CARBOHYDRATE METABOLISM IN THE SHOOTS OF BAMBOO, *LELEBA OLDHAMI*

II. Phosphorylation of Hexoses by the Cell-free Extracts of Bamboo Shoots[1][2]

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Glucose, fructose and sucrose consist the bulk of the dilute ethanol extractable fraction of bamboo shoots (Su, 1965). Since the tissue is nonphotosynthetic, these carbohydrates must be translocated from the mother plant to sustain the rapid growth of the young tissue.

Sucrose is known as the chief carbohydrate translocated in higher plants. The findings (Su, 1965) that a strong invertase activity and a large amount of reducing sugars are present in bamboo shoots suggest that at least part of the sucrose translocated from the mother plant into the young bamboo tissue must be metabolized through the invertase-catalyzed hydrolysis step.

In this investigation, we studied the hexose phosphate forming reactions catalyzed by the crude and partially purified extracts of the shoots of bamboo, *Leleba oldhami*. C^{14}-glucose was used as the substrate to facilitate the identification of the sugar phosphates formed by the enzyme-catalyzed reaction.

Methods and Materials

General Analytical Methods—Column chromatography of sugar phosphates in the homogenate experiment was done according to Khym and Cohn's procedure (1953).

Paper electrophoresis was carried out with an apparatus similar to that described by Crestfield and Allen (1955). Three different buffer solutions were used: 0.15 M ammonium formate, pH 3.5 (I); 0.15 M ammonium acetate, pH 5.6

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(II); 0.05 M sodium tetraborate, pH 9.2 (III). Sometimes, when additional evidence was desired, 0.1 M sodium phosphate, pH 7.0, was also used. The electrophoretic mobility of individual compounds is given relative to that of picric acid (M*pa*) with the endoosmotic flow corrected by the caffeine marker.

Paper chromatography was performed in the following two solvent systems, the ratio of which being expressed as volume per volume: n-butanol-acetic acid-water, 4:1:1, for sugars, sugar acids, and sugar lactones, and methanol-ammonium hydroxide-water, 6:1:3, for sugar phosphates.

Whatman No. 1 filter paper was generally used for paper electrophoresis and paper chromatography. In the case of chromatography of sugar phosphates, the paper was washed with 1 percent versene solution before use.

After separation by paper electrophoresis and paper chromatography, sugar phosphates were detected by the molybdate perchloric acid procedure of Bandurski and Axelrod (1952); sugar acids, by the o-phenylenediamine method (Lanning et al, 1951); and free sugars and sugar lactones, by the alkaline permanganate method (Pacsu et al, 1949).

Aliquots of radioactive fractions from column chromatography in the homogenate experiment were dried in a stainless steel planchet and counted with a 2π gas flow counter. Radioactive substances on the electrophoretogram or paper chromatogram were located by autoradiography or roughly by a G-M tube monitor.

The modified acid-base indicator method of Darrow (1962) was adopted for the hexokinase assay. Protein content was determined by the phenol method (Lowry et al, 1951).

Preparation of Bamboo-shoot Extracts—Some of the bamboo shoots used were obtained from the local market at Nankang, Taipei, but most of them were collected from the hillside near the institute, from September to December, 1964. The sheath and bottom part of the bamboo shoots were first removed. The remaining young tissue was cut to pieces with a stainless steel knife and then ground with aqua regia-cleaned sand in about equal amount (by weight) of 0.12 M Tris buffer, pH 8.1. The sirupy mass was then squeezed through two layers of cheese cloth and centrifuged at 1,000 $\times g$ for 10 minutes. The supernatant liquid thus obtained was used in the homogenate experiment. For enzyme study, the supernatant liquid was further centrifuged at 78,000 $\times g$ and the sedimented pellets discarded.

Preparation of Labeled Glucose—Radioactive glucose used in the enzyme study was purchased from the Volk Radiochemical Company, that used in the homogenate experiment was prepared by the second author, according to the photosynthetic method of Abraham and Hassid (1957). Radioactive fructose was obtained from the invertase hydrolysate of radioactive sucrose which was also

purchased from the Volk Company.

Optical density values were measured with a Beckman Model DU spectrophotometer equipped with a set of thermospacer.

All operations that require low temperature were carried out in a cold room at 2 to 5°C.

Experiments and Results

I. Homogenate Experiment

A mixture solution containing U-C^{14}-glucose (totaling 140,000 cpm) diluted with 17 μmoles of non-radioactive glucose, 20 μmoles of ATP,* 10 μmoles of NaF,

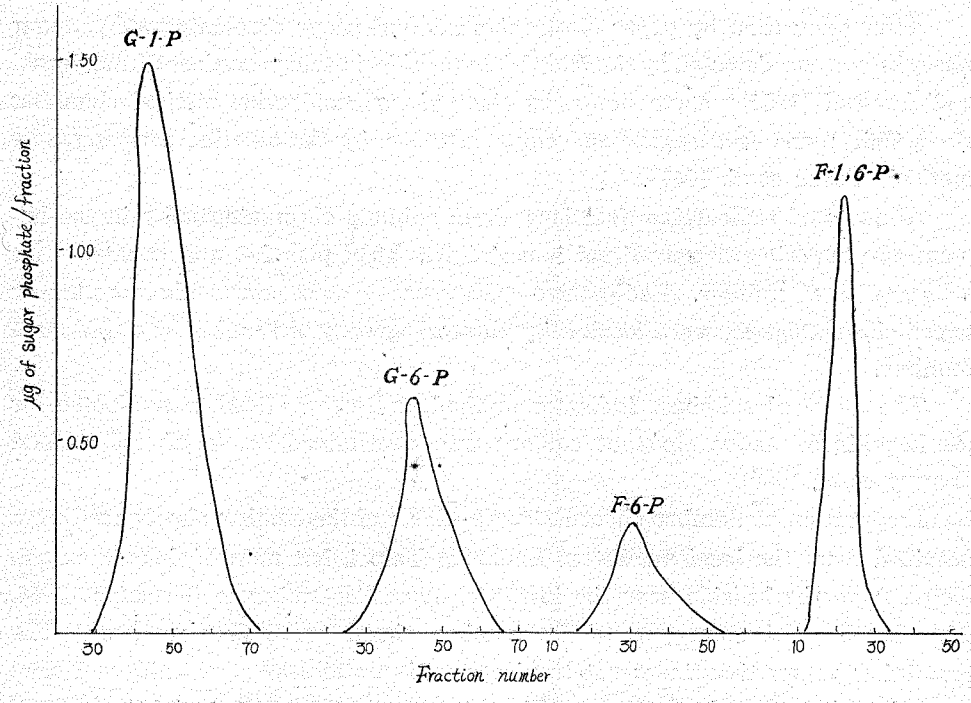

Fig. 1. Elution pattern of authentic sugar phosphates—A pH 8.5 ammoniacal solution of 6 to 10 mg each of G-1-P, G-6-P, F-6-P, and F-1, 6-P was absorbed on a 200 to 400 mesh Dowex-1 (chloride) column, 11.6 × 1.1 cm, and washed with water to remove nonanionic materials. The column was eluted successively, at a flow rate of about 3.5 ml per minute, with the following solutions: (1) 0.025 M NH_4Cl, 0.01 M $K_2B_4O_7$, for G-1-P; (2) 0.025 M NH_4Cl, 0.0025 M NH_4OH, 0.001 M $K_2B_4O_7$, for G-6-P; (3) 0.025 M NH_4Cl, 0.0025 M NH_4OH, 0.00001 M $K_2B_4O_7$, for F-6-P; (4) 0.02 M HCl, 0.02 M KCl. for F-1,6-P. The eluates are analyzed by the anthrone method (Scott, 1953). Fraction volume; 10 ml.

* The following abbreviations are used: ATP, Adenosine triphosphate; ADP, Adenosine diphosphate; G-1-P, Glucose-1-phosphate; G-6-P, Glucose-6-phosphate; F-6-P, Fructose-6-phosphate; F-1,6-P, Fructose-1,6-diphosphate; GTP, Guanosine triphosphate; UTP, Uridine triphosphate; UDP, Uridine diphosphate; UDPG, Uridine diphosphate glucose; Tris, Tris (hydroxymethyl) aminomethane; ATPase, Adenosine triphosphatase; PGA, phosphoglyceric acid; PP, Pyrophosphate.

and 12.5 μmoles of $MgCl_2$, in a total volume of 3.25 ml was incubated with 5 ml of bamboo shoot homogenate at 30°C for one hour. At the end of the incubation, the reaction was stopped by adding 1 ml of 3 M trichloroacetic acid. The supernatant solution collected from centrifugation was extracted with petroleum ether for several times to remove trichloroacetic acid and then condensed to a small volume and neutralized to a slightly alkaline pH (8.5) with dilute ammonium hydroxide. The clear solution obtained was applied to a column of Dowex-1, chloride form (15×1.5 cm, 200 to 400 mesh). Free sugars were removed by washing the column with 100 ml of 0.001 M ammonium hydroxide. A succession of eluting agents, in the order described by the elution curve of Fig. 2, was passed through the column to selectively desorb the different sugar phosphates that might be present in the mixture. A mixture of authentic sugar phosphates was applied on a similar column and the elution was carried out in a similar manner so as to obtain a calibration curve. The calibration curve is shown in Fig. 1.

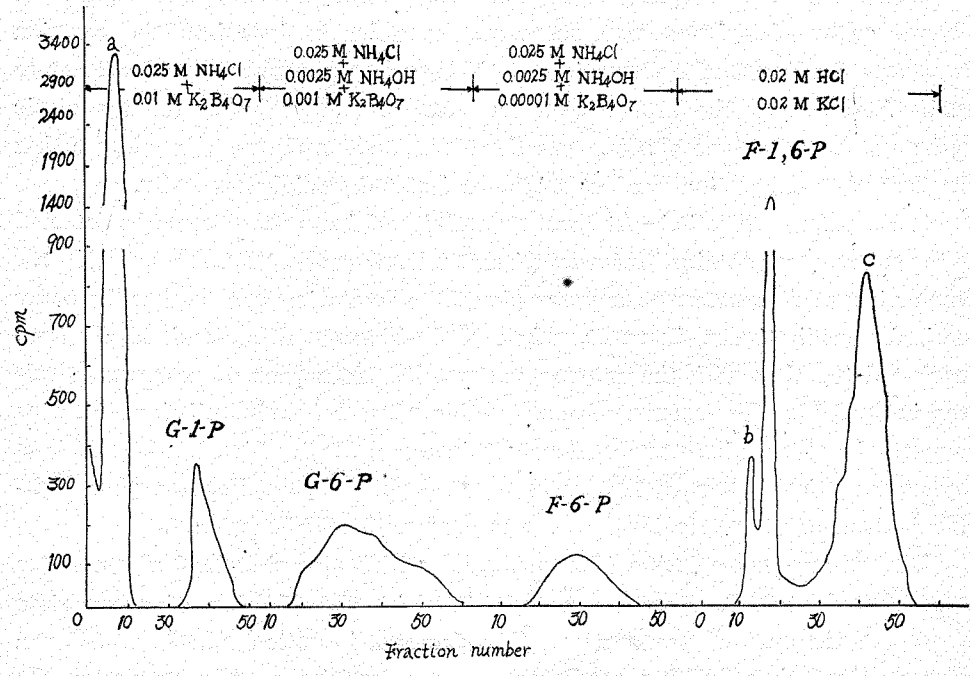

Fig. 2. Ion exchange separation of radioactive sugar phosphates from the reaction mixture (see text). Ion exchanger, Dowex-1 (chloride), flow rate, about 3.5 ml/min., fraction volume, 10 ml.

It is seen from the elution curve in Fig. 2 that, in addition to the four hexose phosphates, there are three unidentified peaks (*a*, *b*, *c*). It was postulated, from a consideration of the different acidity and borate complexing ability of sugar phosphates, that peak *a* and peak *b* might be a triose phosphate and a

PGA, respectively. As to the identity of peak *c*, we are not able to assign any known compound for it for the time being. However, since our main purpose lay on the survey of the possible existence of hexose phosphates in the reaction mixture, the identification of these compounds was not attempted.

Another experiment with unlabeled glucose as the substrate was carried out by the same procedure. Each eluting sample was assayed by the anthrone reaction (Scott, 1953). The peaks of G-1-P, G-6-P, F-6-P and F-1, 6-P appeared in the effluent following the same order and pattern as that in the labeling tests, however, with the positions of the peaks slightly shifted to the right. The three unidentified peaks *a*, *b* and *c*, as shown in Fig. 2, were not detected in this experiment. Since these compounds do not react with anthrone, they can not be hexose phosphates. When the same experiment was repeated with fructose substituting for glucose as the substrate, only the latter three sugar phosphate peaks were produced. No G-1-P was detectable.

II. Enzyme Study

In the experiment using homogenate as the enzyme source, formation of several sugar phosphates was observed. In order to look into the reaction sequences leading to the formation of these sugar phosphates, an attempt to purify hexokinase, the first enzyme for glucose metabolism in the glycolytic pathway, from bamboo shoots, was therefore made with the sole purpose that the first phosphorylation product might be isolated straightforwardly.

1. Enzyme purification—At first, no activity of hexokinase could be detected in the supernatant obtained from centrifuging bamboo shoot extracts at 78,000 $\times g$ when glucose disappearance or sugar phosphate formation was used as the assay method. Qualitatively, however, when U-C^{14}-glucose, ATP, and $MgCl_2$ were incubated together with the enzyme preparation, formation of new compounds was indicated by the appearance of a movable, though heavily tailed, radioactive zone on the electrophoretogram (formate buffer, pH 3.5). Finally, the acid base indicator method (Darrow, 1961) was adopted with the modification that Tris-HCl instead of glycylglycine-NaOH was used as the buffer system.

The stock assay solution (Darrow, 1961) which also contained glucose, was preincubated at 30°C. Reaction was initiated by the addition of appropriate amount of the enzyme preparation. The decrease in optical density at 560 mμ between the 15th and 45th seconds was measured with a spectrophotometer, the thermospacer of which was previously equilibrated at 30°C. This is the total activity resulted from the combined actions of hexokinase and ATPase. The net activity of hexokinase was obtained by subtracting the ATPase activity, measured in the same way by excluding glucose from the assay mixture, from the total activity.

A net decrease in optical density of 0.010 at 560 mμ per minute was arbitra-

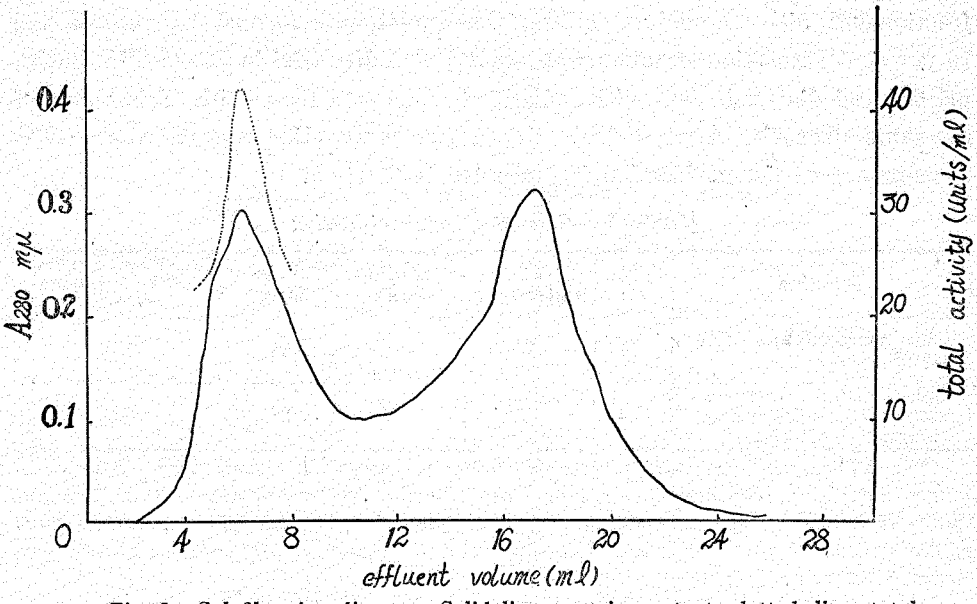

Fig. 3. Gel filtration diagram. Solid line, protein content; dotted line, total activity. Gel, Sephadex G-75. Fraction volume, 1 ml.

rily taken as one unit of hexokinase activity. Specific activity was expressed as units per mg of protein.

Because of the extremely low activity of hexokinase in the crude extract of bamboo shoots, and because of the existence of high ATPase activity, the crude extract was first fractionated with ammonium sulfate. The enzyme activities in the crude extract and in the first ammonium sulfate fractionation step were semiquantitatively determined in the following manner. Appropriate amount of enzyme solution from each fraction was incubated with ATP, $MgCl_2$, NaF, and radioactive glucose(about 500,000 cpm) in a capillary tube for 10 minutes. After the incubation, the content in the capillary tube was applied on a strip of Whatman No. 1 filter paper previously prepared on the glass bridge of the electrophoretic apparatus. Electrophoresis was run in the formate buffer for about 1 hour at a field strength of 40 v./cm. The electrophoretogram was dried and the radioactive sugar phosphate spots directly counted with a G-M tube monitor. The magnitude of the activities of the movable spots was then taken as the measure of the enzyme activity. It was found that the fraction between 50 and 80 per cent saturation of ammonium sulfate was the most active and hence was used as the starting material for further purification.

The active protein precipitated by ammonium sulfate was dissolved in 11 ml of Tris buffer, 0.05 M, pH 7.5, and passed through a column of sephadex G-75, previously equilibrated with the same buffer.

Two peaks were obtained as shown in Fig. 3. The first peak contained all the hexokinase activity. The most active fractions were then pooled and again

fractionated with ammonium sulfate. The precipitate formed between 50 and 75 per cent saturation of ammonium sulfate was dissolved in 0.01 M Tris buffer, pH 6.5, and dialyzed, first with redistilled water and then with Tris buffer of the same strength. After dialysis, the enzyme preparation was stored at −10°C. A summary of the purification is presented in Table 1.

Table 1. *Summary of purification*

Fraction	Vol. ml	Total protein mg	Hexokinase units	ATPase units	Hexokinase specific activity, units/mg protein
First ammonium sulfate fractionation	11	1,100	88	352	0.08
Gel filtration, Second ammonium sulfate fractionation, and dialysis	4	74	68	0*	0.90

*No ATPase activity could be detected under the assay conditions used. After prolonged incubation (10 minutes), however, optical density decrease of about 0.01 to 0.015 was observed, indicating the contamination of small amount of ATPase. The uppermost limit of the optical density change contributed by ATPase in the final preparation was estimated to be 15 per cent.

2, Identification of hexokinase reaction products—U-C^{14}-glucose (about 2,000,000 cpm), ATP, and $MgCl_2$ were incubated with about one unit of the enzyme solution in a capillary tube at 35°C for 1 hour. The reaction mixture was then electrophorased at pH 3.5 (I) and the electrophoretogram autoradiographed. Three spots were distinctly observed, of which the mobilities relative to that of the picric acid marker were respectively: 0.88 (*A*), 0.37 (*B*), and 0.00 (*C*).

On considering the mobilities of the three spots together with the ionization properties of phosphate esters, it was immediately realized that spot *A* could be a sugar phosphate. Accordingly, further experiments were mainly aimed at the identification of this spot.

Examination of the electrophoretic mobility of spot *A* showed that at pH 3.5 (I) and 5.6 (II), it was the same as that of G-1-P and G-6-P. In a borate buffer at pH 9.2, however, it was greater than G-1-P but identical with G-6-P. Results obtained from paper chromatographic analysis also showed that the R_f value of spot *A* agreed with that of G-6-P. Further evidence of spot *A* as a phosphate ester was shown by the fact that when it was hydrolyzed with prostatic phosphatase, its electrophoretic mobility in ammonium acetate (II) changed from 1.0 to zero. The conclusion that spot *A* is identical with G-6-P was then reached. The results are presented in Table 2.

The identification work of spots *B* and *C* has not been extensively done. It was observed, however, that spot *B* formed three new spots when electrophorased at pH 5.6 (II). Also, electrophoresis of spot *C* at pH 9.2 (III) resulted in the formation of as many as six spots. Available information obtained from some electrophoretic and chromatographic experiments seemed to indicate that

there might be gluconic acid in spot *B* and δ-gluconolactone in spot *C*. Owing to the lack of substantial evidence, however, data of these identification work are, nevertheless, not presented here. It is apparent, any way, that the present enzyme preparation is still far from being pure.

Another experiment using radioactive fructose as the substrate was conducted in the same fashion as that with radioactive glucose. Electrophoresis of the reaction mixture at pH 3.5 (I), a spot (*D*) with mobility 0.92 was also located. It was identified to be F-6-P (Table 2).

Table 2. *Identification results of spot A and D.*

Compound	Mobility relative to M_{Pa}			R_f*
	Buffer system			
	I	II	III	
Glucose	0.00	0.00		
G-1-P	0.90	1.10	1.21	0.73
G-6-P	0.90	1.10	1.36	0.61
Spot A	0.88	1.10	1.36	0.61
Spot A, treated with prostatic phosphatase		0.00		
F-6-P	0.92	1.12	1.45	0.52
F-1, 6-P	0.92	1.12	1.45	0.30
Spot D	0.92	1.12	1.45	0.52

*Methanol : ammonium hydroxide : H_2O, 6:1:3

Discussion

The obtained results indicate that the following reactions are catalyzed by the cell-free extract of bamboo shoots.

(1) glucose + ATP ⟶ G-6-P + ADP

(2) fructose + ATP ⟶ F-6-P + ADP

(3) G-6-P ⟷ F-6-P

(4) G-6-P ⟷ G-1-P

(5) F-6-P + ATP ⟶ F-1,6-P + ADP

Further degradation of fructose 1,6-diphosphate through glycolytic pathway is suggested by the findings that radioactive peaks with the elution volumes corresponding to triose phosphates and glyceric acid phosphates were obtained when the incubation mixture of C^{14}-glucose, ATP and the bamboo shoot homogenate was chromatographed on a Dowex-1 column (Fig. 2). These peaks were not found, however, when cold glucose was used instead of C^{14}-glucose in a similar experiment, and the chromatographic eluate assayed with anthrone reagent which is known to give little or no green color with pentose, tetrose or triose phosphates. This finding implies that the suggested identity of the

stated radioactive peaks might be correct.

It was demonstrated in the bamboo shoot extracts the presence of UDPG pyrophosphorylase as well as GDPG pyrophosphorylase activity (Su, J. C., unpublished). It was also found that UDPG comprises almost 50 per cent of the total dilute ethanol soluble nucleotides of bamboo shoots (Su, J. C., unpublished). Furthermore, when a mixture solution containing UDP and sucrose was incubated with bamboo shoot extract, the formation of UDPG was detectable (Su, 1965), indicating the existence of sucrose synthetase activity. It is suggested that the potential glucosyl donor, UDPG, is formed from sucrose in the young bamboo tissue, besides by the reaction catalyzed by sucrose synthetase, through the following reaction sequences:

$$\text{sucrose} + H_2O \longrightarrow \text{fructose} + \text{glucose}$$
$$\text{fructose} + \text{ATP} \longrightarrow \text{F-6-P} + \text{ADP}$$
$$\text{F-6-P} \rightleftharpoons \text{G-6-P}$$
$$\text{glucose} + \text{ATP} \longrightarrow \text{G-6-P}$$
$$\text{G-6-P} \rightleftharpoons \text{G-1-P}$$
$$\text{G-1-P} + \text{UTP} \rightleftharpoons \text{UDPG} + \text{PP}$$

Leleba oldhami 竹筍中之醣代謝

II. 竹筍抽出液中己糖類 (Hexoses) 之磷酸化作用

李建中 盧棟生 丁昌玲 蘇仲卿

用竹筍粗抽出液與 C^{14}- 葡萄糖及三磷酸腺核 (ATP) 作用後，應用離子交換樹脂分析法，證明有葡萄糖及菓糖之磷酸酯生成。

應用蛋白質分離方法，將竹筍抽出液部分淨化後，可使其中之己糖磷酸化酵素 (Hexokinase) 活性提高約十倍，以此與 C^{14}- 葡萄糖及三磷酸腺核反應，可自反應液中直接分離得葡萄糖 —6— 磷酸酯 (G-6-P)。因此可確證竹筍中有醣酵解過程 (Glycolytic pathway) 中之各種酵素反應。

Literature cited

ABRAHAM, S., and W. Z. HASSID. The synthesis and degradation of isotopically labeled carbohydrates and carbohydrate intermediates. in Enzymology, vol. IV, Colowick, S. P., and N. O. Kaplan, (editors), N. Y. Acad. Press, Inc., p. 480, 1947.

BANDURSKI, R. S., and B. AXELROD. Chromatographic identification of some biologically important phosphate esters. J. Biol. Chem., **193**: 405, 1952.

CRESTFIELD, A. M., and F. W. ALLEN. Resolution of ribonucleotides by zone electrophoresis. Anal. Chem., **27**: 422, 1955.

DARROW, R. A., and S. P. COLOWICK. Hexokinase from baker's yeast. In Methods in Enzymology, vol. V, Colowick, S. P. and N. O. Kaplan. (editors), N. Y. Acad. Press, Inc., p. 226, 1961.

KHYM, J. X., and W. E. COHN. The separation of sugar phosphates by ion exchange with the use of the complex borate. J. Am. Chem. Soc., **75**: 1153, 1953.

LANNING, M. C., and S. S. COHEN. The detection and estimation of 2-ketohexonic acids. J. Biol. Chem., **189**: 109, 1951.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. J. Biol. Chem., **193**: 265, 1951.

PACSU, E., T. P. MORA, and P. W. KENT. Science, 110: 446, 1949.

SCOTT, T. A., and E. H. MELVIN, Determination of dextran with anthrone. Anal. Chem., **25**: 1656, 1953.

SU, J. C., Carbohydrate metabolsim in the shoots of bamboo, *Leleba oldhami* I. Preliminary survey of soluble saccharides and sucrose-degrading enzymes. This Bulletin, this issue.