separation of the P_i and organic acids from

the phosphate esters, so that the latter would only be subjected to acid conditions for a short time. This meant that if an excess of solvent was used there was a danger of some phosphate esters being eluted. Under the standard conditions the P_i and the organic acids were completely eluted before any phosphate esters appeared, though in practice the progress of the elution was followed by phosphorus analysis on the eluate. As soon as the P_i had been washed through, the solvent was changed to one in which formic acid had been omitted from the solvent mixture. This washed the formic acid out of the column before the main bulk of the phosphate esters was eluted. A small amount of a phosphate ester (phosphoglyceric acid) was sometimes present and was isolated separately. The advantage of removing the formic acid was that the final elution with aq. methanol gave a solution of the nucleotide and sugar phosphates free from formic acid which could be concentrated to remove the methanol without danger of any breakdown of the phosphate esters.

The recovery of D-glucose 6-phosphate and ATP in control experiments in which the whole procedure was tested was excellent. The same was true of extracts of peas and potatoes in which the amounts present initially were compared with those found finally in the purified extract. It was noticeable that enzymic reactions in the purified extracts showed no evidence of inhibitors being present except in strawberry leaves, which are known to be very rich in polyphenols.

The entire elimination of solutes other than phosphate esters means that the extract can be concentrated to almost any extent to allow for the detection of individual phosphate esters either by chromatography or by enzymic assay.

This means that it is now possible to study in a comprehensive manner the changes in the concentrations of all the phosphate esters in plant tissues and represents a definite advance in the investigation of the physiology or metabolism of plant tissues.

We are grateful to Dr J. Barker, F.R.S., for his interest and helpful discussion.

REFERENCES

- Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
- Anderson, G. (1955). *Nature, Lond.*, **175**, 863.
- Bandurski, R. S. & Axelrod, B. (1951). *J. biol. Chem.* **193**, 405.
- Barker, J., Isherwood, F. A., Jakes, R., Solomos, T. & Younis, M. E. (1962). *Nature, Lond.*, **196**, 1115.
- Barker, J., Jakes, R., Solomos, T., Younis, M. E. & Isherwood, F. A. (1964). *J. exp. Bot.* **15**, 284.
- Bartholomew, W. H. (1949). *Analyt. Chem.* **21**, 527.
- Benson, A. A. (1955). In *Modern Methods of Plant Analysis*, vol. 2, p. 113. Ed. by Paech, K. & Tracey, M. V. Berlin: Springer-Verlag.

- Bergmeyer, H. U. (Ed.) (1963). *Methods of Enzymatic Analysis*, pp. 117, 983. New York and London: Academic Press Inc.
- Bieleski, R. L. & Laties, G. G. (1963). *Plant Physiol.* **38**, 586.
- Bieleski, R. L. & Young, R. E. (1963). *Analyt. Biochem.* **6**, 54.
- Boatwell, P. W. (1917). *J. Amer. chem. Soc.* **39**, 491.
- Craig, L. C. (1950). *Analyt. Chem.* **22**, 1462.
- Fawaz, E. N. & Fawaz, G. (1962). *Biochem. J.* **83**, 438.
- General Medical Council (1953). *British Pharmacopoeia*, p. 581. London: The Pharmaceutical Press.
- Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
- Heslop, J. P. (1964). *Biochem. J.* **91**, 183.
- Isherwood, F. A. & Hanes, C. S. (1953). *Biochem. J.* **55**, 824.
- Isherwood, F. A. & Niavis, C. A. (1956). *Biochem. J.* **64**, 549.
- Khim, J. X. & Conn, W. E. (1953). *J. Amer. chem. Soc.* **75**, 1153.
- Lowry, O. H., Passonneau, J. V., Hasselberger, F. X. & Schultz, D. W. (1964). *J. biol. Chem.* **239**, 18.
- Martell, A. E. & Calvin, M. (1952). *Chemistry of Metal Chelate Compounds*, p. 459. New York: Prentice Hall Inc.
- Minard, F. N. & Davis, R. V. (1962). *J. biol. Chem.* **237**, 1283.
- Newsholme, E. A. & Randle, P. J. (1961). *Biochem. J.* **80**, 655.
- Polakis, E. S. & Bartley, W. (1966). *Biochem. J.* **99**, 521.
- Rappaport, D. A. & Chen, P. T. (1960). *Biochim. biophys. Acta*, **38**, 156.
- Slater, E. C. (1953). *Biochem. J.* **53**, 157.
- Talbert, W. F. & Smith, O. (1959). *Potato Processing*, p. 191. Westport, Conn.: The Avi Publishing Co. Inc.
- Wildman, S. G., Campbell, J. M. & Bonner, J. (1949). *Arch. Biochem.* **24**, 9.