THE JOURNAL OF AGRICULTURE
OF THE UNIVERSITY OF PUERTO RICO

Issued quarterly by the Agricultural Experiment Station of the University of Puerto Rico, for the publication of articles by members of its personnel, or others, dealing with any of the more technical aspects of scientific agriculture in Puerto Rico or the Caribbean Area.

Vol. XLVIII July 1964 No. 3

Sucrose-Enzyme Relationships in Immature Sugarcane as Affected by Varying Levels of Nitrate and Potassium Supplied in Sand Culture

Alex G. Alexander[1]

INTRODUCTION

Prior to 1957 sucrose yields in Puerto Rico sometimes exceeded 12 percent, but since 1958, yields have declined to 10.3 percent with the resultant income loss of about $36,000,000 in 1961 alone (*9*).[2, 3] Very little is understood of the mechanisms responsible for sugar degradation in cane, although a number of nutritional and climatic factors are known to induce fluctuations in sugar content.

Sugarcane nutrition experiments have repeatedly pointed up an influence of potassium and nitrogen on sucrose yield. Samuels and Capó (*58*) reported that significant reductions in sucrose-percent-cane accompanied reduced tonnage from trials in which nitrogen and potassium had been omitted. Humbert (*38*) suggested that a K/N ratio of 4:5 in the 8–10 stalk tissue is optimum for high sucrose yields in Hawaii. Van Dillewijn (*20*) reviewed numerous findings that the content of both reducing sugars and sucrose is strongly affected by the rate of nitrate supplied to sugarcane. High nitrate concentrations tend to promote high reducing sugar and low sucrose concentrations in young cane. The depressing effect of high nitrate on sucrose diminishes beyond 6 months of age.

Low sucrose levels have been found associated with cane potassium deficiency in Puerto Rico (*59*, *60*, *61*). Beauchamp (*6*) reported that potassium applications increased the potassium content of crude chlorophyll of leaves and the percentage of sucrose in the juice of sugarcane. Hartt (*32*) cited numerous reports indicating that potassium was known to be essential for high juice quality prior to 1934. In a subsequent publication (*33*) Hartt provided indirect evidence that sucrose may be synthesized by

[1] Associate Plant Physiologist, Agricultural Experiment Station, University of Puerto Rico, Río Piedras, P.R.

[2] Italic numbers in parentheses refer to Literature Cited, p. 228–31.

[3] With only the Mercedita Sugar Central still grinding, the 1963 yield is reportedly down to 9.66 percent.

invertase, and that potassium is a specific activator of invertase in sugarcane.

Our own objective was to clarify some of the mechanisms involved in the alteration of sucrose content by treatments such as the nutritional variables mentioned above. Our procedures were focused upon enzymes. The initial experiments involved a gradual curtailment of nitrate and potassium supplied to immature cane plants, as well as increasingly high nitrate supplied to others, so that the subsequent sugar variations could be studied in relation to sugar-metabolizing enzymes.

MATERIALS AND METHODS

GROWTH AND SAMPLING OF PLANT MATERIALS

One-eye cuttings of the variety M. 336 were planted in No. 2 quartz sand on September 7, 1962, and all plants received a complete nutrient solution until treatments began on November 12. The sand was previously treated with 0.1 N HCl for 12 hours, and after thorough leaching with tapwater, it was placed in glazed, 2-gallon containers fitted with glass wool over the drainage outlets. Ten to twelve PMA-treated cuttings were planted in each container.[4]

The seedlings were watered each day at 7 a.m. with 600 ml. of tapwater, and again at 1 p.m. with 600 ml. of nutrient solution.[5] Each of the nutrient concentrations proved adequate with the exception of iron. Iron-deficiency symptoms began to appear 4 weeks after planting, but the disorder was corrected for the duration of the study by increasing the iron supply from 0.50 to 1.0 p.p.m.

After 8 weeks of growth, 36 of the most uniform containers were selected for treatment. Nine of these were designated as controls and continued to receive the complete solution throughout the study. The remaining plants were divided into three groups of nine containers each and one group was supplied with decreasing nitrate, another with decreasing potassium, and the third with increasing nitrate at intervals of 21 days (table 1). For example, all plants received 10 meq./l. of nitrate for 9 weeks, at which time the plants of the increasing nitrate group were selected to receive 12.5 meq./l. for the next 3 weeks, 15.0 meq./l. for the following 3 weeks, and

[4] The cuttings were immersed in a 0.25-percent solution of PMA (phenyl mercury acetate) and allowed to remain for 10 minutes. The treatment is a general antiseptic against seedborne micro-organisms.

[5] Nutrient concentrations, expressed as milliequivalents per liter, were provided as follows: Nitrate, 10; phosphate, 6; potassium, 5; calcium, 3; magnesium, 2; and sulfate, 2. Microelements, expressed as parts per million, were supplied as follows: Boron, 0.05; copper, 0.02; manganese, 0.50; zinc, 0.05; molybdenum, 0.01; and iron, 0.50.

so forth until the final treatment period when they received 25 meq./l., as compared with the controls which had continued to receive the original 10 meq./l. Simultaneously another experimental group was gradually reduced to 1.0 meq./l. of nitrate, and a third group to 0 meq./l. of potassium. The experimental design was a randomized block with three replicates for each of the four treatments.

The first samples were harvested on November 12, a few hours prior to initial treatments, and subsequent samples were taken at the conclusion of each 21-day period. Two representative canes from each replicate were cut at the surface of the sand. Sheath material was removed for determina-

TABLE 1.—*Nutrient levels supplied to immature sugarcane in sand culture*

Weeks from planting	Treatment period No.[1]	Nutrient concentrations in milliequivalents per liter for—					
		Experiment 1, increasing NO_3		Experiment 2, decreasing NO_3		Experiment 3, decreasing K	
		Control	Treatment	Control	Treatment	Control	Treatment
0–9	0	10	10.0	10	10.0	6	6
9–12	1	10	12.5	10	8.5	6	5
12–15	2	10	15.0	10	7.0	6	4
15–18	3	10	17.5	10	5.5	6	3
18–21	4	10	20.0	10	4.0	6	2
21–24	5	10	22.5	10	2.5	6	1
24–27	6	10	25.0	10	1.0	6	0

[1] The first samples were taken just prior to initial treatments when the plants were 9 weeks of age. Subsequent harvests were made at the completion of each 21-day treatment period.

tion of sugars and sheath-percent-moisture. Leaf samples were taken for sugars and enzyme protein, and all remaining material was oven-dried to be included in total dry weight. Leaves +1 to +3 were utilized at all harvests.[6] Sheaths were trimmed with scissors, weighed, and subsequently removed to a forced-air oven for drying. The basal 12 to 14 inches of the leaf blades were trimmed with scissors, inserted in stoppered 38 × 200 mm. pyrex culture tubes, and immediately frozen in a mixture of Dry Ice (solidified carbon dioxide) and acetone. The frozen tissues were stored at −20°C. and over a period of several weeks were withdrawn to a cold chamber where they were dried under vacuum, at 0–2°C., with the aid of a Virtis Roto-Freeze drying assembly. When thoroughly dry the samples

[6] The leaf nomenclature employed here is that of Kuijper (*39*), in which the highest leaf bearing a visible dewlap is designated +1.

were ground with a Wiley Mill to pass a 60-mesh screen, sealed in sample jars equipped with screw tops, and stored at 0–2°C. until extractions could be made.

LABORATORY ANALYSES

Sugars

Sheath-sugar assays were conducted with distilled-water extracts of oven-dried tissue which had been ground to pass a 60-mesh screen. Leaf sugars were determined using aliquots of the initial enzyme extracts. Total ketoses, sucrose, and total reducing sugars were assayed directly, while fructose and glucose were determined by calculation.

Total ketoses were measured colorimetrically by the resorcinol method of Roe (*57*). A deep red color is produced when ketose is heated in strongly acid solution in the presence of resorcinol (Seliwanoff's test). The reaction was once considered specific for fructose, but later studies have shown that all sugars bearing a keto group are involved. The sucrose fraction was determined by the method of Cardini, *et al.* (*16*) in which fructose was destroyed by heating the samples for 10 minutes at 100°C. in 0.25 N NaOH. According to Sumner (*68*), who used this technique to distinguish between reducing sugars and nonsugar reducing compounds in urine, heating with alkali completely destroys reducing sugars, including glucose when it is present in small amounts.

A standard curve representing 0.01 to 0.50 mg. of ketose was prepared daily from a 0.1-percent sucrose stock solution in saturated benzoic acid. To 1 ml. of diluted sugar solution was added 1 ml. of alcoholic resorcinol, *i.e.* 0.5 gm. resorcinol in 500 ml. of 95-percent ethyl alcohol, and 2 ml. of 30-percent HCl. The tubes were placed in boiling water and, after 5 minutes, were cooled to room temperature. Percentage transmittance was measured at 525 mμ with a Beckman Model B Spectrophotometer.

Total reducing sugars were measured by the dinitrosalicylic-acid technique of Sumner (*68*). Some small nonsugar-reducing activity was present in the extracts, but this could be estimated by eliminating the reducing-sugar fraction with hot alkali. The interfering activity was slight and generally constant among all samples, so that we were eventually able to contrive a correction factor which eliminated the need for rerunning each sample assay. The fructose values determined previously were substracted from the corrected total reducing-sugar content and the remaining fraction was considered to be predominately glucose, although traces of maltose were undoubtedly present.

Water-Soluble Protein

Distilled-water extracts were used for enzyme assays throughout the study. From each sample, 6 gm. of the powdered, freeze-dried leaf tissue

were placed in a stoppered 125-ml. flask containing 100 ml. of distilled water. The mixture was shaken mechanically for 1 hour. Most of the tissue debris was then removed by expressing the mixture through four layers of grade 10 Curity absorbent gauze. The extracts were chilled to 1°C. and centrifuged at 3,000 r.p.m. for 20 minutes. A clear, amber supernatant liquid was then available from which an aliquot was removed and diluted for sugar analysis.

After bringing the supernatant liquid to 100 ml. with distilled water and adjusting the pH to 7.0 with 2.5 N NaOH, sufficient ammonium sulfate was added to bring the solution to 95-percent saturation. The salt was added slowly over a period of about 40 minutes, while the solution was constantly agitated with the aid of a magnetic stirrer. Protein began to precipitate at 32-percent saturation and, after all the ammonium sulfate was added, the solutions were refrigerated at 6°C. for an additional 8 hours to increase protein yield.

Each precipitate-containing sample was again chilled to 1°C. and centrifuged for 10 minutes at 3,500 r.p.m. The supernatant liquid was discarded. The protein fraction was taken up in 20 ml. of distilled water and dialyzed overnight at 6°C. against 600 ml. of distilled water. A small precipitate appeared during this period and was subsequently removed by centrifuge. One milliliter of the supernatant liquid was withdrawn for protein determination, while the remainder was divided equally among five stoppered test tubes and frozen until required for enzyme assay.

Protein content was determined colorimetrically by the method of Sutherland, *et al.* (*69*), using 30-percent bovine albumen (Nutritional Biochemicals Corp.) for a standard. A reasonably stable blue color is developed which can be measured at 660 mμ with any standard colorimeter. The method is admirably suited for small amounts of protein in the range of 0.025 to 0.50 mg.

Enzyme Assays

When seeking mechanisms responsible for abnormal sugar levels, attention is understandably drawn to the glycolytic and tricarboxylic acid pathways. A number of enzymes known to be active in these cycles were found in cane-leaf extracts. Considerable effort was made to measure their response to the variable nitrate and potassium treatments, and to discover relationships between the enzymes and fluctuating sugar levels.

Enzymes of glycolysis included hexokinase, phosphorylase, phosphatase, phosphohexoisomerase, aldolase, triose-phosphate dehydrogenase, phosphoglyceryl kinase, and pyruvic carboxylase. Enzymes of the TCA or Kreb's cycle included the condensing enzyme, isocitric dehydrogenase, α-ketoglutaric carboxylase, succinic dehydrogenase, fumarase, transaminase, and oxalacetic carboxylase. Several other enzymes were specifically

sought, but could not be found. In spite of strong hexokinase activity when glucose served as phosphate acceptor, no trace of fructokinase or galactokinase could be detected. Glucose-6-phosphate dehydrogenase was conspicuously missing, although other dehydrogenases were present. Neither aconitase nor malic enzyme could be detected. Aspartase was not present, although a transaminase was found which catalyzed the transfer of the amino group of *l*-aspartic to α-ketoglutaric acid, with the subsequent formation of oxalacetic and *l*-glutamic acid.

Measurements of other enzymes of importance in sugar metabolism were also made, including invertase, amylase, and cytochrome-C reductase. Still other enzymes of less direct interest, including peroxidase, catalase, and polyphenol oxidase were likewise measured. Acid phosphatase *per se* was assayed with β-glycerophosphate as a representative substrate. However, phosphatase activity was recorded against numerous substrates ranging from ATP and UDP to 6-phosphogluconate and the phosphoglyceric acids. Virtually every phosphorylated intermediate of the glycolytic system was subject to hydrolysis by cane-leaf acid phosphatase. It was not at first known whether this was the work of one enzyme or several, but the activity curves recorded in response to treatment variables indicate that a number of acid phosphatases were definitely active.

In most instances the activity unit was arbitrarily defined and the action of each enzyme was ultimately expressed as specific activity (units per milligram of protein). No attempt was made to employ the most optimum pH for every enzyme, although near-optimum pH was conveniently achieved in each instance by adjusting the substrate solution with dilute NaOH or acetic acid, or by utilizing a commercial acetate buffer (pH 4.65), succinate buffer (pH 6.25), or a phosphate buffer (pH 7.00 or 7.50). The temperature for all reaction mixtures was maintained at 30°C. with a constant-temperature water bath. Room temperature in the laboratory varied between 19.5 and 21.5°C. Enzyme assays for every sample, including controls, were run in duplicate. Unless otherwise specified all biochemicals were supplied by the Nutritional Biochemicals Corp.

Acid phosphatase was measured colorimetrically by determining the inorganic phosphate enzymatically released from β-glycerophosphate. To each of a series of test tubes, equilibrated to 30°C., was added 1 ml. of 0.1 M[7] acetate buffer, 1 ml. of 0.05-M β-glycerophosphate, and 0.25 ml. of enzyme solution. The reagents were mixed and the reaction was allowed to proceed for 10 minutes. Since the protein solutions contained no detectable phosphorus and the amber color of 0.25 ml. was insufficient to interfere with

[7] The letter M is used throughout this paper as an abbreviation for the word "molar".

colorimetry, satisfactory controls were maintained by supplying distilled water in place of enzyme.

The reaction was stopped with 2 ml. of 10-percent TCA (trichloroacetic acid). Inorganic phosphorus was determined by the phosphomolybdic acid technique (*48*). To 2 ml. of the TCA-phosphate mixture was added 1 ml. of ammonium molybdate solution (25 gm. of ammonium molybdate are dissolved in 375 ml. of distilled water; 75 ml. of concentrated H_2SO_4 are added and the solution is brought to 500 ml.), 1 ml. of hydroquinone solution (1.0 gm. of hydroquinone is dissolved in 200 ml. of distilled water, 3 drops of 2-N H_2SO_4 being added to retard oxidation), and 1 ml. of sodium sulfite solution (40 gm. of Na_2SO_3 dissolved in distilled water, and diluted to 200 ml.). The solutions were mixed upon addition of each reagent. Ten minutes were allowed for color development, at which time percentage transmittance was recorded at 640 mμ and the phosphate present determined by reference to a standard curve representing 0.001 to 0.04 mg. of phosphorus. One unit of activity was defined as the quantity of enzyme catalyzing the release of 0.01 mg. of phosphate under the specified conditions of the assay.

In like manner phosphatase activity was also measured against the following substrates: ATP (0.01 M), UDP (8.70×10^{-3} M), glucose-1-phosphate (0.05 M), glucose-6-phosphate (0.012 M), fructose-6-phosphate (0.01 M), fructose-1,6-diphosphate (0.025 M), 6-phosphogluconate (0.01 M), 2-phosphoglyceric acid (0.02 M), and 3-phosphoglyceric acid (0.01 M). A white precipitate forms upon the addition of TCA and color-development reagents during the tests for glucose-6-phosphatase, fructose-6-phosphatase, fructose-1,6-diphosphatase, and 3-phosphoglyceric phosphatase. The precipitate is readily removed by 5 minutes centrifuging at 2,500 r.p.m., after addition of sulfite, and does not interfere with color development. A 0.5-ml. aliquot of the TCA-phosphate mixture, plus 1.5 ml of distilled water, was used for color development during the fructose-1,6-diphosphatase assay. For UDP phosphatase the volume of reagents was reduced to 0.5 ml. of buffer, 0.25 ml. of UDP solution, and 0.25 ml. of enzyme for a total reaction-mixture volume of 1 ml. In this instance the reaction was stopped with 1 ml. of TCA, and color-development reagents were added directly to the phosphate-TCA mixture.

The assay for hexokinase, an enzyme catalyzing the transfer of phosphorus from ATP to hexoses, was based upon the disappearance of acid-labile phosphate during the reaction. The reaction mixture contained 1 ml. of acetate buffer, 1 ml. of 0.1-M glucose, 0.5 ml. of 0.01-M ATP, 0.25 ml. of enzyme solution, 5 drops of 0.1-M magnesium acetate, and 5 drops of 0.5-M sodium fluoride. Magnesium is a specific activator for this enzyme

and fluoride was required to prevent phosphatase activity. Control tubes received distilled water in place of enzyme. The reaction proceeded for 20 minutes at 30°C. and was stopped with 2 ml. of TCA. One milliliter of each sample was transferred to pyrex tubes containing 2 ml. of HCl (35 ml. of concentrated HCl diluted to 100 ml. with distilled water). After standing in boiling water for 6 minutes the tubes were cooled to room temperature and color was developed as described for phosphatase. One activity unit was defined as the amount of enzyme catalyzing the conversion of 0.01 mg. of phosphate from the ATP form (acid-labile) to the glucose-6-phosphate form (acid-stable) under the specified conditions of the assay.

Phosphorylase was measured by a method similar to that of Whelan (*75*) in which soluble starch is employed as the activating agent for phosphate transfer. Each treatment tube contained 1 ml. of acetate buffer, 0.5 ml. of 0.05-M glucose-1-phosphate, 0.5 ml. of 2-percent soluble starch, 2 drops of 0.5-M sodium fluoride, and 0.25 ml. of enzyme solution. Control tubes received distilled water in place of starch. The reaction proceeded 20 minutes at 30°C. The color was developed by the phosphomolybdic acid technique, as described for phosphatase and hexokinase, but without previous treatment of samples with TCA. One activity unit was defined as the amount of enzyme catalyzing the hydrolysis of 0.01 mg. of phosphate under the specified conditions of the assay.

Amylase was measured by the method of Bernfeld (*7*), in which soluble starch was employed as substrate and the formation of maltose was determined colorimetrically by the dinitrosalicylic acid technique (*68*). The reaction mixture was composed of 1 ml. of succinate buffer (pH 6.5), 1 ml. of 2-percent soluble-starch, and 0.25 ml. of enzyme solution. Control tubes received distilled water in place of enzyme. Reaction time was 20 minutes. From the reaction mixture 0.5 ml. was withdrawn and placed in tubes containing 2.5 ml. of distilled water and 1 ml. of dinitro reagent. The contents were thoroughly mixed and the tubes were allowed to stand in boiling water for 6 minutes. After cooling to room temperature the percentage transmittance of each solution was recorded at 525 mμ, and the quantity of maltose present was determined by reference to a standard curve representing 0.01 to 0.50 mg. of maltose. One activity unit was defined as the quantity of enzyme catalyzing the formation of 0.10 mg. of maltose under the specified conditions of the assay.

Phosphohexose isomerase, an enzyme catalyzing the reversible conversion of glucose-6-phosphate to fructose-6-phosphate, was measured by the method of Macleod and Robinson (*47*), which is based upon the increase of aldose in a solution of fructose-6-phosphate and enzyme extract. The hypoiodite technique was employed for the determination of aldose. Iodine, under controlled conditions, oxidizes aldoses to the corresponding mono-

basic acids while ketoses are only slightly attacked (*11*). When aldose is completely oxidized the test solution is acidified and the excess iodine is titrated with thiosulfate. The difference between the total iodine added and that found at the end of the experiment corresponds stoichiometrically to the quantity of aldose present. While the hypoiodite technique has been used primarily in determining relatively large amounts of aldose, the modification by Macleod and Robinson was developed specifically for quantities in the area of 1 mg.

The reaction mixture for phosphohexose isomerase was composed of 1 ml. of 0.1-M succinate buffer (pH 6.5), 1 ml. of 0.012-M fructose-6-phosphate, and 0.25 ml. of enzyme solution. Control tubes received 2 ml. of TCA before enzyme was added. The reaction continued for 20 minutes and its termination was effected by the addition of 2 ml. of TCA to treatment tubes. All samples were centrifuged for 5 minutes at 3,500 r.p.m. One milliliter of the deproteinized supernatant liquid was placed in each of a series of glass-stoppered, 50-ml. Erlenmeyer flasks. Three milliliters of 0.04-M iodine solution was added, plus 3 drops of chlor-phenol-red indicator, followed by dropwise addition of 10-percent NaOH to bring the pH to 6.8. The last step was accomplished with constant swirling of the contents. In like manner 6 drops of 5-percent Na_2CO_3 were added, the flasks were stoppered, and the aldose-oxidation reaction was allowed to proceed for 10 minutes. The samples were then acidified with 1 ml. of 0.5-N H_2SO_4. Three drops of 2-percent soluble starch were added, and, after thorough mixing, each sample was titrated with 0.06-percent sodium thiosulfate.

Simultaneous with the above procedure, two additional solutions were carried through all steps. Solution A consisted of 1 ml. of buffer, 1 ml. of aldose standard (4.25 mg. of glucose per ml.), and 0.25 ml. of distilled water. Solution B contained distilled water in place of glucose. These solutions were carried through the same steps as those containing enzyme and substrate. One milliliter of the TCA supernatant liquid of solution A represents 1 mg. of glucose in the final titration. The difference in free iodine between solutions A and B represents the iodine required for the oxidation of 1 mg. of aldose, and served as the basis for calculating the aldose formed during the enzymatic reaction. One unit of activity was defined as the quantity of enzyme catalyzing the formation of 0.10 mg. of aldose under the specified conditions of the assay.

Aldolase was measured by the method of Dounce and Beyer (*21*), in which the triose phosphate formed by action of aldolase upon fructose-1,6-diphosphate is converted to acetaldehyde with hot, concentrated H_2SO_4. The acetaldehyde is then measured colorimetrically with *p*-hydroxydiphenyl, as in the Barker-Sumerson method for lactic acid (*3*). The reaction mixture was composed of 1 ml. of acetate buffer, 0.5 ml. of hexose

diphosphate, and 0.25 ml. of enzyme solution. Control tubes received distilled water in place of enzyme. The reaction was terminated after 15 minutes with 2 ml. of TCA. The samples were centrifuged at 3,500 r.p.m. for 5 minutes and 1 ml. of the supernatant liquid was transferred to pyrex test tubes. Two milliliters of concentrated H_2SO_4 were added slowly to each tube with a fine-tipped burette, due caution being taken to avoid local overheating. The sample-acid mixtures were then placed in boiling water for 3 minutes. After cooling to room temperature with tapwater each tube received 2 drops of 2-percent copper sulfate, followed by 3 drops of *p*-hydroxydiphenyl solution (dissolve 1.5 gm. of *p*-hydroxydiphenyl in 10 ml. of 0.5-percent NaOH and bring to 100 ml. with distilled water). The contents were thoroughly mixed and allowed to stand for 20 minutes. The tubes were again placed in boiling water, for 90 seconds only, cooled again to room temperature with tapwater, and percentage transmittance of the solutions was then recorded at 560 mμ. The aldehyde present was determined by comparison with a standard curve representing 0.02 to 4.0 mg. of acetaldehyde. One unit of activity was defined as the quantity of enzyme catalyzing the formation of triose phosphate equivalent to 1 mg. of acetaldehyde.

Triose phosphate dehydrogenase was assayed spectrophotometrically by a modification of the method of Gibbs; coenzyme I was employed rather than coenzyme II; which is based upon the fact that reduced diphosphopyridine nucleotide absorbs light at wave-length 340 mμ while the oxidized form does not (*29*). The reaction was measured directly in 1-cm. pyrex cuvettes with a Beckman Model B Spectrophotometer. Each cell contained 2 ml. of acetate buffer, 0.5 ml. of DPN solution (2 mg. of DPN[8] dissolved in 50 ml. of 0.05-M cysteine hydrochloride solution) and 0.25 ml. of enzyme. A control cell containing these same reagents received 0.5 ml. of distilled water, and the reaction was initiated in treatment cells by adding 0.5 ml. of DL-glyceraldehyde-3-phosphate solution (0.17 gm. of the barium salt dissolved in 100 ml. of distilled water). Initial optical density readings were taken immediately at 340 mμ and recorded as "zero-time values". Readings were recorded at 30-second intervals for 2 minutes. One activity unit was defined as the amount of enzyme causing an optical-density increase of 0.05 in 2 minutes under the specified conditions of the assay.

Isocitric dehydrogenase was measured exactly as was triose phosphate dehydrogenase, with the exception that coenzyme II was used rather than coenzyme I, and that isocitric acid (0.01 M) was supplied in place of DL-glyceraldehyde-3-phosphate.

Phosphoglyceryl kinase, an enzyme involved in the reversible degradation of 1,3-diphosphoglyceric acid, was found to be present in the cane-

[8] Supplied by the California Corp. for Biochemical Research.

leaf extracts. The enzyme is of particular interest in that the formation of diphosphoglyceric acid marks one of the two points in the glycolytic pathway where the energy of glycolysis becomes available for transfer. We employed the assay method of Axlerod and Bandurski (*1*). Notwithstanding the difficulty in preparing 1,3-diphosphoglycerate, these investigators developed a procedure for measuring the enzyme, by the reverse reaction, through the utilization of 3-phosphoglyceric acid as substrate in the presence of ATP. Diphosphoglyceric acid is apparently quite unstable but can be "trapped" with hydroxylamine, and the anhydride thus formed is measured colorimetrically by the hydroxamic acid test of Lipmann and Tuttle (*44*).

The reaction mixture for phosphoglyceryl kinase was composed of 1 ml. of 0.1-M succinate buffer (pH 6.2), 1 ml. of 2-M hydroxylamine (mix equal volumes of 4-percent hydroxylamine hydrochloride with 3.5-percent NaOH, adjust pH to 6.2), 0.5 ml. of 0.01-M ATP, 0.25 ml. of enzyme solution, 1 drop of 0.1-M NaF, and 1 drop of 0.01-M $MgCl_2$. The contents were mixed thoroughly upon the addition of each reagent. At this time the control tubes received 2 ml. of $FeCl_3$-TCA-HCl reagent—made by dissolving 9.3 gm. of $FeCl_3 \cdot 6H_2O$ in 42 ml. of concentrated HCl, adding 20 gm. of TCA and bringing to 500 ml. with distilled water. The reaction was then initiated by adding to each tube 1 ml. of 0.01-M 3-phosphoglyceric acid, barium salt, adjusted to pH 6.2. The reaction proceeded for 30 minutes and was terminated by the addition of 2 ml. of $FeCl_3$-TCA-HCl reagent to the treatment tubes. Ten minutes were allowed for color development. Optical density of the solutions was then measured at 430 mμ. One activity unit was defined as the amount of enzyme causing an increase in optical density of 0.05 under the specified conditions of the assay.

The condensing enzyme was measured by a method similar to that of Ochoa (*53*), with citrate being determined according to the procedures outlined by Stern (*66*). This enzyme is involved in an acetyl-transferring sequence in which the acetyl group from coenzyme A is moved to oxalacetate with the subsequent production of citrate (*67*). The assay of Ochoa is based upon the conversion of a mixture of acetyl phosphate and oxalacetate to citrate and orthophosphate in the presence of coenzyme A and condensing enzyme.

The reaction mixture for the condensing enzyme test was composed as follows: One milliliter of acetate buffer; 0.5 ml. of 0.05-M oxalacetic acid—adjusted to pH 4.65 with 2.5 N NaOH; 0.5 ml. of 0.025 M acetyl phosphate, made up weekly from the silver salt; and 0.25 ml. of coenzyme A[9], 1 mg. of the lithium salt dissolved in a cysteine solution containing 0.153 gm. of *l*-cysteine monophosphate in 25 ml. of distilled water. The reaction

[9] Supplied by the California Corp. for Biochemical Research.

was initiated by adding 0.25 ml. of enzyme solution, and was allowed to proceed for 25 minutes. Control tubes received distilled water in place of enzyme.

The reaction was stopped with 2 ml. of TCA and all samples were centrifuged for 10 minutes at 3,500 r.p.m. Two milliliters of the supernatant liquid were transferred to pyrex tubes containing 0.1 ml. of 18-N H_2SO_4, and the tubes were then allowed to stand in boiling water for 10 minutes. This is intended to destroy materials capable of interfering with the citrate assay. After cooling for 3 minutes in an ice bath, the samples were transferred to tubes equipped with screw caps and received 0.5 ml. of 1-M potassium bromide plus 1 ml. of 5-percent potassium permanganate. The samples were mixed thoroughly and allowed to stand for 5 minutes. Excess permanganate was removed by dropwise addition of 6-percent hydrogen peroxide. Three milliliters of heptane was added to each tube, the tubes were capped and shaken vigorously for 2 minutes, and 2 ml. of the heptane layer were transferred to glass-stoppered tubes containing 4 ml. of thiourea solution (4-percent of thiourea containing 4 gm. of sodium borate per 200 ml.). The samples were again shaken vigorously for 2 minutes and 5 additional minutes were allowed for color development.

Three milliliters of the yellow aqueous layer were transferred to 1-cm. cuvettes and the percentage transmittance of the solutions was measured at 430 mμ. The citric acid present was determined by reference to a standard curve representing 0.005 to 0.30 mg. of citric acid. One activity unit was defined as the quantity of enzyme catalyzing the formation of 0.01 mg. of citric acid under the specified conditions of the assay.

Pyruvic carboxylase was measured by determining the quantity of acetaldehyde formed from the keto acid, using the colorimetric test of Dounce and Beyer (*21*). The reaction mixture was made up of 1 ml. of acetate buffer, 0.5 ml. of 0.05-M pyruvic acid, and 0.25 ml. of enzyme solution. Control tubes received distilled water in place of enzyme, the latter, as in the assay for aldolase, being devoid of detectable aldehyde. The reaction was ended after 20 minutes with 2 ml. of TCA, and 1 ml. of each sample was then carried through all phases of the Dounce and Beyer procedure, as was described for aldolase. One unit of activity was defined as the amount of enzyme causing the formation of 0.1 mg. of acetaldehyde under the specified conditions of the assay.

Additional carboxylase activity was also present in the cane-leaf extracts, as evidenced by the appearance of aldehyde when both α-ketoglutaric and oxalacetic acid were incubated with enzyme solution. Each system was measured by identical procedures as described for pyruvic carboxylase, using 0.05-M solutions of both α-ketoglutaric and oxalacetic

acid. In each instance, however, the activity we recorded can be considered only relative. Succinic semialdehyde was not available to prepare the logical standard curve for α-ketoglutaric carboxylase so acetaldehyde curves were substituted. It appears that at least two enzymes are involved in the assay of oxalacetic carboxylase. Preliminary experiments showed that oxalacetic acid was, in fact, disappearing by enzyme action, but attempts to demonstrate the formation of pyruvic acid, the logical product of a carboxylase reaction, were negative.

It was therefore assumed that the reaction was going forward to pyruvate and continuing on to acetaldehyde, or that the reverse reaction was in progress, and that malic acid was being formed. It was soon found that acetaldehyde was, in fact, forming as a product of the reaction, suggesting the pyruvic carboxylase was an integral part of the system. Tests for pyruvic acid by the method of Friedemann (*27*), in spite of the excellent precision of the test, consistently failed to yield even a trace of the acid, so it was assumed that the pyruvic carboxylase step was not a limiting one, and the oxal-acetic carboxylase assay was thus established on the basis of acetaldehyde formed. The assay procedures for both α-ketoglutaric and oxal-acetic carboxylase are identical with those described for pyruvic carboxylase, and the activity unit for each was defined as the amount of enzyme catalyzing the formation of 0.1 mg. of acetaldehyde under the specified conditions of the assay.

Cytochrome-C reductase was measured by a method similar to that of Wainio, *et al.* (*74*), with the exceptions that acetate buffer of pH 4.63 was employed rather than phosphate buffer of pH 7.4, and activity was initially recorded in units of optical density rather than as time values. The enzyme was originally encountered while trying to detect cytochrome-C oxidase. Using reduced cytochrome C as substrate, the enzyme preparations further reduced rather than oxidized cytochrome C. The reaction was stimulated by TPNH, but was unaffected by DPNH. The reaction proceeded strongly at pH 4.63, but was not detectable at pH 7.5.

The assay method for cytochrome-C reductase is based on the fact that reduced cytochrome C is pink and possesses visible absorption bands at 550, 522, and 415 mμ, while oxidized cytochrome C is yellow and has visible absorption bands at 530 and 400 mμ. The tests were run directly in 1-cm. pyrex cuvettes. Each cuvette contained 1 ml. of acetate buffer, 0.5 ml. of TPNH solution—2 mg. of reduced TPN dissolved in 50 ml. of distilled water—and 0.25 ml. of enzyme. A control cuvette received the same reagents plus 1 ml. of distilled water. The reaction was then initiated in the treatment cuvettes by adding 1 ml. of 0.1-percent oxidized cystochrome C. Optical density was measured immediately at 550 mμ, and again 60 seconds

later. One unit of activity was taken as the amount of enzyme causing an optical density increase of 0.05 in 60 seconds, under the specified conditions of the assay.

Transaminase was measured according to the method of Tonhazy, *et al.* (*71*). The assay is based upon the conversion of aspartic acid to oxalacetic acid by the enzyme, the chemical conversion of the oxalacetate to pyruvate, and the colorimetric determination of the latter. The test was conducted in the presence of excess aspartic and α-ketoglutaric acid. The oxalacetate was converted to pyruvate by means of aniline citrate, and pyruvate was then determined by the Friedemann-Hangen method (*26*).

The reaction mixture for transaminase was made up of 1 ml. of acetate buffer, 0.5 ml of *l*-aspartic acid (0.1-M, adjusted to pH 4.65), 0.5 ml. of α-ketoglutaric acid (0.1-M adjusted to pH 4.65), and 0.25 ml. of enzyme solution. Control tubes received distilled water in place of enzyme. The 20-minute reaction period was terminated with 2 ml. of TCA. Each sample received 0.5 ml. of aniline reagent (80 ml. of aniline added to 50 gm. of citric acid dissolved in 50 ml. of distilled water), and after thorough mixing, was allowed to stand for 5 minutes. Two milliliters of each sample were transferred to test tubes containing 2 ml. of hydrazine reagent—made by dissolving 0.20 gm. of 2,4-dinitrophenylhydrazine in 2 ml. of concentrated HCl and bringing to 100 ml. with distilled water. The solutions were mixed and allowed to stand again for 5 minutes. Each tube then received 2 ml. of spectral-grade benzene and was shaken vigorously. One milliliter of the benzene phase was transferred to another set of tubes containing 4 ml. of 2-N NaOH. The contents were mixed and allowed to stand 5 minutes for development of red color. Three milliliters of the colored layer were transfered to 1-cm. cuvettes and percentage transmittance was measured at 540 mμ. The pyruvate present was determined by reference to a standard curve representing 0.005 to 0.5 mg. of pyruvic acid. One activity unit was defined as the amount of enzyme catalyzing the formation of oxalacetate equivalent to 0.01 mg. of pyruvic acid under the specified conditions of the assay.

The enzyme fumarase catalyzes the reversible conversion of fumaric acid to *l*-malic acid, with the equilibrium lying toward the malate, and thus constitutes a necessary role in the tricarboxylic acid cycle. The fumarase activity of cane-leaf extracts was measured by titration with potassium permanganate in acid media. Fumaric acid which has not been enzymatically hydrolyzed is reduced by permanganate with the simultaneous loss of purple color. The method is a modification of Scott's (*62*), in which the titration end-point is taken as that volume of permanganate at which 1 drop results in a pink color which persists for 20 seconds.

The reaction mixture for fumarase consisted of 1 ml. of phosphate buffer (pH 7.0), 1 ml. of 0.05-M fumaric acid, adjusted to pH 7.0 with NaOH,

and 0.25 ml. of enzyme solution. Control tubes received distilled water in place of enzyme. The reaction was permitted to proceed for 20 minutes, at which time 2 ml. of TCA was added to each tube. Two milliliters of the TCA-fumaric acid mixture were transferred to 50-ml. beakers, 0.5 ml. of 2 N HCl was added, and the samples were titrated with 0.037-M $KMnO_4$. The control titration sample, which had not been acted upon by enzyme, contained 2.73 mg. of fumaric acid, and thus served as a basis for calculating the amount of substrate hydrolyzed by the enzyme. The activity unit was defined as the amount of enzyme causing the hydrolysis of 0.1 mg. of fumaric acid under the specified conditions of the assay.

Peroxidase was assayed spectrophotometrically by measuring the optical-density increase of a solution containing catechol, peroxide, and enzyme. The reaction mixture, contained in 1-cm. pyrex cuvettes, was composed of 1 ml. of acetate buffer, 0.5 ml. of 3-percent hydrogen peroxide, and 0.25 ml. of enzyme solution. A control cuvette received 0.25 ml. of distilled water in place of enzyme. The reaction was initiated by adding 1 ml. of 0.05-M catechol to each cell. Optical density was measured immediately at 550 mμ, and again 3 minutes later. One activity unit was defined as the amount of enzyme causing an optical density increase of 0.10 in 3 minutes under the specified conditions of the assay.

The assay method for polyphenol oxidase was based on the formation of yellow color when catechol is acted upon by the enzyme. The reaction mixture was made up of 2 ml. of acetate buffer, 1 ml. of 0.05-M catechol, and 0.25 ml. of enzyme solution. Control tubes received 1 ml. of distilled water in place of catechol. A second control tube was carried through all steps with distilled water in place of enzyme in order to estimate the auto-oxidation of substrate. The reaction proceeded for 20 minutes, at which time optical density was measured at 430 mμ. One unit of activity was defined as the amount of enzyme causing an optical-density increase of 0.10 under the specified conditions of the assay.

Catalase activity was measured manometrically with Warburg constant-volume manometers equipped with single sidearm reaction vessels. Manometers and vessels were calibrated separately with mercury, by the technique of Santiago Grisolia, as described by Umbreit, *et al.* (*72*). The main compartment of each vessel received 1 ml. of phosphate buffer (pH 7.5), 0.75 ml. of distilled water, and 0.25 ml. of enzyme solution. One milliliter of chilled, 3-percent hydrogen peroxide was placed in the sidearm of each flask. After a 10-minute equilibration period the manometers were tipped so that the peroxide entered the main vessel to initiate the reaction, with stopcocks remaining open. Measurement of the reaction was begun immediately by closing the stopcock on each manometer. The control vessel received distilled water in place of enzyme. The measured reaction time

was only 1 minute and it was found unnecessary to maintain a control for atmospheric pressure changes during this period. Microliters of oxygen produced during the reaction were calculated with the aid of previously determined KO_2 values. One activity unit was defined as the amount of enzyme catalyzing the production of 10 μl. of oxygen per minute under the specified conditions of the assay.

All data collected during these experiments were subjected to analysis of variance, and means were compared by the Student-Newman-Keul's Q test. The results of the statistical analyses are presented in tables 2 and 3.

RESULTS

LEAF AND SHEATH SUGARS

Leaf sucrose declined during the 9- to 15-week period and thereafter increased through 24 weeks (table 4). Sucrose increased most readily among the plants receiving decreasing nitrate (fig. 1). Leaf fructose varied inversely with sucrose as nitrate was decreased, and fructose virtually disappeared among low nitrate samples by the end of the study. By 24 weeks leaf glucose had increased in response to the curtailment of potassium supply.

Sheath-sugar content (table 5) was slightly higher than that of the leaves. Leaf sugars were roughly 70 to 80 percent of sheath quantitites, with the exception of glucose which was found in nearly equal amounts in both types of tissue. The inverse relationship between sucrose and fructose content observed in leaf samples was also apparent in the sheaths, with fructose again declining to its lowest recorded level among the decreasing nitrate group.

Nutrient variables appeared to have less influence on the sugar content of sheaths than leaves. This is taken to mean that leaf-sugar variations were due directly to metabolic processes rather than failure of translocation mechanisms during nutritional stress. The one important exception was a pronounced increase in glucose content by plants supplied with decreasing potassium. This effect was even more pronounced in sheath samples that in leaf material.

TOTAL DRY WEIGHT AND SHEATH-PERCENT-MOISTURE

Little variation appeared between treatment and control groups with regard to total dry weight (table 6). The greatest dry-weight response was recorded among control plants where mean values increased from 17.1 to 194.8 gm. per plant, and the least increase was found among the low nitrate group where mean values increased from 15.7 to 154.9 gm. per plant.

Generally speaking, sheath-percent-moisture was decreasing among all

groups by 27 weeks of age (table 6), although the changes were not statistically significant. The greatest decrease was observed among the low-nitrate plants, where an initial value of 86.2 percent was reduced to 79.4 percent. This is a far cry from the 73 to 75 percent of moisture suggested by Clements (*17*) as being indicative of a mature plant, but it is apparent that this same low-nitrate group which was accumulating the highest leaf-sucrose content was likewise most rapidly approaching a low sheath-moisture value by the end of the study.

WATER-SOLUBLE PROTEIN

Leaf protein gradually increased throughout the study. Grand mean values reveal a protein content of 1.84 mg. per gm. of freeze-dried tissue at 9 weeks and 4.07 mg. per gm. at 27 weeks (table 6). No significant variations were recorded among treatments.

ENZYME ACTIVITY

With a few exceptions the enzymes exhibited their greatest activity at 9 weeks and thereafter declined as the study progressed (table 7). The grand mean of all enzyme activity recorded at 27 weeks was only 30.1 percent of that recorded at 9 weeks.

Several enzymes did not conform to this generalization, including invertase, peroxidase, polyphenol oxidase, and cytochrome-C reductase. With invertase (table 7, item 13) the control activity was highest at 15 weeks but subsequently showed little variation with increasing age. Peroxidase activity (table 7, item 26) likewise rose to a peak at 15 weeks and thereafter declined to its initial level, while polyphenol oxidase (table 7, item 28) was initially high, declined through 24 weeks, and increased at 27 weeks to almost double the original level. The final activity recorded for cytochrome-C reductase (table 7, item 25) was about 9 times as high as the initial activity values measured among plants at 9 weeks of age.

Generally speaking, the overall enzyme response was determined more by the physiological age of the plants than by treatments. Often the magnitude of an enzyme curve was altered by treatment effects, but only in rare instances was the normal curve, as established by control plants, completely reversed or obscured by nutritional variables. Nevertheless a number of enzymes appeared to have some bearing on the leaf-sugar responses already mentioned, particularly those involved in the hydrolysis or transfer of phosphate, and the starch-destroying enzyme amylase.

Phosphate-Metabolyzing Enzymes

None of the enzymes responded more consistently to treatment and increasing plant maturity than did the acid phosphatases which hydrolyze

TABLE 2.—*Summary of significant mean differences among sugar, dry-weight, and enzyme values in response to control, increasing nitrate, decreasing nitrate, and decreasing potassium treatments applied to immature sugarcane in sand culture*[1]

Data classification	Data for cane aged (weeks)—						
	9	12	15	18	21	24	27
Leaf sugars,							
Total ketoses	$-NO_3 >$ Con.* $-NO_3 > +NO_3$** $-K > +NO_3$** Con. $> +NO_3$*		$-K > -NO_3$** $+NO_3 > -NO_3$* Con. $> -NO_3$**			$-K > -NO_3$* $-K >$ Con.** $+NO_3 >$ Con.*	$-K > -NO_3$** $-K > +NO_3$** $-K >$ Con.**
Sucrose	$-NO_3 > +NO_3$* $-K > +NO_3$* Con. $> +NO_3$*	$-K > +NO_3$*					$-NO_3 > -K$** $-NO_3 >$ Con.** $-NO_3 > +NO_3$**
Fructose		$+NO_3 > -NO_3$* $+NO_3 >$ Con.* $+NO_3 > -K$*		$-K > -NO_3$* $+NO_3 > -NO_3$* Con. $> -NO_3$*	$-K >$ Con.* $-K > +NO_3$*		$-K > -NO_3$* $+NO_3 > -NO_3$* Con. $> -NO_3$*
Total reducing					$-K > +NO_3$** $-K >$ Con.** $-K > -NO_3$**		$-K >$ Con.* $-K > -NO_3$** Con. $> -NO_3$** $+NO_3 > -NO_3$**
Glucose					$-K > +NO_3$* $-K >$ Con.** $-K > -NO_3$**	$-K >$ Con.*	$-K > +NO_3$* $-K > -NO_3$** Con. $> -NO_3$**
Sheath sugars,							
Total ketoses					$-K > -NO_3$* $-K > +NO_3$* $-K >$ Con.*		$-K > +NO_3$* $-K >$ Con.** $-NO_3 >$ Con.** $+NO_3 >$ Con.*
Sucrose							$-NO_3 >$ Con.* $-K >$ Con.*

Total reducing	$+NO_3 > -NO_3$* $+NO_3 > -K$*			$-K > -NO_3$** $-K > +NO_3$* $-K >$ Con.*			$-K > -NO_3$** $-K > +NO_3$** $-K >$ Con.**
Glucose	$+NO_3 > -K$* $+NO_3 >$ Con.* $+NO_3 > -NO_3$*		Con. $> -K$*		$-K >$ Con.** $-K > +NO_3$* $-K > -NO_3$*		$-K > -NO_3$** $-K > +NO_3$** $-K >$ Con.** $-NO_3 > +NO_3$** $-NO_3 >$ Con.** $+NO_3 >$ Con.*
Total dry weight					$+NO_3 > -NO_3$*		
Leaf enzymes							
β-glycero-phosphatase	$-NO_3 >$ Con.** $-NO_3 > +NO_3$** $-NO_3 > -K$**	$+NO_3 > -K$* $+NO_3 >$ Con.*	$+NO_3 >$ Con.* $+NO_3 > -K$** $+NO_3 > -NO_3$** Con. $> -K$* Con. $> -NO_3$*			$+NO_3 > -K$* $+NO_3 > -NO_3$* Con. $> -K$*	$+NO_3 > -NO_3$* Con. $> -NO_3$* $-K > -NO_3$*
ATP-ase	$+NO_3 > -K$** $+NO_3 >$ Con.** $-NO_3 > -K$** $-NO_3 >$ Con.** $-K >$ Con.*	$+NO_3 > -NO_3$* $+NO_3 > -K$* $+NO_3 >$ Con.*	$+NO_3 >$ Con.* $+NO_3 > -K$** $+NO_3 > -NO_3$** Con. $> -NO_3$*	$+NO_3 >$ Con.* $+NO_3 > -NO_3$* $-K >$ Con.*		$+NO_3 > -K$*	$+NO_3 > -NO_3$* Con. $> -NO_3$* $-K > -NO_3$*
2-phosphoglyceric phosphatase	$-NO_3 > +NO_3$* $-NO_3 > -K$** $-NO_3 >$ Con.** $+NO_3 > -K$* $+NO_3 >$ Con.**	$+NO_3 >$ Con.*	$+NO_3 >$ Con.** $+NO_3 > -K$** $+NO_3 > -NO_3$**			$+NO_3 > -NO_3$* Con. $> -K$* Con. $> -NO_3$*	$+NO_3 > -NO_3$* $-K > -NO_3$*
3-phosphoglyceric phosphatase	$+NO_3 >$ Con.** $-NO_3 >$ Con.* $-K >$ Con.*		$+NO_3 > -NO_3$*	$+NO_3 > -K$* $+NO_3 > -NO_3$** Con. $> -NO_3$** $-K > -NO_3$**		Con. $> -NO_3$*	
UDP-ase						$+NO_3 > -NO_3$*	$+NO_3 > -NO_3$* Con. $> -NO_3$* $-K > -NO_3$*

TABLE 2.—*Continued*

Data classification	Data for cane aged (weeks)—						
	9	12	15	18	21	24	27
6-phosphogluconate phosphatase	$+NO_3 > -K^*$ $+NO_3 > -NO_3^*$		$+NO_3 > Con.^{**}$ $+NO_3 > -NO_3^{**}$ $+NO_3 > -K^{**}$			$+NO_3 > -NO_3^*$ $-K > -NO_3^*$ $Con. > -NO_3^*$	$+NO_3 > Con.^{**}$ $+NO_3 > -K^{**}$ $+NO_3 > -NO_3^{**}$ $Con. > -NO_3^{**}$ $-K > -NO_3^*$
Glucose-1-phosphatase			$+NO_3 > Con.^*$ $+NO_3 > -NO_3^{**}$ $+NO_3 > -K^*$			$+NO_3 > -NO_3^*$	$Con. > -K^{**}$ $Con. > -NO_3^{**}$ $+NO_3 > -K^{**}$ $+NO_3 > -NO_3^{**}$
Glucose-6-phosphatase		$+NO_3 > Con.^*$ $+NO_3 > -NO_3^*$	$+NO_3 > Con.^*$ $+NO_3 > -K^{**}$ $+NO_3 > -NO_3^{**}$	$+NO_3 > Con.^*$ $+NO_3 > -K^*$ $+NO_3 > -NO_3^*$		$Con. > -K^*$ $Con. > -NO_3^*$	$-K > -NO_3^*$
Fructose-6-phosphatase			$+NO_3 > -NO_3^*$			$Con. > -NO_3^*$ $-K > -NO_3^*$ $+NO_3 > -NO_3^*$	
Fructose-1,6-diphosphatase						$+NO_3 > Con.^{**}$ $+NO_3 > -K^{**}$ $+NO_3 > -NO_3^{**}$	
Catalase	$+NO_3 > -K^*$ $+NO_3 > Con.^{**}$			$Con. > -K^{**}$ $Con. > +NO_3^{**}$ $-NO_3 > +NO_3^*$ $-NO_3 > -K^*$			
Phosphoglyceryl kinase	$+NO_3 > Con.^*$ $+NO_3 > -K^*$ $+NO_3 > -NO_3^{**}$	$Con. > -K^{**}$ $Con. > -NO_3$ $+NO_3 > -K^*$ $+NO_3 > -NO_3^{**}$	$+NO_3 > -NO_3^*$ $+NO_3 > -K$	$Con. > -K^{**}$ $+NO_3 > -K^{**}$ $-NO_3 > -K^*$			
Phosphorylase	$Con. > -K^*$	$-K > +NO_3^*$					$+NO_3 > -NO_3^*$ $Con. > -NO_3^*$ $-K > -NO_3^*$

Hexokinase	$-K > Con.^{**}$ $-K > +NO_3^{**}$ $-NO_3 > Con.^{*}$ $-NO_3 > +NO_3^{**}$			$-K > Con.^{*}$ $-K > +NO_3^{**}$ $-NO_3 > Con.^{**}$ $-NO_3 > +NO_3^{**}$ $Con. > +NO_3^{**}$			$-K > Con.^{**}$ $-K > -NO_3^{**}$ $-K > +NO_3^{**}$
Invertase		$-K > -NO_3^{*}$ $+NO_3 > +NO_3^{*}$ $Con. > -NO_3^{*}$		$Con. > +NO_3^{*}$ $Con. > -K^{*}$ $Con. > -NO_3^{**}$ $+NO_3 > -NO_3^{*}$ $Con. > -NO_3^{*}$			$-K > -NO_3^{*}$
Aldolase					$-K > -NO_3^{*}$ $-K > Con.^{*}$		
Amylase			$+NO_3 > -NO_3^{*}$				$+NO_3 > -NO_3^{*}$ $Con. > -NO_3^{*}$
Phosphohexose isomerase		$-NO_3 > +NO_3^{**}$ $-NO_3 > Con.^{**}$ $-K > +NO_3^{**}$ $-K > Con.^{**}$	$Con. > -NO_3^{**}$ $Con. > -K^{**}$ $Con. > +NO_3^{**}$ $-NO_3 > +NO_3^{**}$ $-K > -NO_3^{**}$	$-NO_3 > -K^{**}$ $Con. > -K^{**}$ $+NO_3 > -K^{**}$			
Fumarase	$Con. > -K^{**}$ $+NO_3 > -K^{*}$ $-NO_3 > -K^{**}$		$-K > +NO_3^{**}$ $Con. > +NO_3^{**}$	$-NO_3 > Con.^{**}$ $-NO_3 > -K^{**}$ $-NO_3 > +NO_3^{**}$ $Con. > -NO_3^{**}$ $-K > +NO_3^{**}$			
Pyruvic acid carboxylase	$Con. > +NO_3^{**}$ $Con. > -NO_3^{**}$ $Con. > -K^{**}$						
α-keto glutaric acid carboxylase	$+NO_3 > -NO_3^{*}$ $+NO_3 > Con.^{*}$ $+NO_3 > -K^{*}$	$-NO_3 > Con.^{*}$ $-NO_3 > -K^{*}$					$-K > +NO_3^{*}$ $Con. > +NO_3^{*}$ $-NO_3 > +NO_3^{*}$
Transaminase			$-K > +NO_3^{*}$ $-K > -NO_3^{*}$ $-K > Con.^{**}$ $-NO_3 > Con.^{*}$		$-K > -NO_3^{*}$ $Con. > -NO_3^{*}$ $+NO_3 > -NO_3^{*}$		

TABLE 2.—*Concluded*

Data classification	Data for cane aged (weeks)—						
	9	12	15	18	21	24	27
Peroxidase			$+NO_3 > -K^{**}$ $+NO_3 > -NO_3^{**}$ $Con. > -K^{**}$ $Con. > -NO_3^{**}$ $-K > -NO_3^{*}$	$Con. > -K^{*}$ $Con. > -NO_3^{*}$ $+NO_3 > -K^{*}$ $+NO_3 > -NO_3^{*}$			$+NO_3 > -NO_3^{**}$ $Con. > -NO_3^{**}$ $-K > -NO_3^{**}$
Polyphenol oxidase	$+NO_3 > -NO_3^{*}$ $+NO_3 > Con.^{*}$ $+NO_3 > -K^{**}$ $Con. > -K^{*}$						$+NO_3 > -NO_3^{*}$ $+NO_3 > Con.^{*}$ $+NO_3 > -K^{*}$
Isocitric acid dehydrogenase	$-NO_3 > Con.^{*}$ $-NO_3 > -K^{*}$	$Con. > -NO_3^{*}$ $Con. > +NO_3^{**}$ $Con. > -K^{**}$					
Triose phosphate dehydrogenase		$-NO_3 > -K^{*}$ $-NO_3 > +NO_3^{**}$ $Con. > -K^{*}$ $Con. > +NO_3^{**}$	$Con. > -NO_3^{**}$ $-K > -NO_3^{**}$	$-NO_3 > +NO_3^{*}$ $-K > +NO_3^{*}$ $Con. > +NO_3^{*}$			
Oxalacetic carboxylase		$Con. > -K^{**}$ $Con. > +NO_3^{*}$ $Con. > -NO_3^{*}$	$-NO_3 > Con.^{*}$ $-K > Con.^{*}$	$-K > +NO_3^{*}$ $Con. > +NO_3^{**}$ $-NO_3 > +NO_3^{**}$			
Condensing enzyme			$+NO_3 > Con.^{*}$ $+NO_3 > -K^{*}$ $+NO_3 > -NO_3^{**}$	$-K > -NO_3^{*}$ $-K > +NO_3^{*}$ $Con. > -NO_3^{*}$ $Con. > +NO_3^{*}$	$+NO_3 > -NO_3^{**}$ $+NO_3 > -K^{*}$	$+NO_3 > -K^{*}$ $Con. > -K^{*}$	
Cytochrome-C reductase			$-NO_3 > Con.^{*}$ $-NO_3 > -K^{*}$ $-NO_3 > +NO_3^{*}$		$-NO_3 > -K^{**}$ $-NO_3 > +NO_3^{**}$ $-NO_3 > Con.^{**}$		

[1] The abbreviations and symbols used to prepare this table are defined as follows: The abbreviation Con. represents mean values from the control treatment, and the symbols $+NO_3$, $-NO_3$, and $-K$ represent mean values from the increasing nitrate, decreasing nitrate, and decreasing potassium treatments, respectively. The symbol > between 2 values indicates that the first mean was of greater magnitude than the second. One asterisk denotes significant mean difference at the 5-percent level, and 2 asterisks at the 1-percent level. For illustration, the notation $-NO_3 > -K^{*}$, appearing at the top of the table for total leaf ketoses, means that decreasing nitrate resulted in more ketose than decreasing potassium, and that the mean difference is significant at the 5-percent level.

TABLE 3.—*Summary of significant F values derived through the variance analyses of means from control, increasing nitrate, decreasing nitrate, and decreasing potassium treatments applied to immature sugarcane in sand culture*[1]

Data classification	Differences for cane aged (weeks)—						
	9	12	15	18	21	24	27
Leaf sugars:							
Total ketoses	**		**			**	**
Sucrose	*	*					**
Fructose		*		*	*		**
Total reducing					**		**
Glucose					**	*	**
Sheath sugars:							
Total ketoses					*		**
Sucrose					*		*
Fructose							
Total reducing	*				*		**
Glucose	*		*		*		**
Total dry weight							
Sheath moisture (percent)	?	?			?		?
Enzymes							
β-glycerophosphatase	**	*	**			*	*
ATP-ase	**	*	**	*		*	*
UDP phosphatase						*	
2-phosphoglyceric acid phosphatase	**	*	**			*	*
3-phosphoglyceric acid phosphatase	*		*	**		*	
6-phosphogluconate phosphatase	*		**			*	**
Glucose-1-phosphatase			**			*	**
Glucose-6-phosphatase		*		**		*	*
Fructose-6-phosphatase			*			*	
Fructose-1,6-diphosphatase						**	
Hexokinase	**			**			**
Aldolase					*		
Amylase			*				*
Invertase		*		**			
Triose phosphate dehydrogenase		**	**	*			
Isocitric acid dehydrogenase	*	**		*			
Pyruvic carboxylase	**						
α-keto glutaric carboxylase	*	*					*
Oxalacetic carboxylase		**	*	**			
Transaminase	**		**		*		
Condensing enzyme			**	*	**	*	
Peroxidase			**	**			**
Polyphenol oxidase							*
Catalase	**			**			
Phosphohexose isomerase							
Phosphoglyceryl kinase		**	**	**			
Fumarase	**		*	**			
Cytochrome-C reductase			*		**		
Phosphorylase	*	*					*

[1] Asterisks indicate significant differences among the means of the 4 treatments: * Significance at the 5-percent level; ** Significance at the 1-percent level.

TABLE 4.—*Mean values for leaf sugars of immature sugarcane supplied with variable nitrate and potassium in sand culture*[1]

Sugar	Data for weeks after planting indicated—							
	9	12	15	18	21	24	27	Mean
Total ketose								
Control	52.1	45.2	46.3	42.5	34.2	47.3	46.4	44.9
Increasing NO_3	41.3	48.4	46.3	49.6	37.9	50.7	47.3	45.9
Decreasing NO_3	62.9	48.1	40.0	40.5	46.5	48.9	48.6	47.9
Decreasing K	57.2	52.8	48.5	43.1	52.0	52.3	52.0	48.0
Mean	53.4	48.6	45.3	43.9	42.6	49.8	48.6	46.7
Sucrose								
Control	36.1	26.5	13.7	25.8	27.9	31.0	27.0	26.9
Increasing NO_3	26.4	19.6	23.3	30.8	33.1	36.3	26.3	27.9
Decreasing NO_3	39.0	27.6	25.9	29.1	35.4	43.8	46.6	35.3
Decreasing K	38.8	35.8	25.2	28.9	32.5	38.0	29.2	32.6
Mean	35.1	27.4	22.0	28.5	32.2	37.3	32.3	30.7
Fructose								
Control	15.9	18.7	14.6	22.6	6.6	13.8	19.8	16.0
Increasing NO_3	14.4	28.8	18.8	23.0	4.7	14.4	21.0	17.7
Decreasing NO_3	23.8	20.5	11.5	14.1	11.1	5.2	2.0	12.6
Decreasing K	18.3	17.1	14.2	23.3	19.4	14.3	22.8	18.5
Mean	18.1	21.3	14.8	20.8	10.5	11.9	16.4	16.2
Total reducing sugar								
Control	18.7	26.8	34.0	20.4	18.8	22.0	40.9	25.9
Increasing NO_3	18.0	36.7	32.5	26.3	18.9	36.2	39.1	29.7
Decreasing NO_3	27.9	27.5	29.9	23.0	18.7	12.2	14.8	22.0
Decreasing K	21.1	29.8	33.6	24.9	42.9	35.5	49.6	33.9
Mean	21.4	30.2	32.5	23.6	24.8	26.5	36.1	27.9
Glucose								
Control	2.7	8.1	17.1	16.7	12.0	8.2	11.5	10.9
Increasing NO_3	2.9	8.5	11.5	4.7	14.5	21.7	18.1	11.7
Decreasing NO_3	4.2	7.0	16.1	5.7	7.6	7.0	12.7	8.6
Decreasing K	2.7	12.7	17.1	17.9	23.5	21.2	26.8	17.4
Mean	3.1	9.1	15.4	11.3	14.4	14.6	17.3	12.2

[1] Sugar content is expressed as milligrams per gram, dry-weight. Each figure represents the computed mean of 3 replicates.

the phosphorylated intermediates of the glycolytic pathway. Regardless of the phosphorylated substrate employed, enzyme activity was high at 9 weeks and declined to a relatively low level at 27 weeks. In the instance of glucose-6-phosphatase (table 7, item 4), those plants receiving decreasing nitrate experienced phosphatase decline to a mere 4.7 percent of its original level.

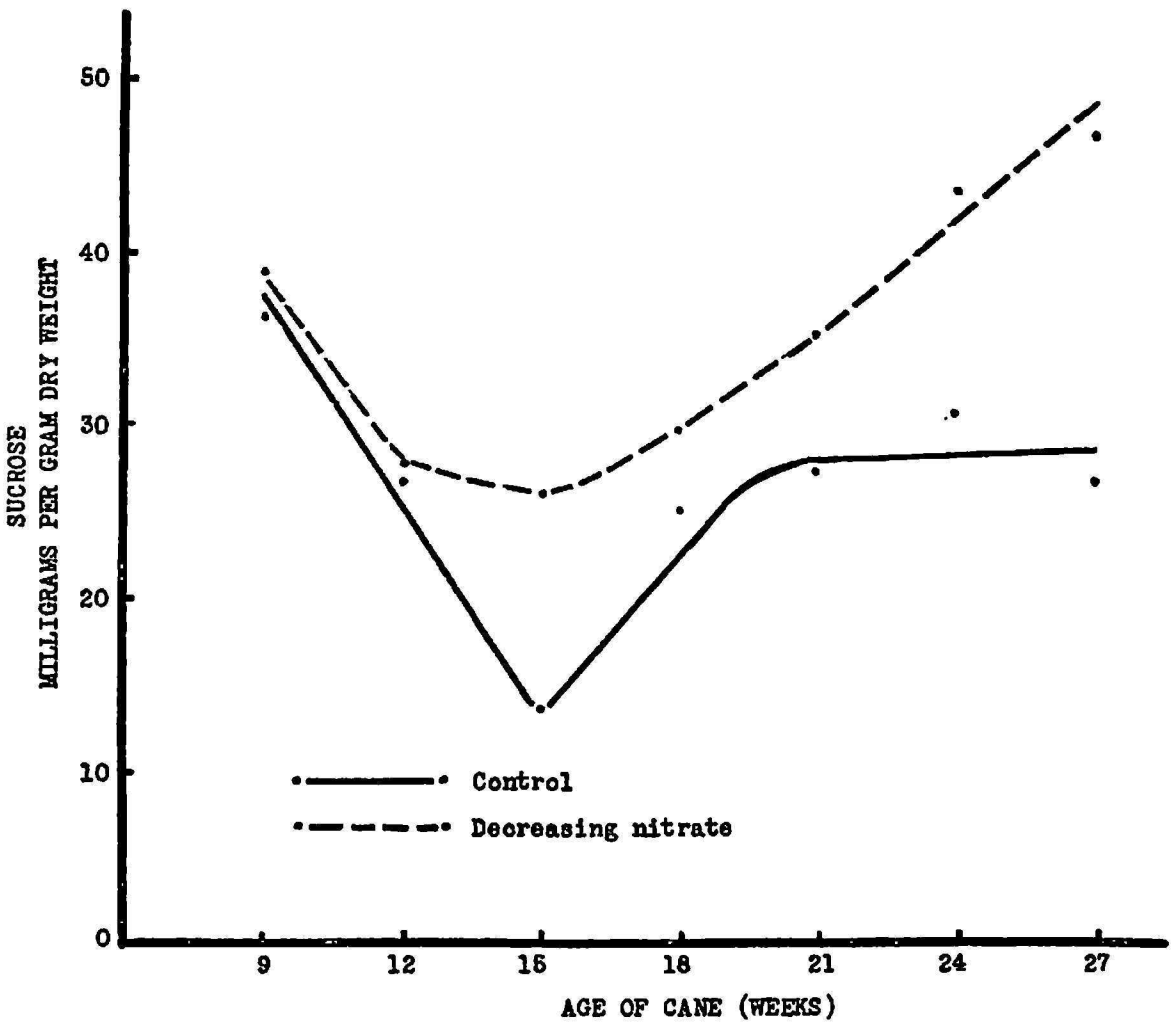


FIG. 1.—Influence of decreasing nitrate on the leaf-sucrose content of immature sugarcane.

Each of the phosphatases measured was depressed by decreasing nitrate (table 7, items 1–9). This effect became apparent by 12 or 15 weeks and usually increased in severity through 27 weeks. The smoothed curves presented by figures 2, 3, and 4 illustrate the low-nitrate effect upon representative phosphatases.

Yet, in spite of the general suppressing effect of low nitrate, this response was far more evident among the enzymes hydrolyzing glucose phosphates than among fructose phosphates (table 7, items 3 and 4 *vs.* items 5 and 6).

TABLE 5.—*Mean values for sheath sugars of immature sugarcane supplied with variable nitrate and potassium in sand culture*[1]

Sugar	Data for weeks after planting indicated—							
	9	12	15	18	21	24	27	Mean
Total ketose								
Control	46.7	62.9	58.8	51.3	57.5	77.9	55.4	58.6
Increasing NO_3	46.7	76.3	55.4	70.0	59.6	81.7	66.7	65.2
Decreasing NO_3	50.4	74.6	55.8	55.8	65.8	77.1	72.9	64.6
Decreasing K	46.3	73.3	60.0	55.0	79.2	81.3	79.6	67.8
Mean	47.5	71.8	57.5	58.0	65.5	79.5	68.7	64.1
Sucrose								
Control	22.5	30.4	31.3	34.2	31.3	63.8	44.2	36.8
Increasing NO_3	19.2	42.1	23.8	46.3	33.8	66.7	59.6	41.5
Decreasing NO_3	22.9	38.8	24.6	32.5	35.8	67.9	68.3	41.5
Decreasing K	24.2	41.7	24.6	36.3	49.2	70.0	62.9	44.1
Mean	22.2	38.3	26.1	37.3	37.5	67.1	58.8	41.0
Fructose								
Control	24.2	32.5	27.1	17.1	26.3	14.2	11.3	21.8
Increasing NO_3	27.5	43.7	31.6	23.8	25.8	15.0	7.1	24.9
Decreasing NO_3	27.5	35.8	31.3	23.3	30.0	9.2	4.6	23.1
Decreasing K	22.1	31.7	35.8	20.5	30.0	11.3	16.7	24.0
Mean	25.3	35.9	31.5	21.17	28.0	12.4	9.9	23.5
Total reducing sugar								
Control	30.9	43.6	35.6	25.7	14.0	27.3	19.8	28.1
Increasing NO_3	46.3	60.7	35.6	39.8	17.8	37.8	19.8	36.8
Decreasing NO_3	31.7	53.2	34.0	24.4	19.8	21.9	23.2	29.7
Decreasing K	31.1	57.8	37.8	29.4	49.8	37.6	51.1	42.1
Mean	35.0	53.8	35.8	29.8	25.4	31.2	28.5	34.2
Glucose								
Control	6.7	11.1	8.4	8.6	1.1	14.1	8.6	8.4
Increasing NO_3	18.8	16.9	5.2	16.1	1.0	22.7	12.8	13.3
Decreasing NO_3	4.2	17.3	3.5	1.3	0.7	12.6	18.6	8.3
Decreasing K	8.8	26.1	2.0	8.9	21.8	26.3	34.4	18.3
Mean	9.6	17.9	4.8	8.7	6.2	18.9	18.6	12.1

[1] Sugar content is expressed as milligrams per gram dry weight. Each figure represents the computed mean of 3 replicates.

TABLE 6.—*Mean values for total dry weight, sheath-percent-moisture, and water-soluble protein of immature sugarcane plants supplied with variable nitrate and potassium in sand culture*[1]

Analysis	Data for weeks after planting indicated—							
	9	12	15	18	21	24	27	Mean
Total dry weight (grams)								
Control	17.1	32.0	50.7	81.2	106.3	132.8	194.8	87.38
Increasing NO_3	15.7	26.7	40.3	67.7	92.3	151.4	154.9	78.43
Decreasing NO_3	16.4	30.3	45.2	72.7	87.9	109.5	161.3	74.76
Decreasing K	15.7	31.9	50.1	77.2	98.5	132.2	181.1	83.96
Mean	16.2	30.2	46.6	74.7	96.3	131.5	173.0	81.1
Sheath-percent-moisture (percentage of fresh weight)								
Control	86.1	86.0	85.5	85.4	85.1	83.4	83.6	85.0
Increasing NO_3	86.4	86.1	86.2	86.0	85.9	83.1	82.8	85.2
Decreasing NO_3	86.2	86.5	85.0	84.9	84.4	82.5	79.4	84.3
Decreasing K	84.7	86.4	84.9	85.3	84.5	82.4	82.6	84.4
Mean	85.9	86.3	85.4	85.4	84.9	82.9	82.1	84.7
Water-soluble protein (milligrams per gram dry weight)								
Control	2.00	2.18	1.44	2.17	2.85	2.28	3.45	2.34
Increasing NO_3	1.73	2.45	1.44	2.88	2.87	1.91	3.93	2.46
Decreasing NO_3	1.65	2.50	1.97	2.26	3.87	2.40	4.72	2.77
Decreasing K	1.98	2.40	1.59	2.55	3.07	2.55	4.16	2.61
Mean	1.84	2.38	1.61	2.47	3.17	2.28	4.07	2.54

[1] Each figure represents the computed mean of 3 replicates.

Hydrolysis of glucose-1 and glucose-6-phosphate was reduced to 37.3 and 31.9 percent of control activity at 27 weeks, whereas fructose-6-phosphate and fructose-1,6-diphosphate were hydrolyzed at 66.8 and 62.9 percent of controls at the same period. ATP-ase was likewise severely curtailed among low-nitrate plants at the end of the study, with activity recorded at 39.7 percent of controls, while the UDP and 6-phosphogluconate-hydrolyzing enzymes were depressed to intermediate levels of 58.4 and 50.0 percent. Phosphatase was generally a little more active among the high-nitrate plants than among controls. For example, the mean β-glycerophosphatase activity value for all harvests was 20.5 percent greater than the control, when nitrate was increased, and ATP-ase was 34.8 percent more active among plants receiving high nitrate (table 7, items 1 and 2).

TABLE 7.—*Mean specific-activity values for leaf enzymes of immature sugarcane supplied with variable nitrate and potassium in sand culture*[1]

Item No.	Enzyme	Data for weeks after planting indicated—							
		9	12	15	18	21	24	27	Mean
1	β-glycerophosphatase								
	Control	59.5	27.2	35.0	18.6	24.3	29.0	21.4	31.2
	Increasing NO_3	68.3	43.0	45.8	24.2	23.2	34.7	24.2	37.6
	Decreasing NO_3	70.0	33.8	23.7	17.5	13.0	14.4	9.2	26.0
	Decreasing K	53.1	28.9	24.6	19.4	24.0	14.9	19.3	24.8
	Mean	62.7	33.2	32.3	19.9	21.1	23.2	18.5	29.9
2	ATP-ase								
	Control	34.0	23.4	33.7	18.2	24.9	30.1	22.8	26.7
	Increasing NO_3	49.3	40.5	55.0	25.9	23.2	30.0	28.2	36.0
	Decreasing NO_3	37.8	29.7	13.5	16.8	12.2	13.2	9.0	18.8
	Decreasing K	40.2	26.3	26.6	24.6	25.1	15.3	22.4	25.8
	Mean	40.2	30.0	32.2	21.4	21.3	22.1	20.6	26.8
3	Glucose-1-phosphatase								
	Control	14.9	16.9	9.2	7.2	6.5	5.4	4.7	9.2
	Increasing NO_3	13.6	18.4	11.4	7.7	6.9	7.0	3.9	9.8
	Decreasing NO_3	15.0	15.2	7.5	6.2	4.8	4.2	1.7	7.8
	Decreasing K	12.2	15.3	7.3	7.7	6.3	5.7	2.7	8.2
	Mean	13.9	16.4	8.8	7.2	6.1	5.6	3.2	8.7
4	Glucose-6-phosphatase								
	Control	24.4	9.2	11.5	10.6	12.5	10.0	4.5	11.8
	Increasing NO_3	33.1	14.9	15.4	15.6	10.7	6.3	3.9	14.3
	Decreasing NO_3	30.9	8.5	5.7	6.5	7.1	2.7	1.4	8.9
	Decreasing K	30.1	8.5	9.2	9.9	13.6	4.1	6.3	11.7
	Mean	29.6	10.3	10.4	10.6	10.9	5.8	4.0	11.7
5	Fructose-6-phosphatase								
	Control	56.1	31.1	36.5	26.7	24.1	19.3	9.7	29.1
	Increasing NO_3	59.7	32.8	44.8	22.6	23.7	15.9	11.4	30.1
	Decreasing NO_3	51.3	31.4	20.9	16.3	15.4	6.3	6.5	21.1
	Decreasing K	57.3	43.6	31.8	17.7	23.3	16.1	9.8	28.5
	Mean	56.1	34.7	33.5	20.8	21.6	14.4	9.3	27.2
6	Fructose-1,6-diphosphatase								
	Control	139	107	63.0	67.3	32.2	37.5	33.3	68.6
	Increasing NO_3	153	113	61.3	55.4	29.8	61.5	34.7	72.8
	Decreasing NO_3	150	119	47.2	61.4	26.4	24.8	21.0	64.3
	Decreasing K	128	110	63.4	54.8	42.7	34.0	33.9	66.8
	Mean	142	112	58.7	59.2	32.8	39.4	30.7	68.1

TABLE 7.—*Continued*

Item No.	Enzyme	Data for weeks after planting indicated—							
		9	12	15	18	21	24	27	Mean
7	2-PGA phosphatase								
	Control	38.7	23.6	16.5	16.3	18.3	23.2	16.6	21.9
	Increasing NO_3	51.0	37.9	24.6	19.2	17.0	23.8	18.9	27.5
	Decreasing NO_3	57.0	29.8	12.1	15.1	10.9	9.7	6.8	20.2
	Decreasing K	44.3	26.4	12.4	20.1	19.0	13.7	16.3	21.7
	Mean	47.8	29.4	16.4	17.7	16.3	17.6	14.7	22.8
8	3-PGA phosphatase								
	Control	59.3	46.0	41.8	46.2	27.3	34.2	25.3	40.0
	Increasing NO_3	90.5	60.8	57.8	53.8	25.6	21.6	28.5	48.4
	Decreasing NO_3	72.0	50.8	31.7	26.5	17.7	6.8	12.1	31.1
	Decreasing K	81.0	42.1	43.6	40.0	27.1	22.5	25.8	40.3
	Mean	75.7	49.9	43.7	41.6	24.4	21.3	22.9	39.9
9	UDP phosphatase								
	Control	12.3	10.2	5.0	—[2]	4.5	5.5	4.2	6.9
	Increasing NO_3	14.8	12.1	4.2	—	4.5	6.9	4.2	7.8
	Decreasing NO_3	14.4	10.9	4.1	—	2.7	3.4	2.0	6.3
	Decreasing K	12.9	10.2	2.7	—	4.1	4.1	3.8	6.3
	Mean	13.6	10.9	4.0	—	3.9	5.0	3.6	6.8
10	Phosphorylase								
	Control	3.6	3.6	2.1	0.9	1.1	1.2	1.1	1.9
	Increasing NO_3	3.4	2.5	1.6	0.7	1.0	1.8	1.5	1.8
	Decreasing NO_3	3.2	4.1	2.0	1.0	0.8	2.6	0.7	2.1
	Decreasing K	2.7	5.6	1.6	1.0	1.0	1.6	1.0	2.1
	Mean	3.2	3.9	1.8	0.9	1.0	1.8	1.1	1.9
11	Hexokinase								
	Control	63.1	27.8	18.6	13.3	15.1	10.3	8.4	22.2
	Increasing NO_3	43.9	21.0	19.9	6.1	14.5	5.7	6.0	15.8
	Decreasing NO_3	95.7	31.5	15.6	19.1	14.2	12.3	7.9	28.0
	Decreasing K	113.2	41.9	17.8	21.5	21.6	14.4	12.7	34.7
	Mean	53.9	30.5	17.9	15.0	16.3	10.7	8.7	25.2
12	Amylase								
	Control	299	186	106	105	192	99	91	154
	Increasing NO_3	309	198	142	101	187	103	96	162
	Decreasing NO_3	340	165	70	61	85	48	43	116
	Decreasing K	286	216	82	96	141	78	60	137
	Mean	309	191	100	91	151	82	73	142

TABLE 7.—*Continued*

Item No.	Enzyme	Data for weeks after planting indicated—							
		9	12	15	18	21	24	27	Mean
13	Invertase								
	Control	3.5	3.0	4.7	3.8	2.9	3.0	2.8	3.4
	Increasing NO_3	9.9	3.9	3.8	3.0	2.8	3.1	2.6	4.1
	Decreasing NO_3	7.7	1.5	4.2	1.6	2.2	2.6	1.9	3.1
	Decreasing K	7.0	3.9	2.6	2.4	2.7	4.4	3.3	3.7
	Mean	7.0	3.1	3.8	2.7	2.7	3.3	2.9	3.6
14	Aldolase								
	Control	40.0	25.3	95.0	24.2	14.0	14.1	14.0	32.3
	Increasing NO_3	39.6	21.0	86.2	21.8	16.6	11.6	12.9	29.9
	Decreasing NO_3	40.1	25.6	65.1	17.3	14.1	17.1	11.7	27.3
	Decreasing K	37.4	33.1	89.1	13.6	19.9	22.7	11.1	32.4
	Mean	39.3	26.2	83.8	19.2	16.2	16.4	12.4	30.5
15	Triose phosphate dehydrogenase								
	Control	12.5	11.1	9.7	7.1	3.7	9.0	5.8	8.4
	Increasing NO_3	13.5	3.9	7.2	3.7	4.0	15.8	3.2	7.3
	Decreasing NO_3	21.5	11.5	3.0	8.0	3.4	12.5	3.1	9.0
	Decreasing K	13.6	6.4	8.9	7.7	5.9	10.4	4.7	8.2
	Mean	15.3	8.2	7.2	6.6	4.3	11.9	4.2	8.2
16	Isocitric dehydrogenase								
	Control	20.3	17.9	11.5	6.1	8.4	14.4	10.4	12.7
	Increasing NO_3	22.8	11.0	9.0	6.1	8.5	18.3	9.8	12.5
	Decreasing NO_3	27.6	13.2	9.2	6.4	6.1	13.0	7.4	11.8
	Decreasing K	19.7	11.0	10.4	9.1	8.8	13.0	8.4	11.5
	Mean	22.6	13.3	10.0	6.9	7.9	14.7	9.0	12.1
17	Phosphoglyceryl kinase								
	Control	11.0	7.7	12.5	9.5	4.0	3.3	10.6	8.3
	Increasing NO_3	15.2	7.3	12.5	8.9	3.5	5.0	13.5	9.4
	Decreasing NO_3	6.7	2.6	8.4	7.5	2.2	8.1	8.8	6.3
	Decreasing K	10.3	3.5	7.0	4.3	2.3	4.0	11.3	6.1
	Mean	10.8	5.3	10.1	7.6	3.0	5.1	11.1	7.5
18	Pyruvic carboxylase								
	Control	24.3	50.9	53.6	23.7	19.8	34.5	11.3	31.1
	Increasing NO_3	21.1	24.1	42.1	18.7	27.3	34.9	10.3	25.5
	Decreasing NO_3	19.3	33.6	54.3	23.6	14.7	32.0	8.6	26.6
	Decreasing K	17.6	49.1	60.9	16.8	16.5	32.7	7.8	28.8
	Mean	20.6	39.4	52.7	20.7	19.6	33.5	9.5	28.0

TABLE 7.—*Continued*

Item No.	Enzyme	Data for weeks after planting indicated—							
		9	12	15	18	21	24	27	Mean
19	Condensing enzyme								
	Control	56.5	—[2]	54.3	19.1	12.1	27.8	—[2]	33.9
	Increasing NO_3	74.5	—	87.3	10.2	20.5	38.6	—	46.2
	Decreasing NO_3	53.0	—	30.8	10.3	5.1	17.8	—	23.4
	Decreasing K	60.1	—	47.4	19.3	4.0	6.0	—	27.3
	Mean	61.0	—	54.9	14.7	10.4	22.5	—	32.7
20	α-ketoglutaric carboxylase								
	Control	30.0	25.3	29.8	29.8	18.9	28.1	18.7	25.8
	Increasing NO_3	46.1	24.2	18.8	25.3	22.2	37.0	10.4	26.3
	Decreasing NO_3	36.0	34.2	26.5	34.6	17.0	28.5	17.9	27.8
	Decreasing K	27.9	17.8	30.5	28.7	20.2	26.9	26.6	25.5
	Mean	35.0	25.4	26.4	29.6	19.6	30.1	18.4	26.3
21	Oxalacetic carboxylase								
	Control	8.7	21.5	38.9	14.5	—[2]	30.5	17.4	21.9
	Increasing NO_3	9.8	28.8	46.9	5.2	—	52.4	16.5	26.6
	Decreasing NO_3	10.3	26.1	62.4	13.3	—	32.6	8.6	25.5
	Decreasing K	8.6	30.3	60.9	16.5	—	36.5	14.0	27.8
	Mean	9.3	26.7	52.3	12.4	—	38.0	11.6	25.5
22	Phosphohexose isomerase								
	Control	43.8	8.0	37.4	76.0	56.6	9.4	13.6	34.9
	Increasing NO_3	35.9	10.6	3.7	69.1	89.3	36.9	5.4	35.8
	Decreasing NO_3	28.9	42.4	20.2	81.1	44.3	38.9	13.8	38.5
	Decreasing K	31.3	39.1	20.3	21.6	25.0	16.5	5.6	22.7
	Mean	34.9	25.0	20.4	61.9	53.8	25.4	9.6	32.9
23	Fumarase								
	Control	59.5	50.7	73.4	10.7	18.3	56.9	6.8	39.5
	Increasing NO_3	52.8	55.1	43.6	1.7	18.9	41.3	11.3	32.1
	Decreasing NO_3	52.7	66.2	62.9	23.9	13.4	73.4	9.2	43.1
	Decreasing K	31.2	68.6	86.1	8.5	17.1	85.6	11.8	44.1
	Mean	49.1	60.2	66.5	11.2	16.9	64.3	9.8	39.7
24	Transaminase								
	Control	75.7	—[2]	29.9	27.4	17.9	18.9	14.8	30.8
	Increasing NO_3	84.9	—	51.8	20.3	17.6	29.3	17.3	36.8
	Decreasing NO_3	99.9	—	51.6	25.2	12.4	19.9	12.8	36.9
	Decreasing K	114.5	—	81.6	25.4	18.7	19.7	23.3	47.2
	Mean	93.7	—	53.7	25.3	16.7	21.9	17.1	37.9

TABLE 7.—*Continued*

Item No.	Enzyme	Data for weeks after planting indicated							
		9	12	15	18	21	24	27	Mean
25	Cytochrome-C reductase								
	Control	2.6	3.8	13.4	15.9	12.2	14.0	23.0	12.1
	Increasing NO_3	2.4	5.0	11.1	21.0	13.2	8.6	25.2	12.3
	Decreasing NO_3	2.6	4.9	24.6	13.7	26.8	16.6	20.4	15.7
	Decreasing K	2.0	4.2	12.9	18.5	13.6	14.8	21.7	12.5
	Mean	2.4	4.5	15.5	17.3	16.4	13.5	22.6	13.2
26	Peroxidase								
	Control	8.2	13.9	24.6	13.6	11.6	6.8	7.8	12.3
	Increasing NO_3	7.1	13.5	24.8	13.5	10.9	8.8	8.8	12.5
	Decreasing NO_3	8.2	12.3	16.5	8.8	7.6	4.2	2.4	8.6
	Decreasing K	6.8	11.6	19.4	9.3	11.8	6.8	6.3	10.3
	Mean	7.6	12.8	21.3	11.3	10.5	6.7	6.3	10.9
27	Catalase								
	Control	135	120	64.2	29.3	20.9	54.2	25.5	64.1
	Increasing NO_3	203	115	66.9	10.6	19.8	70.3	21.3	72.3
	Decreasing NO_3	173	123	48.7	23.1	14.5	46.5	18.5	63.9
	Decreasing K	160	129	42.7	15.1	20.8	43.3	31.8	63.2
	Mean	168	122	55.6	19.5	19.0	53.6	24.3	65.9
28	Polyphenol oxidase								
	Control	13.3	7.2	7.3	3.7	8.2	2.3	24.5	9.5
	Increasing NO_3	18.2	8.4	8.2	5.6	10.3	3.0	34.6	12.6
	Decreasing NO_3	13.5	7.5	6.2	4.2	7.3	2.8	28.6	10.0
	Decreasing K	9.4	10.3	7.6	5.9	9.0	2.7	23.6	9.8
	Mean	13.6	8.3	7.3	4.9	8.7	2.7	27.8	10.5
29	6-phosphogluconate phosphatase								
	Control	9.1	—[2]	29.6	27.6	15.3	16.9	13.2	18.6
	Increasing NO_3	10.1	—	34.1	30.9	15.0	11.4	18.9	20.1
	Decreasing NO_3	6.6	—	17.0	27.5	9.7	3.8	6.6	11.9
	Decreasing K	7.6	—	18.0	30.8	18.1	12.1	11.5	16.3
	Mean	8.4	—	24.7	29.2	14.5	11.1	12.6	16.7
	Grand mean	49.9	35.8	31.9	21.4	20.2	21.5	15.0	28.0

[1] Each figure represents the mean specific-activity value computed from 3 replicates.

[2] Data not available.

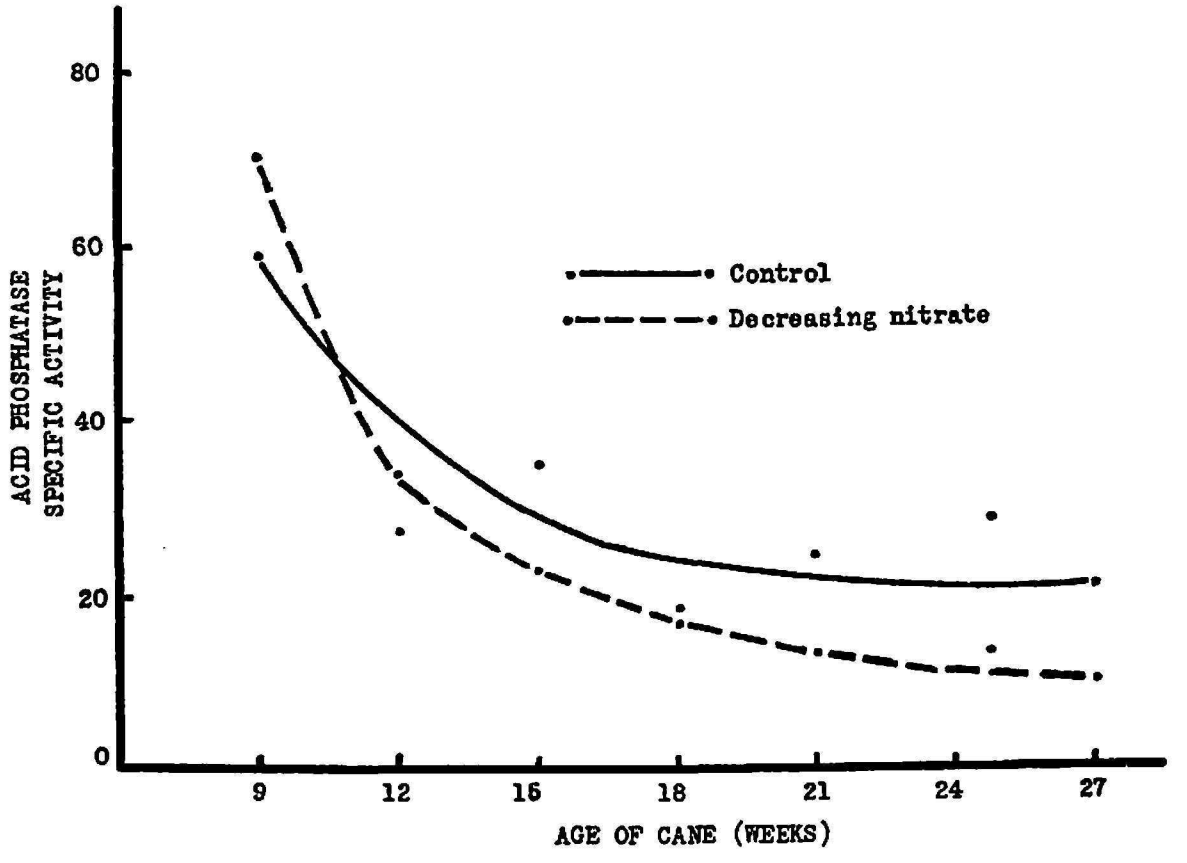


FIG. 2.—Influence of decreasing nitrate supply on acid phosphatase from leaves of immature sugarcane. β-glycerophosphate was used as substrate.

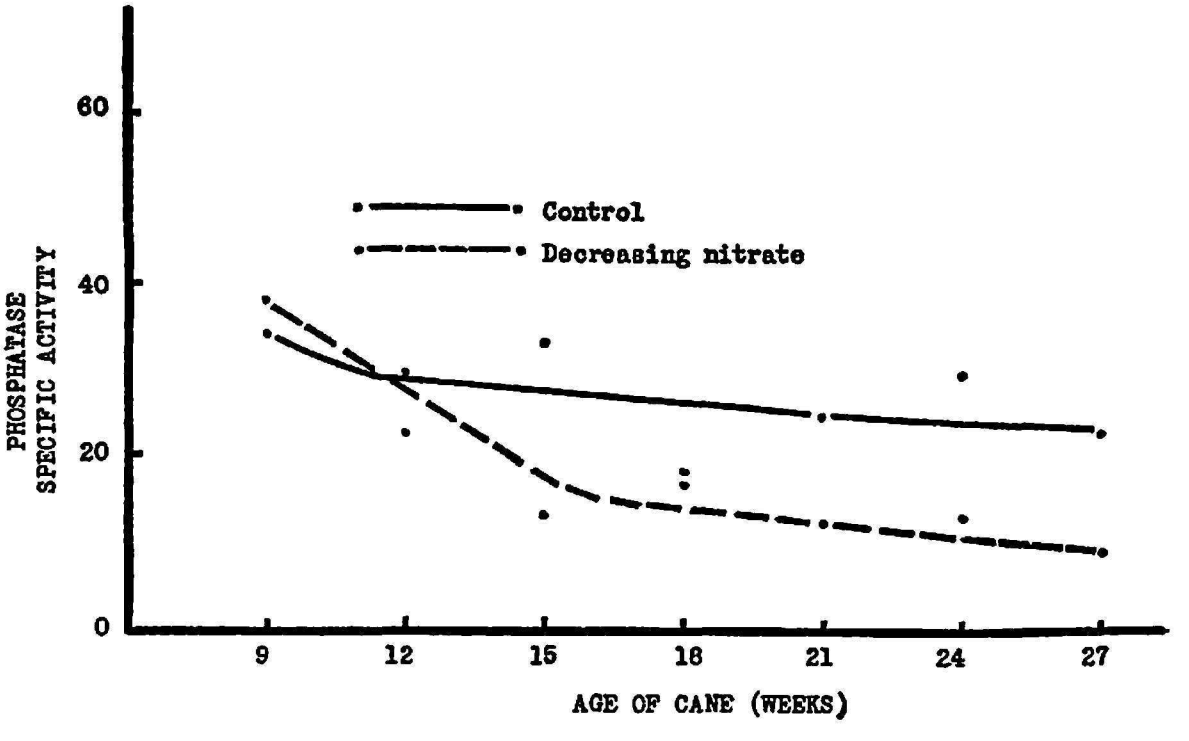


FIG. 3.—Influence of decreasing nitrate supply on the ATP-hydrolyzing phosphatase from leaves of immature sugarcane.

The most striking feature of the hexokinase activity recorded during this study was its rapid decline after the initial harvest (table 7, item 11). Undeniably one of the most critical of the sugar-metabolizing enzymes, hexokinase, had declined by 15 weeks to less than a third of its initial activity among control plants, and by the end of the study was active at approximately one-eighth of the original level. With the exception of the 15-week harvest, when both treatment and control values were virtually equal,

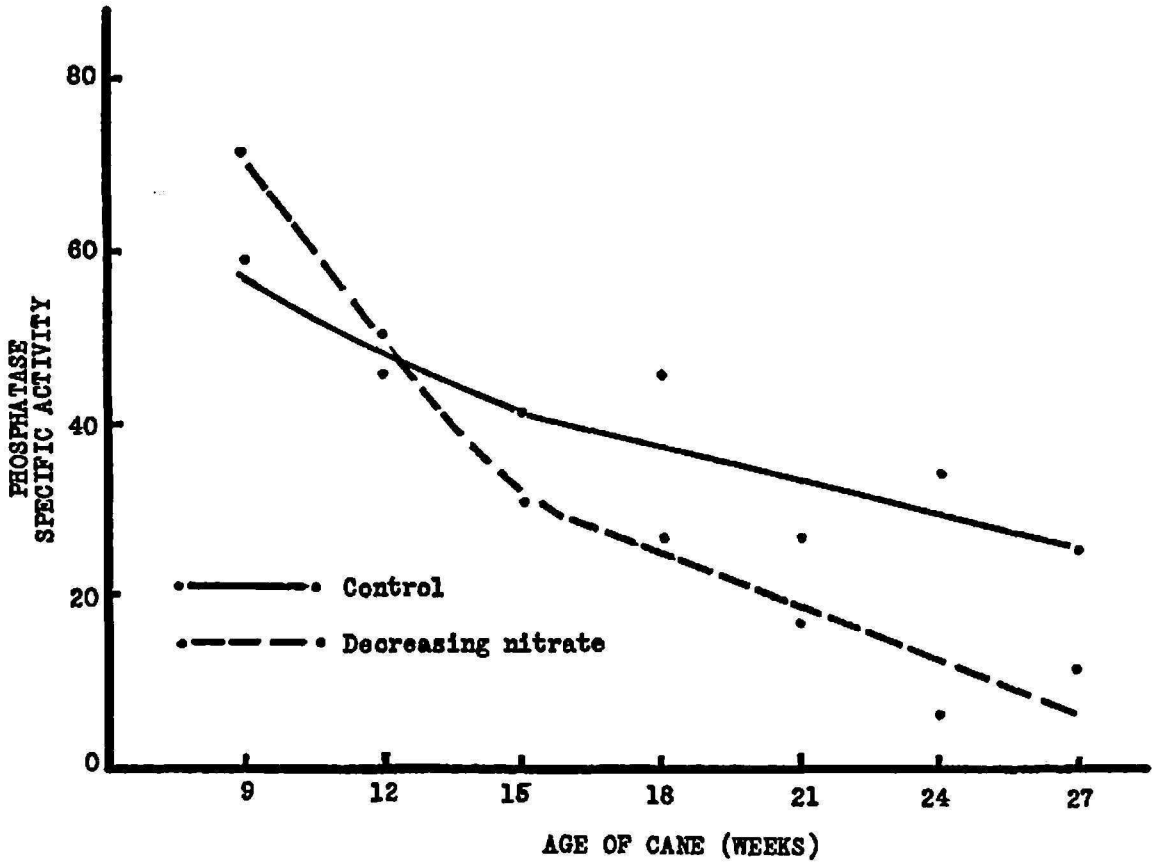


FIG. 4.—Influence of decreasing nitrate supply on the 3-phosphoglyceric acid-phosphatase system from leaves of immature sugarcane.

hexokinase activity was consistently higher among low-potassium than the control plants. A depressing effect by increased nitrate was evident at the 18-week period, and to a lesser extent at the 24-week period, which terminated 21-day treatments of 17.5 and 22.5 meq./l. nitrate, respectively, and in each instance, reduced hexokinase values to approximately half of controls.

Phosphorylase activity was extremely weak throughout the experiments. Among control plants the enzyme declined to about 25 percent of initial values by 18 weeks and showed little variation from this level through the remainder of the harvests (table 7, item 10).

Invertase vs. Amylase

Possibly the most significant observation of these experiments was the pronounced discrepancy between invertase and amylase potential in sugarcane leaves (fig. 5). Both enzymes were measured according to the amount of reducing sugar formed, and although conditions of the respective assays theoretically favored invertase, the reducing sugar produced by action of amylase far exceeded that produced at any time via the invertase system

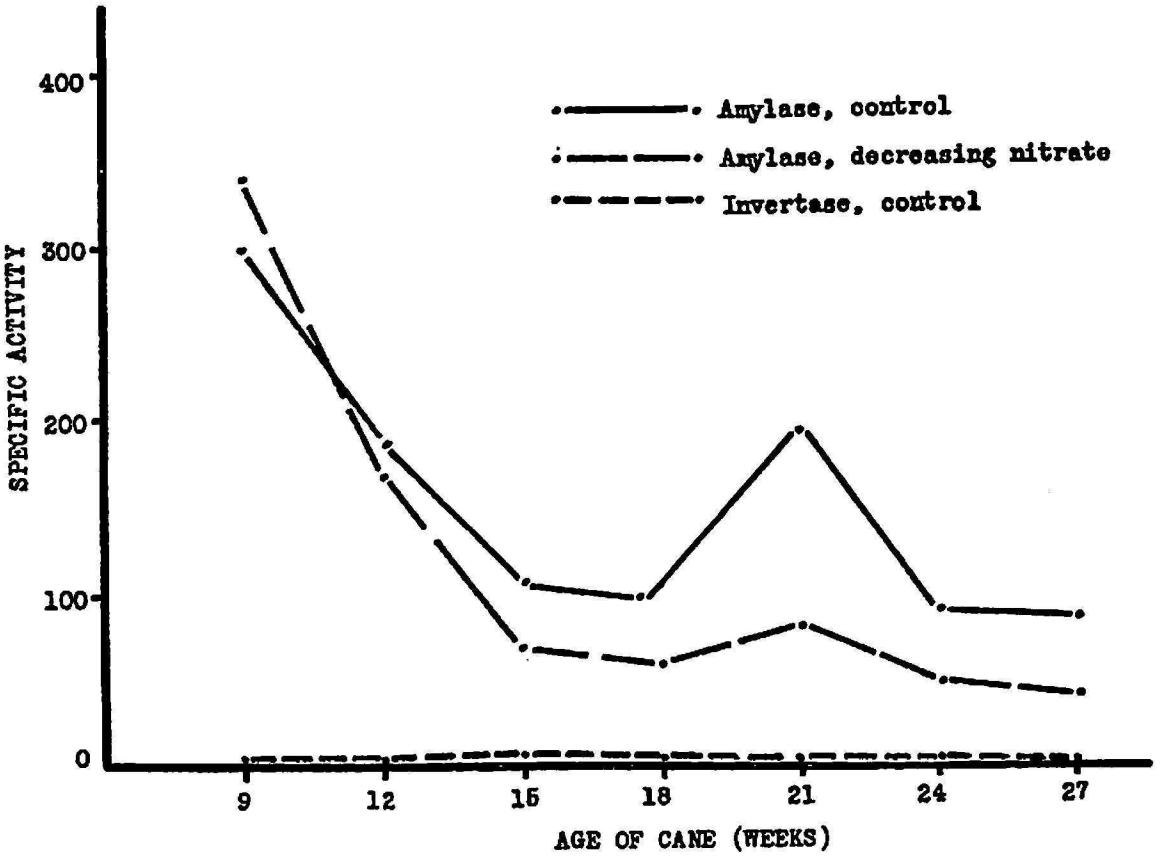


FIG. 5.—A comparison of control values for amylase and invertase, and the effects of decreasing nitrate supply on amylase from leaves of immature sugarcane.

(table 7, items 12 and 13). Mean values for all treatments reveal that amylase activity was some 44 times greater than that of invertase at 9 weeks. Both enzymes declined to their lowest levels at 27 weeks, but at that time amylase still exceeded invertase by twenty-fivefold.

Of particular interest is the fact that both invertase and amylase were depressed by the same decreasing nitrate treatments which promoted leaf sucrose, decreased sheath moisture, and depressed the acid phosphatases. The inhibitory effect of low nitrate was most pronounced against amylase. At 27 weeks amylase activity among low-nitrate plants was only 46.8 percent of controls, whereas invertase was recorded at 69.2 percent of controls.

A possible interrelationship of amylase activity with leaf-fructose content and nitrate level became apparent at 21 weeks. At that time the fructose of control plants dropped sharply to its lowest recorded level (table 4), and amylase increased among the same plants to a new peak completely out of proportion with the remainder of the amylase curve (table 7, item 12). Lowering nitrate supply alleviated the amylase rise at 21 weeks (table 7), while the marked fructose decline observed among control plants was totally absent among those receiving low nitrate.

Decreasing potassium affected invertase activity, but the response was inconsistent (table 7, item 13). At 15 and 18 weeks, which terminated potassium treatments of 4 and 3 meq./l. respectively, invertase was suppressed, whereas by 24 weeks the enzyme had increased beyond control values in response to 1 meq./l.

The Dehydrogenases

The isocitric acid and triose phosphate dehydrogenases exhibited somewhat similar curves during the study (table 7, items 15 and 16). Initially high activity dropped to a low at 18 or 21 weeks and rose to a secondary peak at 24 weeks. Both enzymes were affected by varying nitrate levels in the range of 10 to 25 meq./l. Triose phosphate dehydrogenase declined some 84 percent when nitrate was increased from 10 to 12.5 meq./l., while control dehydrogenase dropped only 11.4 percent (fig. 6). The early depressing effects of increased nitrate disappeared for both enzymes by 21 weeks when the plants were receiving 20 meq./l. nitrate, and activity was stimulated beyond control levels when nitrate was further increased to 22.5 meq./l.

Cytochrome-C Reductase

The response of cytochrome-C reductase to increasing plant maturity was virtually opposite from that of the majority of the enzymes measured. Among control plants, for example, activity increased by 900 percent during the study in contrast to the gradual decline usually observed for other enzymes (table 7, item 25). The only deviations from control values were caused by decreasing nitrate (fig. 7). Lowering nitrate supply from 8.5 to 7.0 meq./l., and later from 5.5 to 4.0 meq./l., caused sharp increases in the enzyme activity recorded at 15 and 21 weeks, respectively.

Peroxidase

Peroxidase activity increased through 15 weeks and thereafter declined during the remainder of the study (table 7, item 26). This was another of the enzymes markedly curtailed by decreasing nitrate, and by 27 weeks this treatment had suppressed activity to 34 percent of the controls. A com-

parison of the control and low-nitrate curves of peroxidase with the corresponding curves for leaf sucrose indicates that the physiological conditions most conducive to sucrose production had just the opposite effect upon peroxidase activity (figs. 8 and 9).

Phosphohexose Isomerase

Decreasing potassium exhibited the greatest influence on phosphohexose isomerase activity (table 7, item 22) and this influence was apparently a

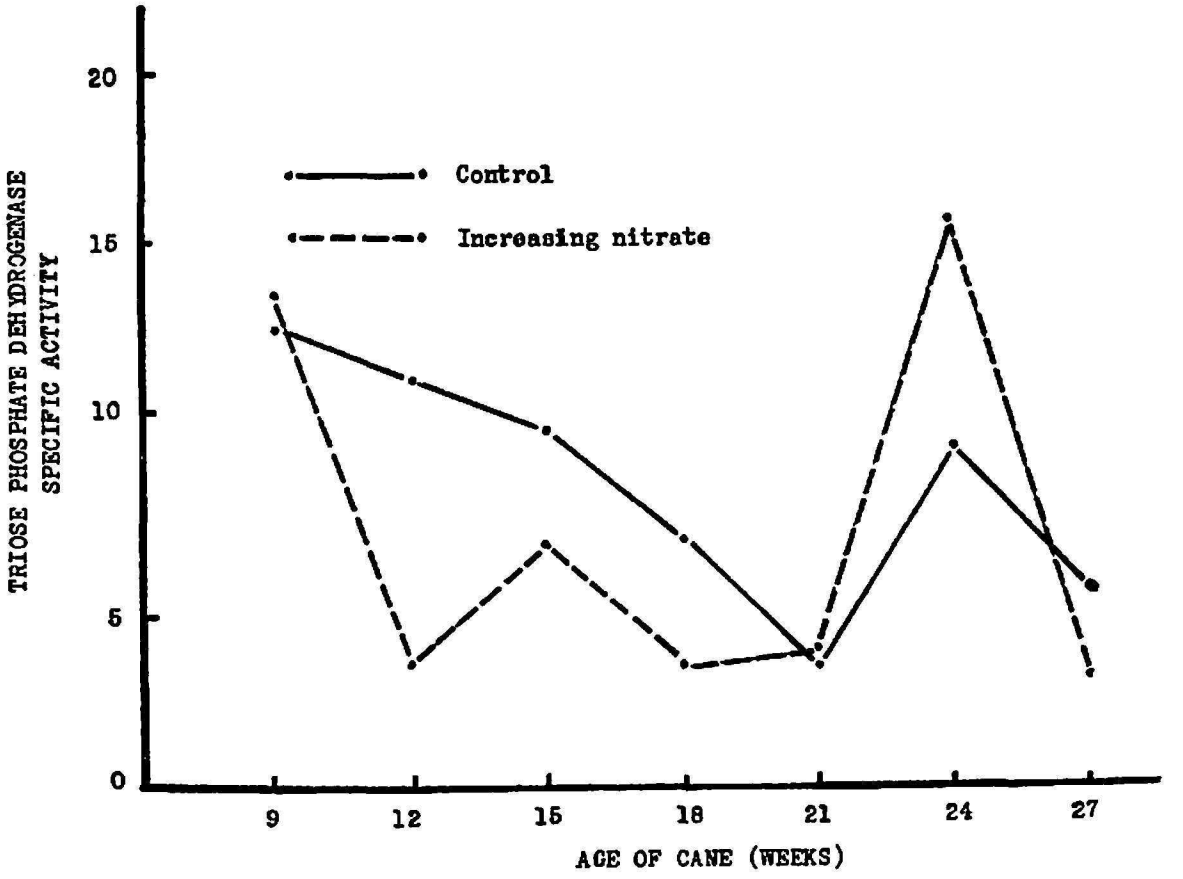


FIG. 6.—Influence of increasing nitrate supply on the enzyme triose phosphate dehydrogenase from leaves of immature sugarcane.

stabilizing one (fig. 10). This was one of the few instances where a treatment response bore no resemblance whatsoever to the control curve. With decreasing potassium, isomerase made neither the abrupt decline at 12 weeks nor the twofold activity increase at 18 weeks which was characteristic of the control group, but rather responded with a seemingly independent activity curve throughout the study.

Condensing Enzyme

We were able to measure the condensing enzyme at only five of the seven sampling periods, but these data point up treatment effects of both de-

creasing nitrate and potassium (table 7, item 19). By comparison with control values the lowering of nitrate supply from 10 to 7 meq./l. caused a depression of the enzyme which remained roughly proportional during the following 63 days (fig. 11). Decreasing potassium, on the other hand, had no effect on the enzyme through 18 weeks, by which time the potassium supply had been reduced from 6 to 3 meq./l. When this element was decreased from

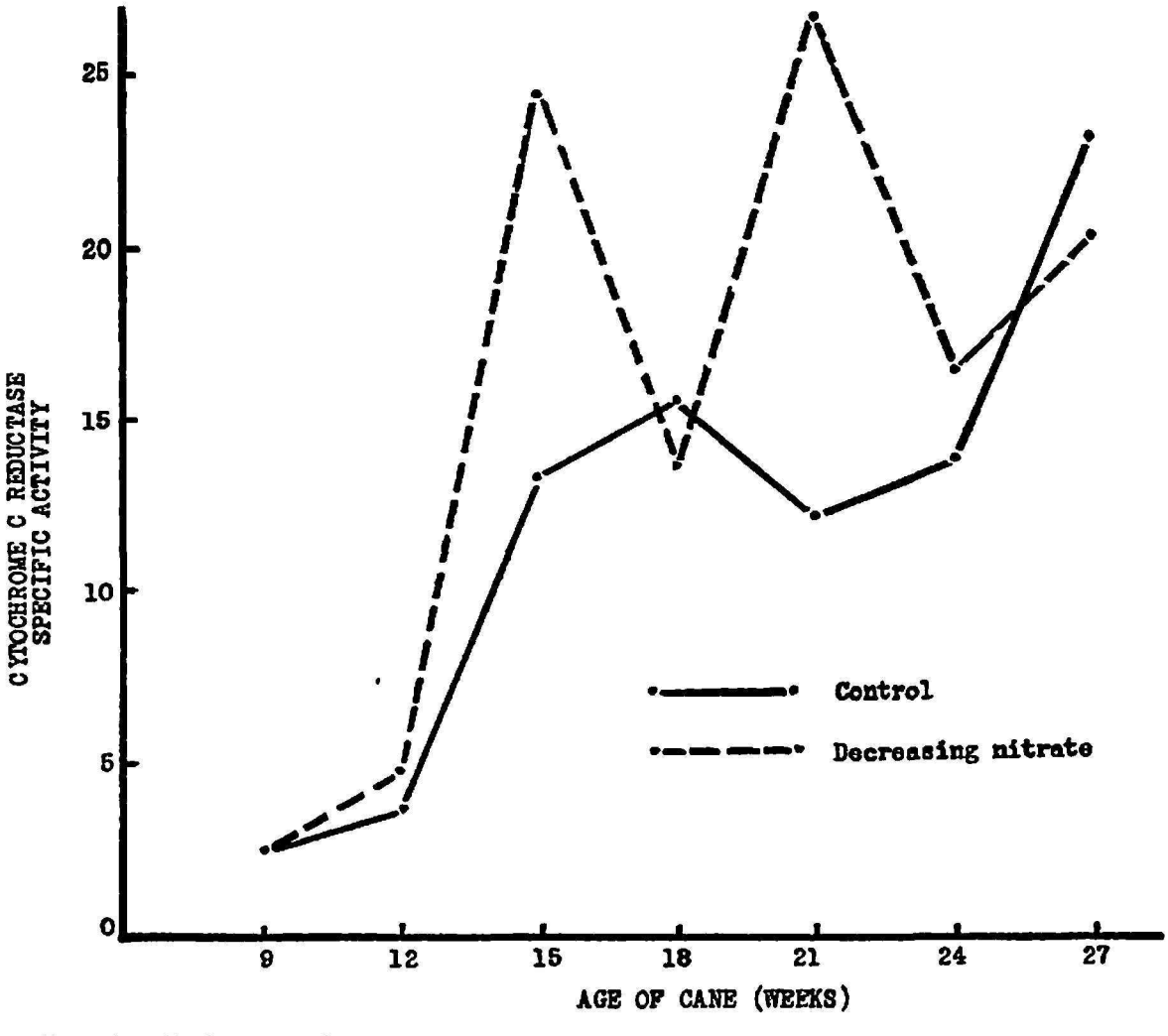


FIG. 7.—Influence of decreasing nitrate on the enzyme cytochrome-C reductase from leaves of immature sugarcane.

3 to 2 meq./l. the condensing enzyme activity dropped below control levels, and following the reduction of potassium from 2 to 1 meq./l. the enzyme was further depressed to only 22 percent of controls.

Phosphoglyceryl Kinase

The suppressing effects of both decreasing nitrate and potassium treatments were again apparent against the enzyme phosphoglyceryl kinase during the 12–21 week period (table 7, item 17). In this case decreasing

potassium did not act to smooth away the broad fluctuations established by control plants, and both decreasing nitrate and potassium enzyme curves lie more or less parallel to controls (fig. 12). This is, in fact, an admirable example of normal physiological requirements dictating the nature of an enzyme's response, even though treatment factors are at work to change the magnitude of the response.

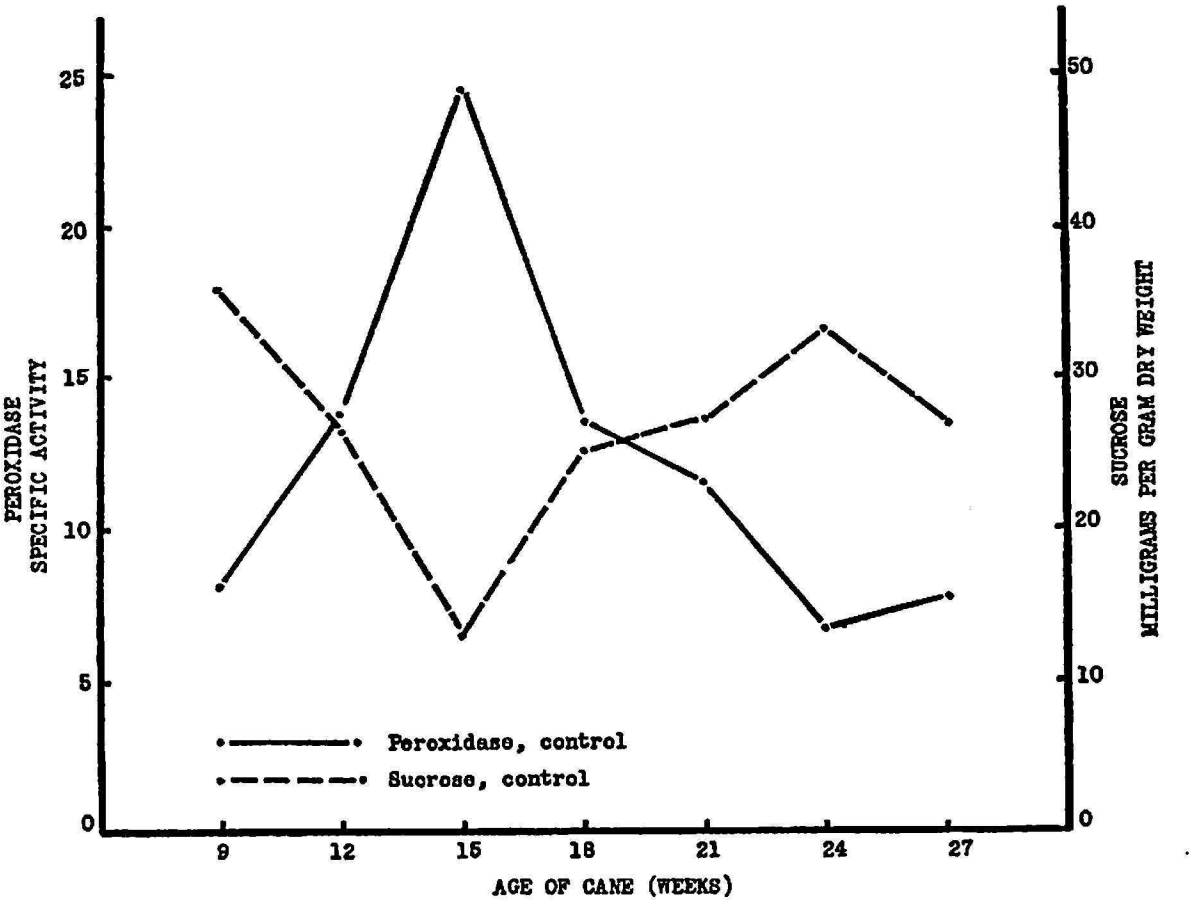


FIG. 8.—The inverse relationship between sucrose and the enzyme peroxidase from leaves of immature sugarcane supplied with complete nutrient solutions.

Aldolase

The enzyme aldolase seemed literally to run wild at 15 weeks (fig. 13). This action was clearly evident in both control and treatment groups (table 7, item 14), although the enzyme was partly suppressed at this time by decreasing nitrate.

Enzymes Unrelated to Sugar Levels or Treatments

Several of the enzymes measured showed no apparent relationships with sugar levels and little or no response to treatments. These systems include the carboxylases, transaminase, fumarase, catalase, and polyphenol oxidase.

DISCUSSION

The enzyme responses herein reported have fallen into three general categories. Of greatest importance is a broad group of phosphate and glucose metabolizing systems which can bear heavily on the sucrose physiology of cane. A second group includes enzyme activity which was likewise altered by changing levels of nitrate and potassium, while their importance to carbohydrate metabolism remains obscure. We can only advance this

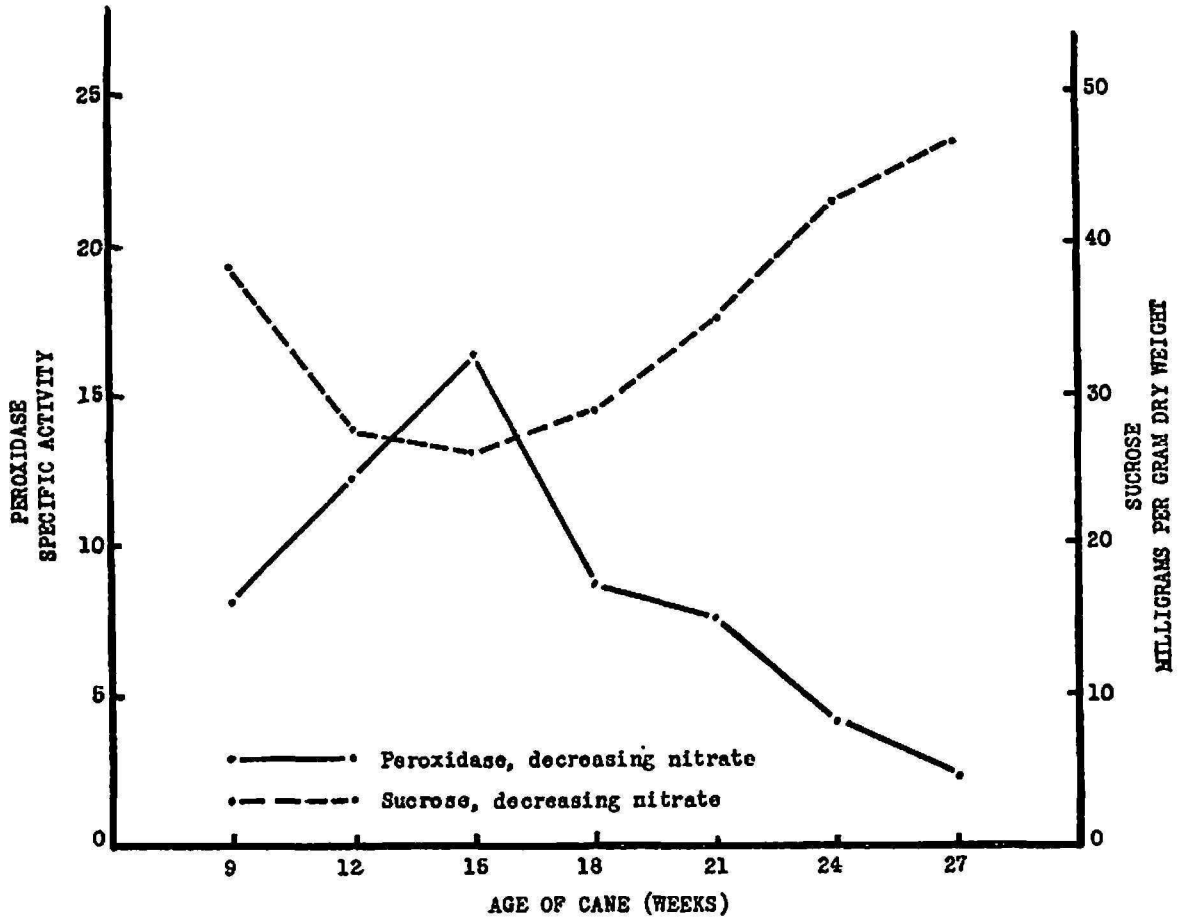


FIG. 9.—The inverse relationships between sucrose and the enzyme peroxidase from leaves of immature sugarcane supplied with decreasing levels of nitrate.

new information looking toward a day when their ultimate functions are clearly understood. The third group is a small one and, important though these enzymes may be to the physiology of cane, they apparently functioned independently of any treatment or constituent level studied during these experiments.

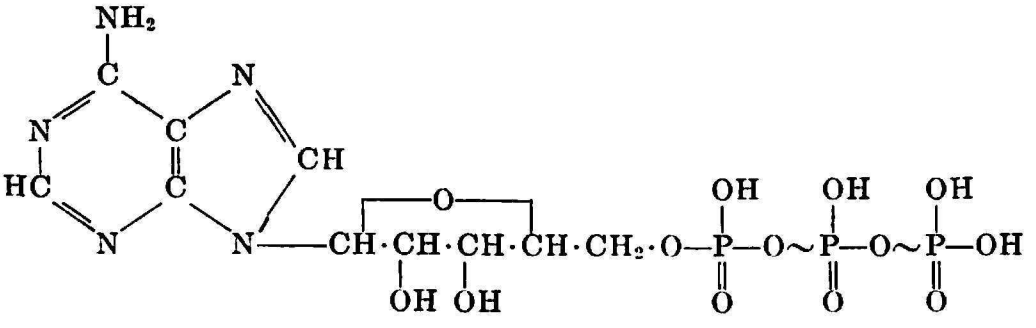
ENZYMES RELATED TO SUCROSE METABOLISM

The phosphate and glucose metabolyzing enzymes are given particular attention because of the far-reaching influence they can bring to bear on cane physiology. Perhaps the single most important finding of this study is

the unqualified capability of leaf phosphatases to attack any of the organic phosphates tested. Two distinct areas are open for such a group of acid phosphatases to affect sucrose metabolism, the adenylic acid system[10] and the supply of phosphorylated metabolites involved in sucrose biosynthesis.

The Adenylic Acid System

In recent years much information has been gathered concerning the importance of adenosine triphosphate (ATP) in carbohydrate metabolism, particularly as a source of energy and inorganic phosphate. Included in its structure are adenine, ribose, one ordinary ester-phosphate linkage, and two high-energy pyrophosphate linkages:



Adenosine triphosphate (ATP)

ATP was first isolated in 1929 by Lohmann (*45*) who obtained it from muscle. He also proved its structure and demonstrated its stepwise degradation to adenosine diphosphate and adenylic acid. The compound has since been proved to be a common constituent of living material which traps the energy of exergonic reactions. This energy is subsequently employed to drive the endergonic processes of life. Unquestionably, adenosine-triphosphate is one of the most important substances to occur in living organisms, and enzymes which catalyze its degradation thus assume an equal importance.

In 1932 Barrenschsen and Lang (*5*) reported the hydrolysis of ATP by liver preparations. Liebknecht (*43*), in 1939, referred to similar hydrolysis as the work of bone "phosphatase". The investigations by Engelhardt and Liubimova (*22*) first indicated that two enzymes may be required to hydrolyze ATP and AMP (adenosinemonophosphate).

The potential importance of an ATP-hydrolyzing enzyme in glucose metabolism was vividly demonstrated by Meyerhof (*50*) in 1949. He found that the fermentation of glucose stops in yeast, when inorganic phosphate is depleted, because of the inactivation of an essential ATP-hydrolyzing enzyme. During the normal fermentation of glucose, ATP is not allowed to

[10] The term "adenylic acid system" refers to adenylic acid, adenosinediphosphate, adenosinetriphosphate, and the enzymes necessary to effect the interconversions of these compounds.

accumulate, but rather it is broken down by a specific phosphatase (ATP-ase) to ADP and inorganic phosphate. Consequently, the action of this enzyme ensures a source of inorganic phosphate needed in the formation of 1,3-diphosphoglyceric acid, which, in turn, is essential for the continuing metabolism of glucose. It was found that, by adding purified ATP-ase to an extract which had stopped fermenting, the process was rapidly revived, but that the addition of too much enzyme caused the destruction of all avail-

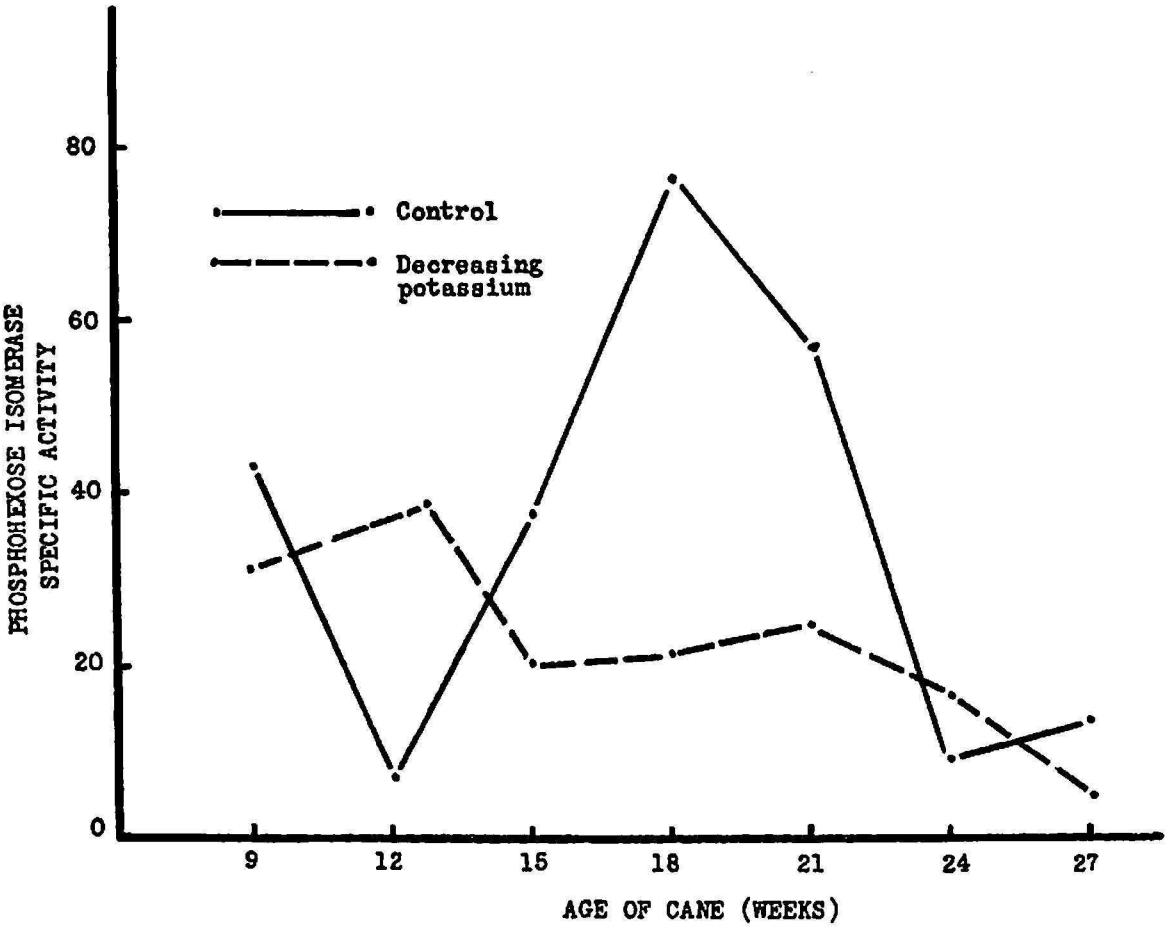


FIG. 10.—Influence of decreasing potassium supply on the enzyme phosphohexose isomerase from leaves of immature sugarcane.

able ATP. It was thus apparent that the presence of a moderately active ATP-ase is essential in yeast cells to maintain a balance between the phosphorylation of glucose, for which ATP is required, and the phosphorylation of glyceraldehyde-3-phosphate, for which inorganic phosphate is required.

The importance of the ATP-ase reaction is not confined to the fermentative breakdown of hexoses, and it must also be considered in its effects upon the synthesis of hexoses from smaller units. The conversion of 1 mol of glucose to 2 mols of pyruvate is an exergonic process which can be reversed only if the overall degradative pathway is coupled with reactions which

provide energy (*28*). A close study of any classical glycolytic diagram indicates that during the anaerobic breakdown of glucose to pyruvic acid a total of 4 ATP molecules are formed from ADP, each of which bears a single high-energy (~) bond available to the system. One of these bonds must be utilized in the phosphorylation of glucose (glucose → glucose-6-phosphate), and another in the phosphorylation of fructose-6-phosphate (fructose-6-phosphate → fructose-1,6-diphosphate). A net synthesis of only two high-

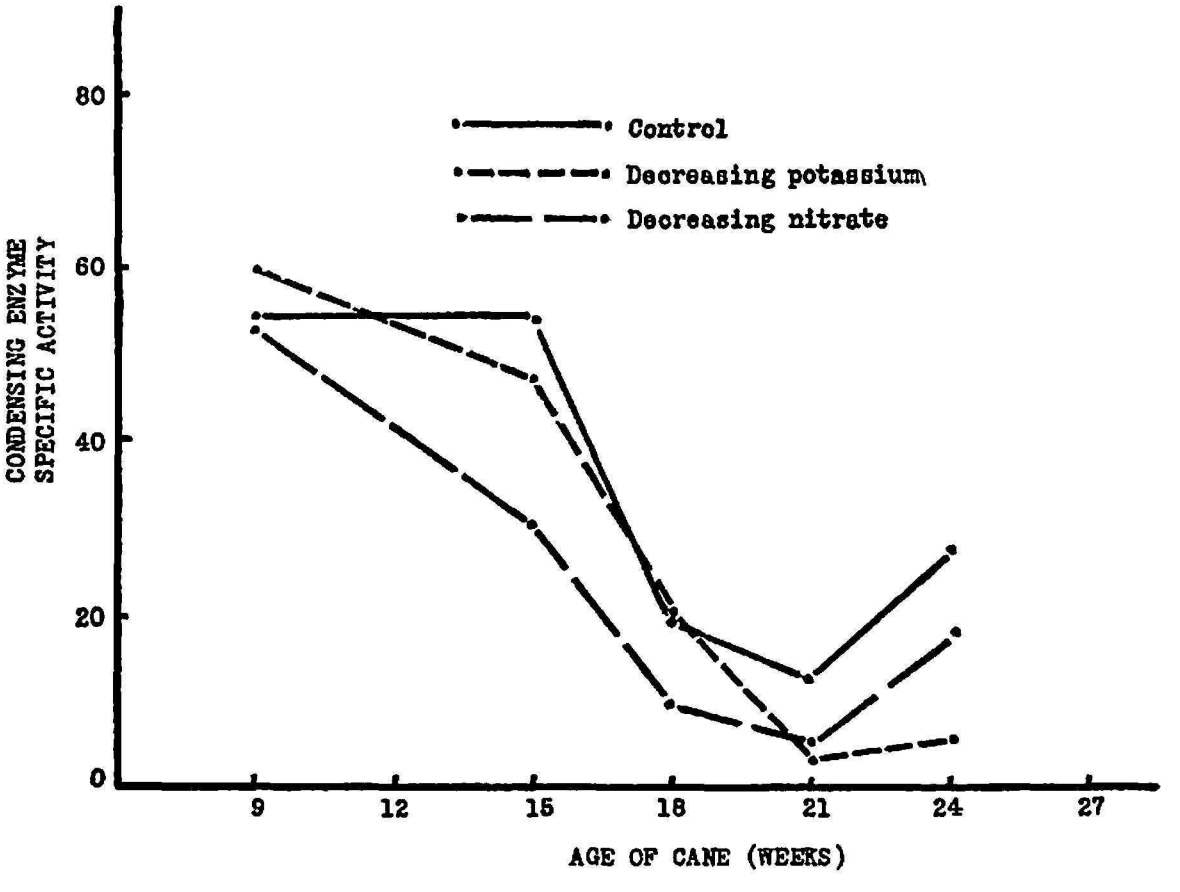


FIG. 11.—Influence of decreasing nitrate and decreasing potassium on the condensing enzyme from leaves of immature sugarcane. Data are not available for harvests 2 and 7.

energy bonds is realized. Consequently, no great excess of energy reserves could be expected for energy-requiring reactions such as the synthesis of hexoses from smaller units. A powerful ATP-ase, such as the enzyme we have encountered in the leaves of sugarcane, might readily deplete ATP reserves through indiscriminate hydrolysis of this substrate. It is possible that during our experiments the accumulation of leaf sucrose at low nitrate levels was effected through the reversal of the glycolytic pathway, and was made possible by a more ample supply of energy reserves realized through the curtailment of the ATP-hydrolyzing system.

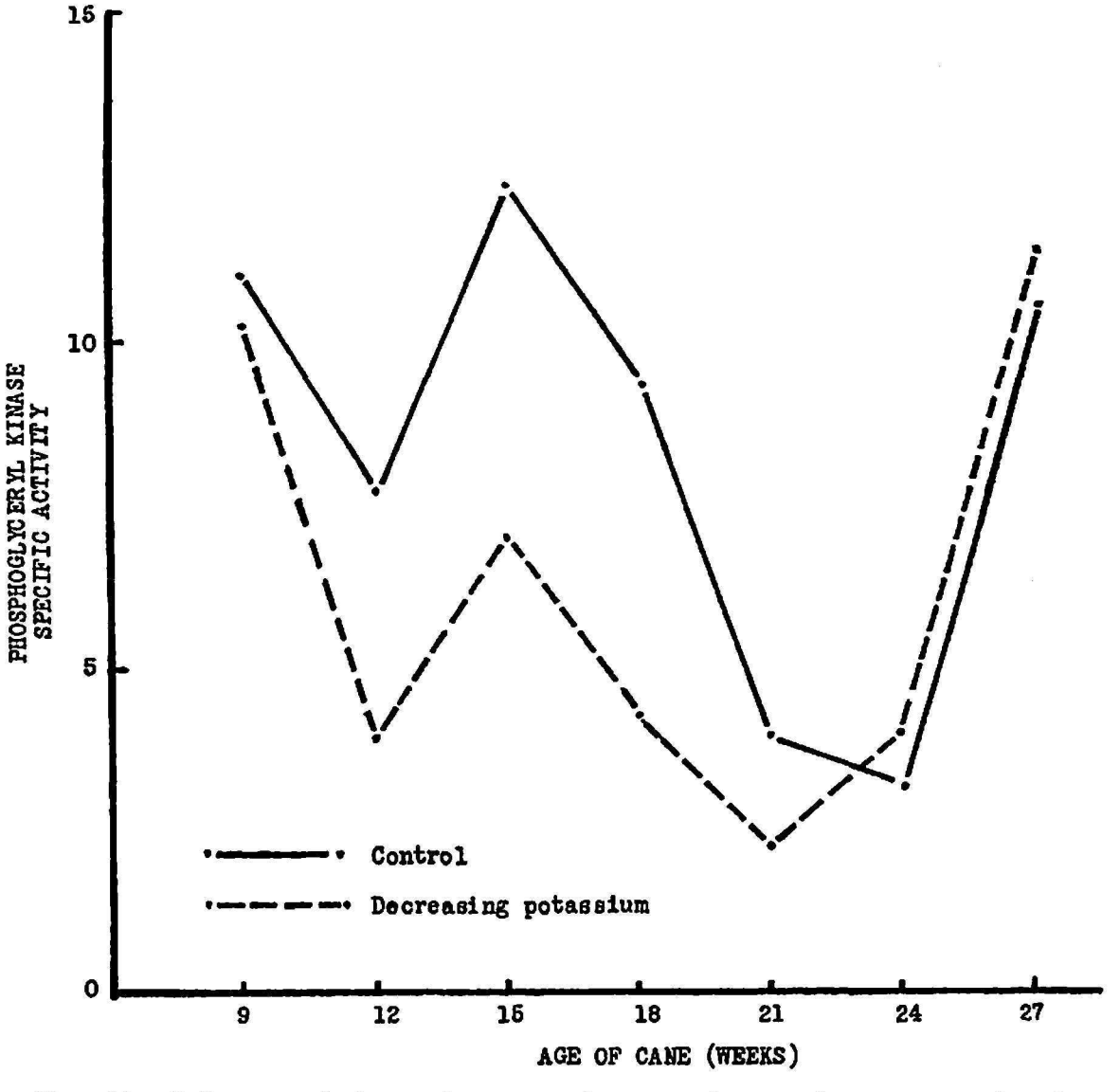


FIG. 12.—Influence of decreasing potassium supply on the enzyme phosphoglyceryl kinase from leaves of immature sugarcane.

These views of ATP-ase action and its relationship to hexose production can be diagramed as follows:

Hexose

Reversal of glycolysis: Endergonic reactions in which ATP energy would be utilized in hexose synthesis

Through glycolysis: Exergonic reactions in which a net synthesis of 2 (~) bonds are realized in the formation of ATP from ADP

Pyruvic acid

Further evidence that a reversal of the glycolytic pathway was in effect during sucrose accumulation will be discussed later, but an appropriate example of such evidence is the aldolase reaction as it was recorded among control and decreasing-nitrate groups (fig. 15). Aldolase is extremely im-

portant to sugar metabolism in that it catalyzes the reversible reaction in which hexose diphosphate is converted to 3-carbon compounds. Our assay procedures were based upon the appearance of triose phosphate, and it was found that the reaction was far more pronounced at 15 weeks than at any other period, which, in turn, corresponds to the lowest sucrose level recorded (fig. 1). This suggests that sucrose was in fact disappearing through the glycolytic system, and that, as a potential "bottleneck" for the process the aldolase equilibrium lay far to the right during the period of low sucrose

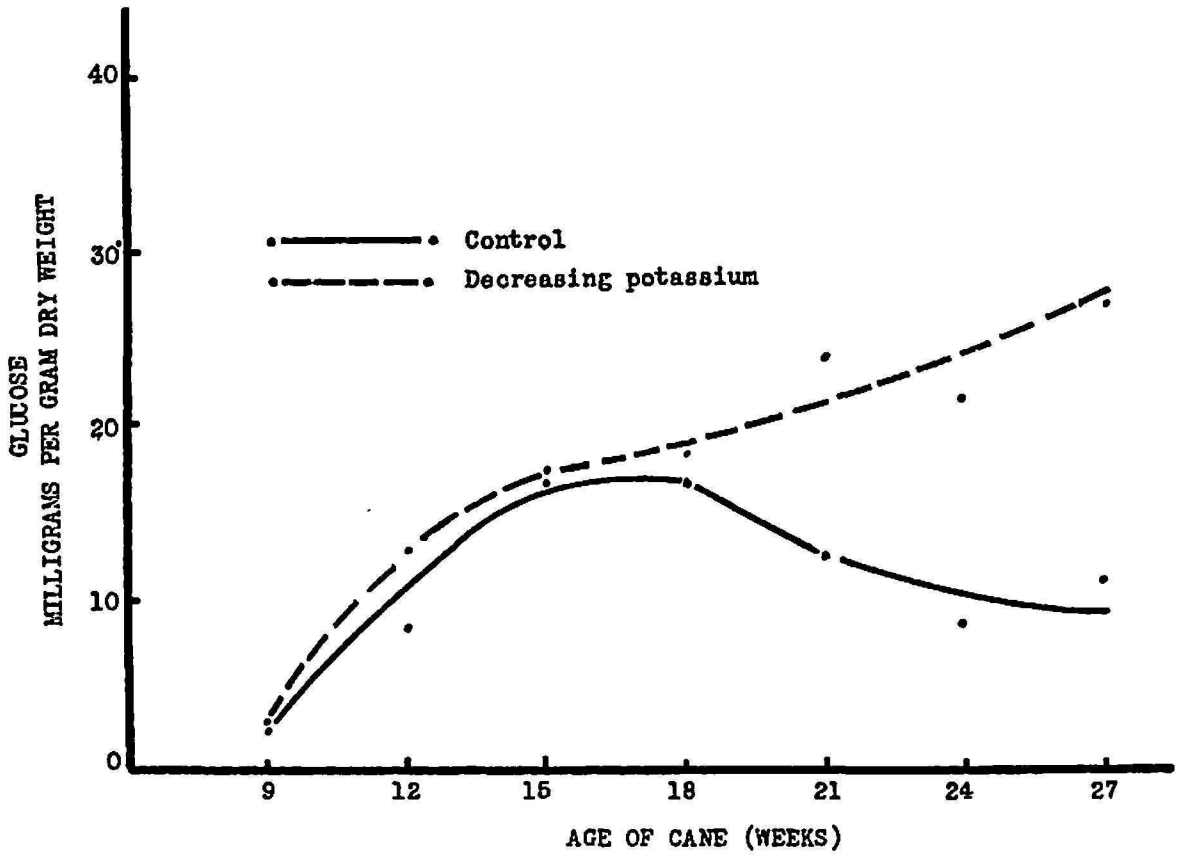


FIG. 13.—Effects of control and decreasing-nitrate treatments on the enzyme aldolase from leaves of immature sugarcane.

and to the left during periods of high sucrose. Figure 1 also portrays an alleviating effect of low nitrate upon the sucrose depression, and it is quite apparent from figure 15 that low nitrate was suppressing aldolase at this same time.

With our present knowledge it is not possible to state precisely the roles of the adenylic acid system in higher plants, not to mention sugarcane. We can only surmise that the painstaking studies with extracts of muscle, yeast, and a few higher plants provide insight into mechanisms common to most living organisms. In any event the reactions which already claim an ATP involvement are formidable. Numerous workers now presume that the

breakdown of ATP provides the source of energy for muscular contraction (*23*, *37*, *55*). Apparently the adenylic acid system is further involved in lactic acid formation (*67*, p.394), the phosphorylation of thiamine (*44*, *76*), the phosphorylation of carboxyl groups with the subsequent easing of their reduction (*42*), the formation of peptide bonds (*18*), fatty-acid oxidation (*40*), fatty-acid synthesis (*65*), action of growth substances (*8*, p.459), amide formation (*64*), and sucrose synthesis (*8*, p.40). It is evident that the

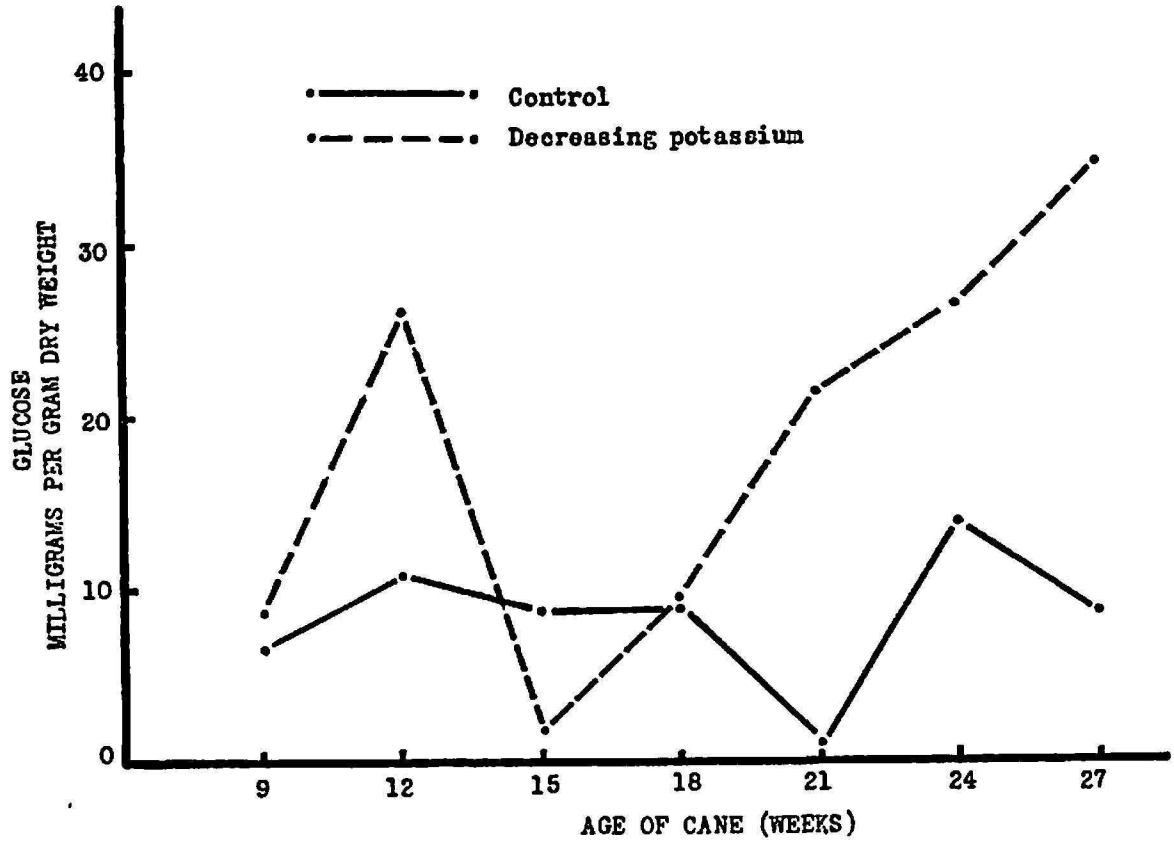


FIG. 14.—Influence of decreasing potassium supply on the glucose content of leaves from immature sugarcane.

importance of the adenylic acid system cannot be overemphasized, and that this area in itself deserves intensive study in sugarcane.

Supply of Sucrose Biosynthesis Metabolites

Leloir and coworkers (*16*, *41*, *54*) have developed several enzyme reactions involving uridine compounds and reducing sugars which account for the biosynthesis of sucrose. These reactions can be summarized as follows:

1. Uridine triphosphate (UTP) + glucose-1-phosphate (G-1-P) → uridine diphosphate glucose (UDPG) + pyrophosphate (PP)
2. A, UDPG + fructose → sucrose + uridine diphosphate (UDP)

   B, UDPG + fructose-6-phosphate (F-6-P) → sucrose phosphate + UDP

Our own studies have dealt with six enzymes which could possibly shift the equilibrium of these reactions and hence affect sucrose production. These enzymes include hexokinase, phosphohexose isomerase, and each of the phosphatases which attack UDP, the glucose phosphates, and fructose-6-phosphate. It should also be remembered that ATP-ase could enter the scheme via its ability to regulate the supply of ATP for the phosphorylation of glucose, and as a source of energy.

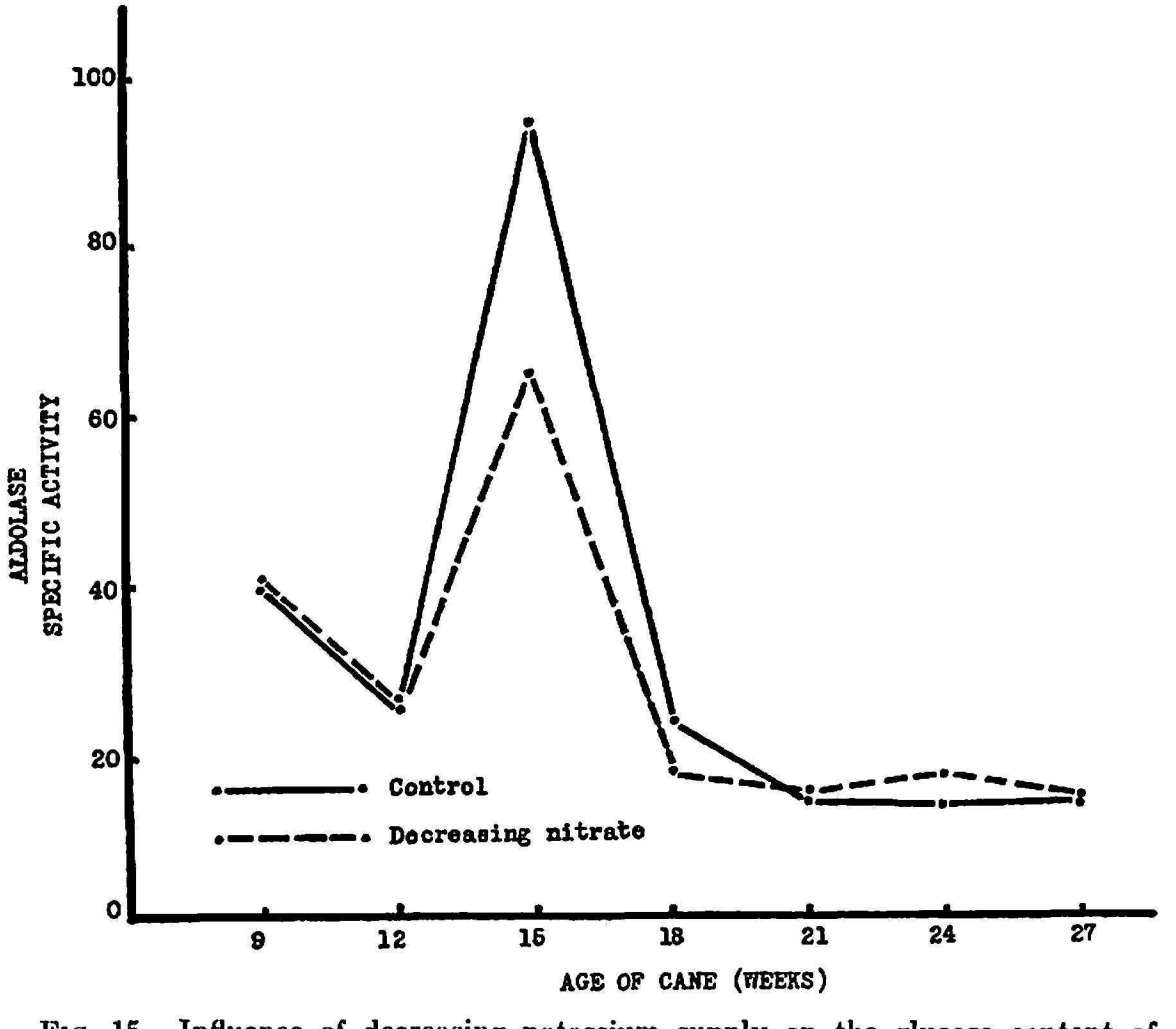


FIG. 15.—Influence of decreasing potassium supply on the glucose content of sheaths from immature sugarcane.

Although for some time we suspected that the enzyme hexokinase would have played a key role in affecting sucrose synthesis, we now feel that the most probable influence upon sucrose biosynthesis was effected through the glucose-1- and glucose-6-phosphate hydrolyzing systems. The hexokinase of our preparations required glucose as the specific acceptor of phosphate from ATP. Neither fructose, fructose-6-phosphate, nor galactose served in this capacity. In the absence of any other hexokinase this enzyme assumed considerable importance as the lone apparent means for providing the phosphorylated glucose needed for sucrose production.

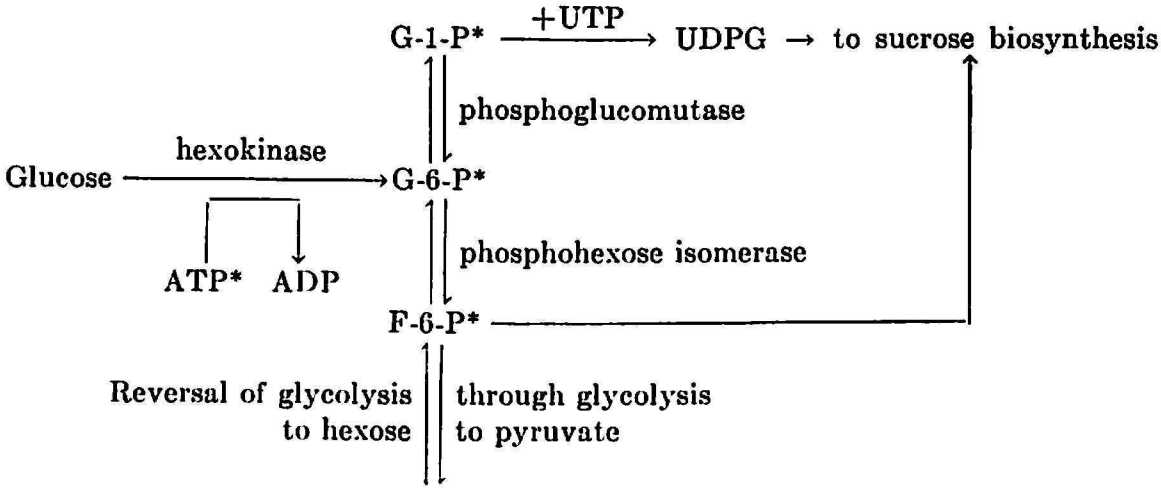
It will be recalled that the hexokinase reaction was significantly altered by the decreasing potassium treatment which brought about an overall increase in the enzyme's activity. However, decreasing potassium itself had no great influence upon sucrose level, while a very definite relationship existed between increased sucrose and the suppression of phosphatase by low-nitrate treatments. Therefore we feel that, while hexokinase theoretically could have promoted sucrose synthesis among low-potassium treatments through an increased supply of glucose-6-phosphate, the failure of sucrose to increase by this treatment suggests that the supply of glucose phosphate was still being limited by some other system, and that the latter system was partly eliminated by the low-nitrate treatment. Consequently, the nitrate-induced suppression of the glucose phosphatases seems to be the critical step which was needed for the most optimum production of sucrose. Bearing in mind our primary contention that the phosphatase effect upon sucrose could have been achieved through a suppression of ATP-ase, it is now apparent that a second critical reaction involved the suppression of the glucose phosphatases, and, subsequently, a more favorable supply of glucose phosphate must have been available to those plants receiving low nitrate.

Further evidence favoring the critical nature of the glucose phosphates is suggested by the lack of a direct source of fructose-6-phosphate (the second eligible hexose phosphate for the Leloir equations), and the high specificity of the phosphatase suppression for the glucose phosphates. In the absence of fructokinase the possiblity of fructose-6-phosphate entering reaction II-B seems unlikely, unless the compound entered via the hexose isomerase reaction, and this, in turn, would be dependent upon an initial source of glucose-6-phosphate.[11] Again, the relative specificity of phosphatase suppression for the glucose phosphates, as compared to the fructose phosphates, implies that this was a primary area of the low-nitrate effect.

Taking into account the sucrose-synthesizing reactions of Leloir and coworkers, coupled with the above suggestions as to the sucrose-affecting roles of phosphatase, we may add to our diagram the areas in which hexokinase and glucose phosphatase may have affected sucrose production among low-nitrate treatments.

In the formula below (p. 213) * indicates compounds known to be hydrolyzed by cane-leaf phosphatase:

[11] The source of fructose required by the sucrose biosynthesis-reactions is not clearly understood. However, fructose does not need to be phosphorylated to take part in these reactions and therefore the isomerase reaction discussed above would be essential for the provision of fructose only in the absence of an active invertase, which would provide free fructose, or in the absence of a photosynthetic source which theoretically could supply both the free and phosphorylated forms.



Further Significance of Sugarcane Phosphatases

Before leaving the subject of cane-leaf phosphatases it is well to place these enzymes in a more general perspective. It was stated earlier that phosphatase activity was not confined to ATP and glucose phosphates alone—indeed, every organic phosphate employed as substrate was subject to hydrolysis. This universal action should be borne in mind while planning studies in related areas of sugarcane physiology.

A case in point is the failure of several workers to detect intermediate phosphates, such as phosphoglyceric acid, during studies of the photosynthetic mechanisms of sugarcane (*14*, *15*). Dr. Calvin made the following comment on this point in a letter to Dr. J. A. Bonnet[12] dated August 14, 1961:

> The failure of a number of workers to catch the intermediate phosphate seems to me to be due to the presence of a highly active phosphatase enzyme system which resists the destruction by either temperature or solvents (or both) long enough to hydrolyze the very small pools of intermediate organic phosphates leading to the free sugars and sugar acids .....

The acid phosphatases which we have encountered during our studies would serve this function admirably. As mentioned earlier, both 2-phosphoglyceric and 3-phosphoglyceric acid were strongly attacked.

Until now we have discussed the phosphatases of sugarcane as if they were generally harmful, and a suppression of their activity must be basically good. In all fairness we must realize that either an excess or extreme suppression of their activity would be harmful to carbohydrate metabolism. It is evident that, if sugars are to be broken down to pyruvic acid, or a reversal of the glycolytic system is to take place for the production

[12] Soil Scientist and Head, Department of Soils, Agricultural Experiment Station, University of Puerto Rico, Río Piedras, P.R.

of hexoses from smaller units, some provision must be made for the liberation of phosphate from the organic intermediates after they have achieved their points of progression in the phosphorylated state. Phosphatases undoubtedly play an important role here. A pertinent example is cited by the work of Buchanan (*12*) in which phosphatase was found to take part in the production of sucrose from the sucrose phosphate of the sugar beet.

Amylase: Starch vs. Sucrose Metabolism

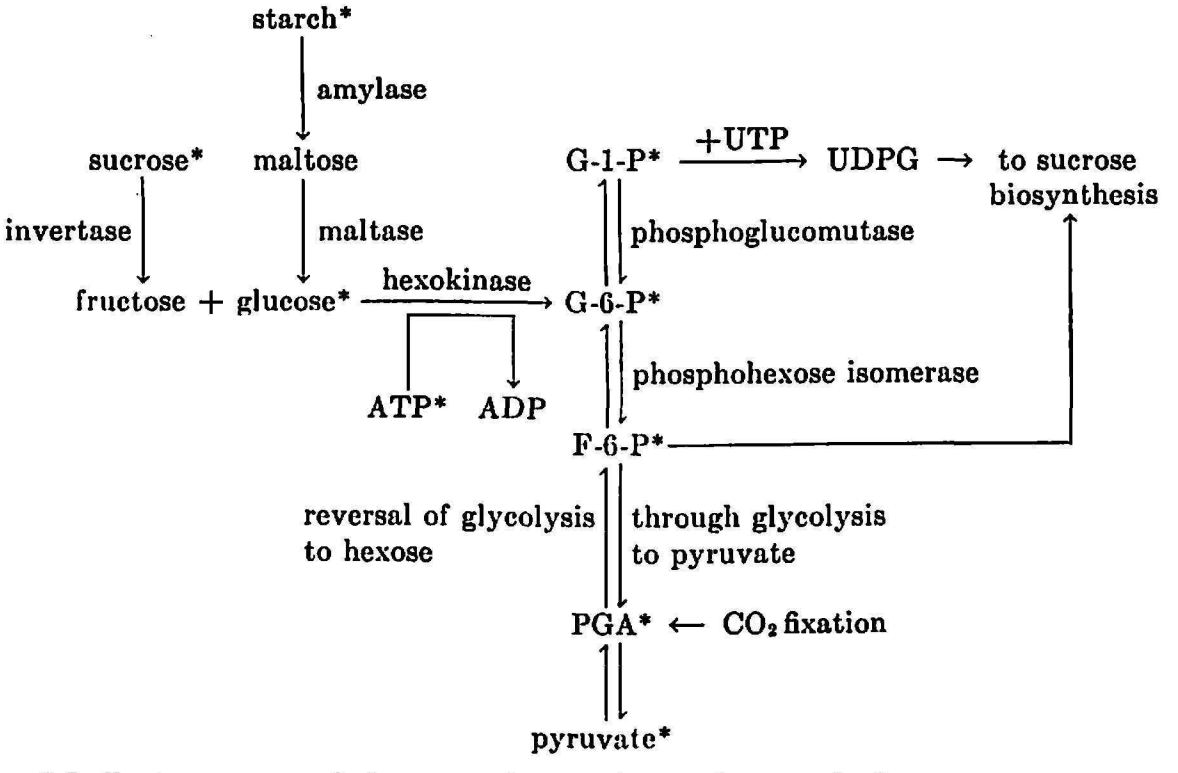
The striking discrepancy between amylase and invertase potential in the leaf extracts is difficult to explain for a plant the greatest claim to fame of which is its ability to produce sucrose. We feel that, insofar as starch may be regarded as a temporary storage form of glucose in sugarcane, the amylase activity is at least justified. On the other hand, the weak invertase activity simply adds support to the thesis that this enzyme had little importance, if any at all, in sucrose metabolism.

It seems highly probable that starch is a natural source of glucose for both the glycolytic and sucrose-synthesizing pathways in sugarcane. We feel that the strong amylase activity encountered during these studies consists of both α and β types acting to form the disaccharide maltose, which, in turn, must be broken down to free glucose by maltase. No attempt has yet been made, however, to demonstrate maltase activity in the M. 336 variety.

The coupling of sucrose synthesis with the CO_2 fixation pathway, as exemplified by the work of Buchanan (*12*), Burma (*13*), and Basham (*2*), appears to hold true in sugarcane (*34*, *35*, *49*, *52*, *56*). It seems unlikely, however, that any plant should need a fresh photosynthetic source of sugars, from the standpoint of growth and development requirements, after it has accumulated several percent of its own weight in carbohydrate. Some mechanism for the reemployment of glucose from polysaccharides must logically exist, and whenever such a system is established the enzyme amylase will likely assume major importance. Thus, assuming the possibility of glucose supply via starch degradation, we complete our proposed outline with the addition of starch-metabolizing enzymes (see diagram p. 215).

The invertase reaction is included because of its theoretical potential rather than for any data we have. Hartt (*33*) claims evidence that sucrose is formed in sugarcane through the action of invertase, but this view is not widely held. The reaction equilibrium for invertase apparently lies too far toward hydrolysis to permit appreciable sucrose formation (*8*,p.40), and our own preparations possessed too little activity to be of consequence.

The relatively low levels of glucose in leaves of the low-nitrate group, as compared to the low-potassium group (table 4), brings our attention to the suppression of amylase by the decreasing-nitrate treatment (table 7, item 12). It is evident that following the 9-week harvest the action of amylase



* Indicates compounds known to be acted upon by cane-leaf enzymes.

was generally greater among the low-potassium plants than those receiving low nitrate. If in fact glucose is being released for metabolism via a starch-degradating system, then the decline of glucose among the low-nitrate group could have been due to the suppression of amylase by this treatment.

Whereas the enzyme amylase seems to have important potential in glucose metabolism, further consideration of our data indicate that this system was not a limiting one either in the supply of glucose for glycolysis or for sucrose biosynthesis. From table 7 (item 12) we see that even though amylase was more active in the low-potassium plants than in the low-nitrate group, the low-potassium values themselves were generally below those recorded for the control plants, and the control group did not contain as much glucose as did the plants receiving decreasing potassium (table 4). Furthermore, the outstanding rise in amylase activity among control p'ants at 21 weeks was not accompanied by a corresponding increase of glucose.[13]

If starch is a source of glucose for sucrose synthesis, and if low nitrate is favorable for sucrose production, why then is amylase suppressed by low nitrate? If amylase is instrumental in supplying glucose, then any treatment

[13] The effect of increased amylase at this period is a good example of what may happen when the level of only one of two critical sugars is increased. The sharp decline of fructose among control plants, while glucose failed to increase, leads us to suspect that some of the anticipated excess glucose was combining with a more limited supply of fructose for sucrose production.

suppressing the enzyme should likewise limit sucrose production. The obvious answer here lies in the vast work capability of the amylase system. We repeat out contention that low nitrate promoted sucrose synthesis by retarding the acid phosphatases, but it should be remembered that the phosphatases can attack critical intermediate phosphates which may never exist in quantities greater than a few micrograms per gram of leaf tissue. Enzyme activity here can be highly critical, and equally so are treatments which curtail or promote such activity. On the other hand, the amylase reaction produced not micrograms of maltose, but milligrams, a great many milligrams as a matter of fact. In our test tubes where substrate supplies were far more optimum than could be expected in living tissues, the total phosphate released by all glucose-6-phosphatase reactions measured during the study came to 19.6 mg., equivalent to 44.1 mg. of free glucose, whereas the total maltose product of the amylase reaction exceeded 2,200 mg. Under such circumstances the reduction of amylase by one-half would still undoubtedly leave ample supplies of maltose for the glucose-releasing sequence.

From the processor's standpoint excessive starch is undesirable in any cane variety. Both the filtration and crystallization rates of cane juices are retarded by starch (*4*, *25*) and the classical example of this was the Uba variety of South Africa (*77*). Hartt (*36*) found that amylase was less active in the meristematic tissue of the Uba variety than other varieties tested. Boyes (*10*) investigated enzyme hydrolysis as a means of removing excess starch from cane juices in the factory.

It has been reported that field deficiencies of potassium and nitrogen lead to starch accumulation in cane, and that application of these nutrients to the soil generally brings about a starch decrease whenever there follows an increased yield of cane (*77*). From the standpoint of enzymes it is tempting to speculate that the low-nitrogen effect is due to a suppression of amylase, and that potassium acts by removing a low-potassium suppression of glycolytic enzymes. However, the direct application of sand-culture data to the findings of field studies is obviously hazardous.

Potassium-Induced Glucose Accumulation

Another question that has not been resolved concerns the mechanism responsible for glucose accumulation among low-potassium plants, an effect which obviously cannot be accounted for by the amylase reaction previously discussed. It has already been pointed out that decreasing potassium stimulated the hexokinase reaction (the formation of glucose-6-phosphate from hexose plus ATP), but that sucrose did not accumulate excessively as a result of the potassium treatment. Hence from this information we could only assume that a large share of the glucose phosphate was passing away through the glycolytic pathway toward the ultimate produc-

tion of pyruvate, or that glucose was being immobilized by glucose phosphatase. However, our tissue analyses showed that total reducing sugars were, in fact, accumulating in response to decreasing potassium, and that glucose accounted for most of the increase. This was true of both leaf and sheath tissue with the sugar accumulation being most pronounced toward the end of the study (figs. 13 and 14).

A review of the glycolytic enzymes which we had measured showed that aside from hexokinase, two systems had been altered by potassium treatment. In each instance the anticipated consequence was a curtailment of hexose degradation at some point prior to the formation of 1,3-diphospho-D-glycerate. The first enzyme, phosphohexose isomerase, catalyzes the following reaction:

Glucose-6-phosphate (70 percent)
⇅
Fructose-6-phosphate (30 percent)

Ordinarily the glycolytic pathway would provide a continuous drain of the fructose phosphate, in spite of the enzymatic equilibrium favoring glucose. The enzyme was suppressed strongly at 15, 18, 21, and 27 weeks (fig. 10).

The second enzyme, phosphoglyceryl kinase, catalyzes the following phosphate-transfering sequence:

1,3-diphosphoryl-D-glycerate
Mg^{++} ⇓ → ADP, → ATP
3-phosphoryl-D-glycerate

Decreasing potassium suppressed this enzyme at 12, 15, 18, and 24 weeks (fig. 12) and must temporarily have caused a bottleneck against hexose degradation. We find that enzyme activity was suppressed as early as 12 weeks (fig. 12), although major glucose accumulations appeared at 24 weeks and later (table 4). Such a timelag could be accounted for in many ways, including an alternate pathway or shunt mechanism handling initial glucose accumulations, the temporary conversion of hexose to undetectable triose or glyceraldehyde forms, or the storage of initial glucose accumulations in the form of starch.

ADDITIONAL ENZYME RESPONSES TO VARYING LEVELS OF NITRATE AND POTASSIUM

The protein nature of enzymes, with all the physical and chemical properties this entails, renders them sensitive to many kinds of experimental treatments, in which varying nutrient supply plays no small role. These experiments were basically set up as nutrition studies, even though sugar-

cane nutrition was not in itself our primary area of interest. Consequently the enzyme analyses have provided a kind of information not ordinarily available in nutrition studies, and which needs to be reviewed both in the light of possible indirect relationships with carbohydrate metabolism, and from the standpoint of its contribution to the understanding of sugarcane nutrition.

Having discussed nitrate and potassium effects upon enzymes directly involved in sugar metabolism, the next logical system for review is peroxidase. This enzyme was suppressed by decreasing nitrate, it exhibited an inverse relationship with sucrose content (figs. 8 and 9), and it has no established role either in the breakdown or synthesis of sugars. It is evident that whatever factors were at work to inhibit sucrose formation among control plants at 15 weeks, these must also have been favorable for high peroxidase activity (fig. 8). Furthermore, the factors which lessened this inhibition at 15 weeks and favored subsequent sucrose formation, under conditions of decreasing nitrate supply, likewise favored the suppression of peroxidase (fig. 9).

Fruton and Simmonds proposed that plant peroxidase catalyzes the oxidation of metabolites by means of H_2O_2 formed in the reaction of reduced flavins with oxygen (*28*, p.364). In the presence of H_2O_2 plant peroxidases are known to catalyze the oxidation of an indefinite number of phenols and aromatic amines (*67*, p.221). The best proposal we can put forward as to the function of the peroxidase recorded during these studies is that, through the oxidation of certain phenols, it may serve as a terminal oxidase in cane respiration. Whereas cytochrome oxidase or polyphenol oxidase is usually implicated in this role (*19*, *30*, *63*), the employment of peroxidase by sugarcane would clearly explain the excessive enzyme action when sucrose was low (equivalent to rapid glycolysis), and severe depression of peroxidase when sucrose was high (equivalent to suppression or reversal of glycolysis). In other words, this would support our earlier suggestion that hexoses, and subsequently sucrose, were accumulating during these experiments via a reversal of the glycolytic pathway. It should also be pointed out that cytochrome-C oxidase was not detected in our preparations, thus exemplifying a need for some alternate mechanism, such as peroxidase, to provide for terminal oxidation.

From a strictly nutritional standpoint the suppression of peroxidase by decreasing nitrate has provided a type of information which could be very useful to cane growers. The suppression of peroxidase was easily measurable at 12 weeks, following the initial nitrate decline from 10.0 to 8.5 meq./l., while nitrogen-deficiency symptoms were not visible until after 24 weeks. Thus we have a physiological condition, wholly a characteristic part of the low nitrate physiology, which can be measured 84 days prior to any visible

evidence of nitrogen starvation. The author has made considerable effort in the past to demonstrate that certain enzyme systems can be employed to detect approaching nutrient deficiencies in economic plants, and that this detection can be realized many weeks in advance of that made by present tissue-analysis techniques and by diagnosis of visual symptoms. This type of analysis would have the additional advantage of measuring only that fraction of a nutrient concentration which is actually taking part in biochemical reactions, and would automatically tell something of the physiological area in which a nutritional disorder is developing. The peroxidase reaction herein reported is an example of this principle.

Triose phosphate dehydrogenase, isocitric acid dehydrogenase, and cytochrome-C reductase each exemplified a need for a more thorough investigation of nitrogen-enzyme relationships. Figure 6 illustrates the fact that an increase of nitrate supply by as little as 2.5 meq./l., following the 9-week harvest, caused a loss of 75 percent of the triose phosphate dehydrogenase activity. Further 2.5 meq./l. increments caused no appreciable enzyme change until 24 weeks when an increase from 20.0 to 22.5 meq./l. caused a fourfold rise in activity. Similar responses to increasing nitrate were recorded for isocitric acid dehydrogenase (table 7, item 16), although the enzyme changes were of lesser magnitude.

When setting up the variable nitrate experiments we considered that an adequate nitrate supply lay in the range of 6 to 10 meq./l., and that any level above 10 meq./l. would provide for luxury consumption. Ou r dry-weight data indicate that this was so, but even if we had been mistaken, the fact remains that within an area of "high" nitrate supply there resides a nitrate capacity completely to transmute the action level of a critical enzyme. Furthermore, whatever causes we put forward to account for one of the nitrate-dehydrogenase relationships, some other factor has to be found to explain the reverse response.

We again emphasize that this is not the type of nitrogen-enzyme relationship observed with peroxidase, where a nitrate effect once established held true indefinitely. It is rather a highly sensitive, fluctuating relationship whose ultimate resolution would require a series of gradual treatment increments, each with its own reference group, maintained in closely controlled sand or solution culture. In soils the critical nutrient-enzyme levels would be far more elusive, while the introduction of other nutrient variables to interact with nitrogen under field conditions would provide grounds for a completely new project. For reasons such as this we sometimes feel that the study of nitrogen nutrition among economic crops, so often felt to be virtually exhausted, is in reality scarcely touched.

The stimulation of cytochrome-C reductase by decreasing nitrate (fig. 7) presents a nutrient-enzyme relationship intermediate between those of

peroxidase and dehydrogenase discussed above. Like peroxidase, the cytochrome-C reductase level can go but one way in response to decreasing nitrate, and in this case it is upward. But, as was true of dehydrogenase, this nitrate-induced response appears highly unstable, and the enzyme activity measured at one period leaves us with no basis whatsoever for predicting the enzyme action at a later date.

The accepted role of cytochrome-C reductase is to catalyze the transfer of electrons from reduced pyridine nucleotides to cytochrome C (*28*, p.341). It is a diaphorase in that it employs reduced TPN or DPN as its substrate, and at the same time is rapidly reoxidized by cytochrome C (*8*, p.178). It is not appreciably reoxidized by molecular oxygen. Since the initial oxidation of many metabolites involves hydrogen transfer to DPN^+ or TPN^+, the enzymes which effect rapid transfer of electrons from DPNH and TPNH to oxidized cytochrome C thus assume great potential importance.

As of this moment we have insufficient information about the cytochrome-C reductase of sugarcane adequately to discuss its significance. Nevertheless our attention is drawn to the fact that, throughout the study, this enzyme generally increased activity while most of the others declined, and that it is apparently susceptible to stimulation by the decreasing nitrate treatment which almost invariably suppressed other nitrate-sensitive enzymes. We must also take into account the possibility that a strong cytochrome-C-reductase activity in sugarcane could simply be masking the action of cytochrome-C oxidase, *i.e.*, instantaneously resupplying the oxidized cytochrome C with electrons. It is not possible at this time to know what effect the reductase system would have on the equilibrium constant of a "normal" cytochrome-C oxidase reaction. It can only be said that an apparently strong alternate mechanism, the peroxidase system, is present to catalyze the terminal oxidation reactions should cytochrome-C oxidase by lacking or in any way curtailed.

The suppression of condensing enzyme by decreasing nitrate and potassium again represents two distinct kinds of nutrient-enzyme relationships (fig. 11). The nitrate effect, which was readily apparent by 15 weeks and continued throughout the remainder of the study, belongs in the same category with the peroxidase-nitrate relationship. The potassium effect did not appear until 18 weeks, or, in other words, until the potassium supply had been reduced from 6 to 2 meq./l. Further lowering of potassium from 2 to 1 meq./l. suppressed the enzyme activity to only 22 percent of controls, an effect far more severe than that induced by low nitrate. This is one of the few examples of a typical enzyme-activator[14] role revealed

[14] The term "activator" here refers to any ionic substance capable of enhancing enzyme activity without actually being required by the enzyme.

during the study. We have no evidence to show that potassium is required for condensing enzyme activity, but it is evident that under the conditions of these experiments normal activity levels require potassium supplies of at least 2 meq./l.

Condensing enzyme initiates the tricarboxylic acid cycle in cellular metabolism by catalyzing the formation of citric acid. It also makes possible the donation of acetyl groups, by citrate, toward many other reactions (*67*, p.353). The important role of the enzyme, plus the fact that it is sensitive to varying nitrate and potassium supply in sugarcane, sets this system apart as another for which we shall be seeking additional information in the future.

PHYSIOLOGICAL BASIS OF NITRATE-ENZYME RELATIONSHIPS

For convenience we have been speaking of nitrate-enzyme relationships as if the nitrate anion itself were directly suppressing or stimulating a given enzyme. Obviously this is an oversimplification and, although nitrate metabolism is beyond the range of our present work, a few suggestions can be put forward to help account for the nitrate-enzyme relationships observed. The easiest way to explain the enzyme suppression by low nitrate, as for example the curtailment of the acid phosphatases and amylase, is to conclude that there was insufficient nitrogen available for normal synthesis of enzyme protein. However, our analyses did not reveal any apparent effect of treatments upon protein content.

Another consideration is the energy requirement of the nitrate reduction sequence. It has been pointed out that the conversion of nitrate to nitrite, hydroxylamine, and ammonia are energy-requiring processes which must be coupled to carbohydrate breakdown (*28*, p.680). This was clearly demonstrated by Hamner (*31*) who found that the application of nitrate to nitrogen-starved tomato plants led to the formation of nitrite, the depletion of carbohydrate reserves, and a marked increase in respiration. From the standpoint of enzymes involved in carbohydrate metabolism, it thus appears that a low-nitrate status would favor weak enzyme activity coupled with carbohydrate accumulation, which agrees well with out own data. In this sense, however, the sucrose accumulation recorded among low-nitrate treatments could only be reviewed as an absence of an active carbohydrate metabolism needed for the conversion of nitrate, rather than a direct influence by low nitrate upon an enzyme. In other words it is possible that during these studies the amylase and phosphatase systems were not actually suppressed when nitrate was withheld, but rather there was no longer a physiological cause for their "normal" activity. A point which perhaps has been unjustly overlooked in this report, but is surely exempli-

fied by the work of Hamner, is the fact that a plant receiving new nitrate supplies above some critical minimum has no apparent choice but to vastly increase a series of far-reaching physiological reactions.

The physiological importance of nitrate as the anion, and not simply as a source of nitrogen, was pointed out by the work of Vickery (*73*), who found that the organic-acid content of tomato and tobacco was many times greater when nitrogen was supplied as nitrate rather than as ammonia. The acids citric and malic, each of which is an essential component of the TCA cycle, were particularly high as a consequence of nitrate being the only nitrogen source. Since these organic acids in plants are derived through carbohydrate metabolism, this again suggests, as did the work of Hamner, that a close relationship exists between the processes of nitrate reduction and the stimulation of sugar breakdown.

It seems likely that this relationship requires hexose degradation both for the supply of energy needed for nitrate reduction, and as a source of building units needed for organic-acid synthesis. In sugarcane this can be accounted for first by the relatively high phosphatase activity at high-nitrate levels which could discourage the accumulation of hexose phosphates needed for maximum sucrose synthesis, and secondly by maintaining a more balanced ATP-ase reaction needed so that the phosphorylation of glucose (ATP-requiring) and the phosphorylation of glyceraldehyde-3-phosphate (inorganic-phosphate-requiring) should continue at an equilibrium near unity. The logical consequence of these high-nitrate effects would include more rapid glycolysis with the increased provision of ATP and building material for organic acid synthesis.

To a cane grower interested in sucrose production the latter situation might not seem very desirable. Yet from the standpoint of the plant itself this is more likely a desirable condition than that already discussed for low-nitrate levels, where sucrose apparently accumulates in the absence of normal phosphatase action against the phosphorylated building units and against the ATP reserves.

A more direct effect of nitrate upon enzyme systems can be accounted for by its ability to take part in electron transfer. Very likely it is this characteristic of nitrate which facilitated the suppression of peroxidase, and which would assume considerable importance in any terminal oxidation reactions involving the peroxidase system. In organisms containing nitrate reductase, nitrate can serve as the terminal electron acceptor during the oxidation of succinic acid, lactic acid, and formic acid under anaerobic conditions (*28*, p.680). In photosynthetic reactions the reduction of nitrate is promoted by illumination, and nitrate can serve as an alternate electron acceptor (*24*). According to Taniguchi (*70*), nitrate participates in electron

transfer during the oxidation of metabolites by numerous organisms, and this transfer is accompanied by the formation of ATP.

Nitrate metabolism in sugarcane lies beyond the scope of our own work and cannot be discussed further here. However, if this area were to be investigated with sugarcane as a test plant, the interrelationships of nitrate metabolism with terminal oxidation and with the adenylic acid system would be extremely promising points of departure.

BASIS FOR FUTURE SUGAR-ENZYME STUDIES WITH SUGARCANE

There is much to be said both for and against a broad, screening-type enzyme study such as the one here completed, but experiments cannot be continued on this scale if the most needed information is to be gathered. It is only fair to point out that some of our most promising results (*i.e.*, the peroxidase, amylase, hexokinase, and cytochrome-C-reductase data) could not have been achieved had we confined our work to enzymes directly involved in sucrose metabolism. And yet the wealth of new questions left suspended by a broad approach begins to channel attention to specific areas which must be clarified. For example, was the ATP-ase system encountered here strictly a hydrolyzing enzyme or is it involved in phosphate transfer between ATP and "pool" phosphates? How many acid phosphatases are at work in cane leaves, and what are their distinguishing properties? Was invertase actually weak in the M. 336 variety or did we fail to extract the system properly? Is there in fact a maltase system working in conjunction with amylase to provide both a source and a storage mechanism for glucose? Is an α or β amylase responsible for the very considerable starch hydrolysis observed? What specifically comprises the terminal oxidation mechanism in cane, and does peroxidase actually play an important part here? These and many other questions are brought to the fore, but not answered, by ground-breaking studies of this type.

Once the promising points of departure are uncovered, the ultimate approach to be taken is dictated by the kind of information most needed. If, for example, the nitrate-induced enzyme changes are characteristic of variable nitrate in sand culture only, then this information is of little value to us. If these are characteristic responses to nitrogen variables in general, they are of more value. If these enzyme changes are characteristic of any cane approaching maturity, and were simply hastened by one of any number of treatments which could have done the same, then the information can be extremely useful. The ultimate objective therefore should be the gathering of information characteristic of a physiological state, such as maturity *vs.* immaturity, or a cane under stress *vs.* the normal condition. Only by taking this approach can we eventually hope to isolate key mecha-

nisms in sugarcane which are regulating its behavior, and to avoid the traditional near-misses which follow whenever basic causes are assigned to any one of a long series of triggering treatments.

As a parting comment on the future basis of nutrient-enzyme studies in sugarcane we would make the following observation: From the standpoint of both basic and applied sugarcane research there is a crying need for a review of the main effects and interactions of the macronutrients under precisely controlled conditions. From the very beginning our own work was hampered by an almost complete lack of data, in terms of milliequivalents per liter, as to where our levels should be set and in what proportions in order to duplicate nutritional responses observed in the field. In this respect our own efforts were only partly successful. This type of work should be done in sand or water culture, with experimental designs capable of bringing out first and second order interactions of all macronutrients and in conjunction with thorough sugar analysis of the cane tissues. Such information would add greatly to the general understanding of nutrition responses in the field, and without doubt would provide more basic studies with promising points of departure.

SUMMARY AND CONCLUSIONS

Immature sugarcane plants grown in sand culture were subjected to conditions of potassium and nitrate stress in order that abnormal carbohydrate levels would be induced. The objective was to learn what areas of sugar metabolism were involved in the degradation of sucrose. The methods centered upon leaf enzymes, of which the following were identified and measured: Amylase, invertase, hexokinase, acid phosphatases, phosphorylase, phosphohexose isomerase, aldolase, triose phosphate dehydrogenase, phosphoglyceryl kinase, pyruvic carboxylase, condensing enzyme, α-ketoglutaric carboxylase, isocitric dehydrogenase, cytochrome-C reductase, fumarase, transaminase, oxalacetic carboxylase, peroxidase, catalase, and polyphenol oxidase. The enzyme preparations consisted of dialyzed water-soluble protein extracted from freeze-dried leaf tissue and precipitated with ammonium sulfate between 32- and 95-percent saturation.

Sugar determinations for leaf and sheath tissue included total ketoses, sucrose, fructose, total reducing sugars, and glucose. Sheath-percent-moisture, total dry weight, and leaf protein were also measured. Treatments were applied from 9 weeks to 27 weeks of age and a total of seven harvests were made at 21-day intervals.

The following results were obtained:

1. Highest leaf sucrose content was found among plants supplied with decreasing nitrate. Decreasing potassium resulted in glucose accumulation

when potassium was lowered from 3 to 0 meq./l. Sugar content was generally greater among sheath tissues than leaf tissues.

2. Sheath-percent-moisture declined most readily under low-nitrate treatments.

3. Leaf protein gradually increased throughout the study and was unaffected by treatments.

4. Enzyme activity was generally highest at 9 weeks and declined as the plants became older. The physiological age of the plants was more critical than treatments in determining the shape of enzyme activity curves. Treatment effects were generally confined to altering the magnitude of enzyme action rather than formulating the shape of an enzyme curve.

5. A number of acid phosphatases were encountered which hydrolyzed all organic phosphates tested. These compounds included nearly all phosphorylated intermediates of the glycolytic pathway, plus ATP, β-glycerophosphate, UDP, and 6-phosphogluconate. The phosphatases exhibited a pH optimum near 4.6 and were readily inhibited by fluoride.

6. Phosphatases were generally curtailed by decreasing nitrate. This effect was most pronounced against ATP-ase and the glucose phosphatases.

7. The enzyme preparations exhibited a powerful amylase action which, on the basis of reducing sugar formed, exceeded invertase by as much as ninetyfold. Amylase was suppressed by the decreasing nitrate treatment.

8. A relationship appeared to exist between the increase of sucrose and the suppression of phosphatase among low-nitrate treatments. The suspected mechanisms are discussed in detail, particularly the role of ATP-ase and the glucose phosphate hydrolyzing systems.

9. Decreasing potassium stimulated hexokinase and retarded both phosphohexose isomerase and phosphoglyceryl kinase. The increased glucose in low-potassium plants is accounted for by the suppression of the latter two systems.

10. Decreasing potassium exhibited a variable influence on invertase, causing a suppression of the enzyme at 4 meq./l. and stimulating it at 1 meq./l.

11. Glucose was the only detected hexose acceptor of phosphate from ATP in the leaf preparations. Other hexoses tested were fructose, fructose-6-phosphate, and galactose.

12. An inverse relationship was found between sucrose and fructose levels, especially among the decreasing nitrate plants in which fructose virtually disappeared.

13. Glucose-6-phosphate dehydrogenase could not be detected in the enzyme preparations, hence the existence of the hexose monophosphate shunt system in cane leaves is doubtful.

14. A definite inverse relationship was found between peroxidase activity and sucrose content. Peroxidase was inhibited strongly by decreasing nitrate. The possibility that peroxidase serves in terminal oxidation is discussed.

15. Cytochrome-C oxidase could not be detected in the leaf extracts, although a strong cytochrome-C reductase system was encountered which was stimulated by reduced TPN. Cytochrome-C reductase was particularly sensitive to increased nitrate supply.

16. Condensing enzyme was suppressed moderately over a broad range of decreasing nitrate levels. Decreasing potassium severely depressed the enzyme in the range of 2 to 1 meq./l. potassium, suggesting that at certain levels potassium may serve as an activator of the condensing enzyme.

17. A number of additional nutrient-enzyme relationships were encountered and these are discussed in relation to their value to growers and physiologists.

18. Indirect evidence suggests that starch serves as a source of glucose for the glycolytic pathway and sucrose synthesis in cane leaves. Amylase and maltase would be essential components of such a system. The low magnitude of invertase activity renders this enzyme of little consequence either in catalyzing the supply of reducing sugars, or in sucrose synthesis.

19. A diagram is presented to account for the phosphatase-amylase-nitrate interrelationships as they may affect sucrose production.

RESUMEN Y CONCLUSIONES

Plantas inmaduras de caña de azúcar sembradas en arena se sometieron a un tratamiento excesivo de potasio y nitrato, con el propósito de inducir niveles anormales de hidratos de carbono. El objetivo era descubrir qué fases del metabolismo del azúcar estaban relacionadas con la reducción de la sacarosa. Los m´todos se centraron sobre las enzimas foliares, de los cuales los siguientes se identificaron y midieron: amilasa, invertasa, hexoquinasa, fosfatasas ácidas, fosforilasa, fosfohexosa, isomerasa, aldolasa, dehidrogenasa trio-fosfatada, quinasa fosfoglicerada, carboxilasa pirúvica, enzima para condensar, carboxilasa alfaquetoglutárica, dehidrogenasa isocítrica, reductasa, citocroma C, fumarasa, transaminasa, carboxilasa oxalacética, peroxidasa, catalasa y oxidasa polifenólica. Las preparaciones enzimáticas consistieron de proteína dializada, soluble en agua, extraída de tejido foliar seco-congelado y precipitado con sulfato amónico a una saturación entre 32 y 95 por ciento.

Los ensayos para las determinaciones de azúcares en el tejido de la hoja y la yagua incluyeron las quetosas totales, sacarosa, fructosa, azúcares reductores totales y glucosa. También se midió el porcentaje de humedad en la yagua, su peso total cuando seca, y el contenido de proteína en la

hoja. Se aplicaron tratamientos a cañas de 9 hasta 27 semanas de edad y se hicieron siete cosechas a intervalos de 21 días.

Se obtuvieron los siguientes resultados:

1. Se encontró el contenido más alto de sacarosa en las plantas a las que se les suministró nitrato reducido. Al reducirse el potasio se produjo una acumulación de glucosa cuando el potasio se redujo de 3 a 0 meq./l. El contenido de azúcar fue generalmente mayor en los tejidos de la yagua que en los foliares.

2. El porcentaje de humedad en la yagua se redujo más rápidamente en las cañas con tratamientos bajos en nitrato.

3. El contenido de proteína de la hoja aumentó gradualmente mientras duró el estudio y no se afectó por los tratamientos.

4. La actividad enzimática fue generalmente más alta a las 9 semanas y se redujo a medida que las cañas envejecían. La edad fisiológica de las plantas fué factor más decisivo que los tratamientos en determinar la forma de las curvas de la actividad enzimática. El efecto de los tratamientos se limitó generalmente a alterar la magnitud de la acción enzimática antes que la forma de la curva enzimática.

5. Se encontró un número de fosfatasas ácidas que hidrolizaron todos los fosfatos orgánicos probados. Estos compuestos incluyeron casi todos los intermediarios fosforitados del proceso glitolítico, más ATP, β-glicerofosfato, UDP y fosfogluconato-6. Las fosfatasas mostraron un pH máximo de alrededor de 4.6 y fueron rápidamente restringidas con fluoruro.

6. La acción de las fosfatasas se restringeron, generalmente reduciendo el nitrato. Este efecto se pronunció más contra ATP-asa y las fosfatasas de glucosa.

7. Las preparaciones enzimáticas mostraron una poderosa acción de la amilasa, la cual excedió la acción de la invertasa más de 90 veces, a base de los azúcares reductores que se formaron. Se reprimió la acción de la amilasa reduciendo el nitrato.

8. Parecía existir una relación entre el aumento de sacarosa y la supresión de la fosfatasa en los tratamientos bajos en nitrato. Los mecanismos que se sospechan se discuten en detalle, particularmente el papel que desempeña la ATP-asa y los sistemas hidrolizadores del fosfato glucoso.

9. Al reducirse el potasio se estimuló la hexoquinasa y retardó la fosfohexosa isomerasa y la quinasa fosfoglicerada. El aumento de glucosa en las plantas con bajo contenido de potasio se explica por la supresión de los últimos dos sistemas.

10. La reducción del potasio causó una influencia variable en la invertasa, resultando en una supresión de la enzima al nivel de 4 meq./l y estimulándola al nivel de 1 meq./l.

11. La glucosa fué la única hexosa que recibió fosfato del ATP en las

preparaciones de la hoja. Las otras hexosas que se probaron fueron: fructosa, fructosa-6- fosfato y galactosa.

12. Se encontró que existe una relación inversa entre la sacarosa y los niveles de fructosa, especialmente en las plantas con nitrato reducido, en las que virtualmente desapareció la fructosa.

13. En las preparaciones enzimáticas, no se encontró la hidrogenasa de glucosa-6- de fosfato, por lo cual es dudosa la existencia del sistema desviatorio monofosfatado en las hojas de caña.

14. Se encontró una definida e inversa relación entre la actividad de la peroxidasa y el contenido de sacarosa. La peroxidasa resultó fuertemente restringida con la reducción de nitrato. También se discute la posibilidad de que la peroxidasa actúe en la oxidación terminal.

15. En los extractos foliares no pudo encontrarse oxidasa citocroma C, aunque sí se evidenció un fuerte sistema de reductasa citocroma C, el cual se estimuló reduciendo la TPN. La reductasa citocroma C fue particularmente sensitiva al aumento del nitrato.

16. La enzima condensadora fue suprimida moderadamente en una amplia variedad de reducidos niveles de nitratos. La reducción del potasio limitó la enzima al nivel de 2 a 1 meq./l, sugiriendo que a ciertos niveles el potasio puede actuar como agente activante de la enzima condensadora.

17. Se encontraron otras relaciones entre los nutrimentos y las enzimas, las cuales se discuten en relación con su importancia para agricultores y fisiólogos.

18. La evidencia indirecta sugiere el hecho de que el almidón sirve como fuente de glucosa para el proceso glicolítico y la síntesis de la sacarosa en las hojas de la caña. La amilasa y la maltasa serían componentes esenciales de tal sistema. La baja magnitud de actividad de la invertasa hace que esta enzima sea de muy poca importancia tanto para catalizar el suministro de azúcares reductores como para la síntesis de la sacarosa.

19. Se presenta un diagrama que explica las interrelaciones de la fosfatasa, la amilasa y el nitrato, en la medida en que podrían afectar la producción de sacarosa.

LITERATURE CITED

1. Axelrod, B., and Bandurski, R. S., Phosphoglyceryl kinase in higher plants, *J. Biol. Chem.* **204** 939, 1953.
2. Basham, J. A., and Calvin, M., The Path of Carbon In Protosynthesis, Prentice-Hall, Inc., Englewood Cliffs, N.J. p. 69, 1957.
3. Barker, S. B., and Summerson, William H., The colorimetric determination of lactic acid in biological material, *J. Biol. Chem.* **138** 535, 1941.
4. Balch, R. T., Further notes on starch in Louisiana canes, juices and raw sugars, *Sugar J.* **15**(8) 11-5, 1953.
5. Barrenscheen, H. K., and Lang, S., *Biochem. Z.* **253** 395, 1932.
6. Beauchamp, C. E., The composition of the crude chlorophyll of cane leaves and

its relationships with cane growth, *Proc. Int. Soc. Sugar Cane Tech.* **6** 811–32, 1939.
7. Bernfeld, Peter, Amylase, α and β, *Methods in Enzymology* **1** 149, 1955.
8. Bonner, James, Plant Biochemistry, Academic Press Inc., New York, N.Y., 1950.
9. Bonnet, Juan A., Chemical concept about sucrose formation and maturity status of harvested sugarcane in Puerto Rico, *Sugar J.*, pp. 45–52, June 1962.
10. Boyes, P. M., Quantitative determinations of some non-sugars and the partial removal of one in particular—starch, *Proc. S. A. Sugar Technol. Ass'n.* (32nd Ann. Cong.) p. 37, 1958.
11. Browne, C. A., and Zerban, F. W., Physical and Chemical Methods of Sugar Analysis, 3d ed. p. 895, 1941.
12. Buchanan, J. G., The path of carbon in photosynthesis; XIX, The Identification of sucrose phosphate in sugar beet leaves, *Arch. Biochem. & Biophys.* **44** 140–9, 1953.
13. Burma, D. P., and Mortimer, D. C., The biosynthesis of uridine diphosphate glucose and sucrose in sugar beet leaf, *Arch. Biochem. & Biophys.* **62** 16–28, 1956.
14. Burr, G. O., and Kortschak, H. P., Hawaiian Sugar Planters Association Rept., Expt. Sta. Comm. 1–80, 1954.
15. Burr, G. O., Hartt, C. E., Brodie, H. W., Tanimoto, Y., Kortschak, H. P., Ashten, F. M., and Coleman, R. E., The sugarcane plant, pp. 275–308, *Ann. Rev. Plant Physiol.* **8** 477, 1957.
16. Cardini, C. E., Leloir, Luis F., and Chiriboga, J., The biosynthesis of sucrose, *J. Biol. Chem.* **214** 149, 1955.
17. Clements, H. F., Managing the production of sugar cane, Hawai. Sugar. Technol. Rept. 6 1–15, 1948.
18. Cohen, P. P., and McGilvery, R., Peptide bond synthesis; I, The formation of p-aminohippuric acid by rat liver slices, *J. Biol. Chem.* **166** 261, 1946; II, The formation of p-aminohippuric acid by liver homogenate, **169** 119, 1947; III, On the mechanism of p-aminohippuric acid synthesis, **171** 121, 1947.
19. Darby, R. T., and Goddard, D. R., The effects of cytochrome oxidase inhibitors on the cytochrome oxidase and respiration of the fungus *Myrothecium verrucaria*, *Physiol. Plantarum* **3** 435, 1950.
20. Dillewijn, C. Van, Botany of Sugar Cane, The Cronica Botanica Co., Waltham Mass., pp. 213–15, 1952.
21. Dounce, A. L., and Beyer, G. T., A new method for the determination of the enzyme aldolase, *J. Biol. Chem.* **173** 159, 1948.
22. Engelhardt, V. A., and Liubimowa, M. N., Myosine and adenosinetriphosphatase, *Nature* **144** 668, 1939.
23. ——, Adenosinetriphosphatase properties of myosine, *Advan. in Enzymology* **6** 147 1946.
24. Evans, H. J., and Nason, A., Pyridine nucleotide-nitrate reductase from extracts of higher plants, *Plant Physiol.* **28** 233, 1954.
25. Feuilherade, L., Starch in Uba juices, *S. A. S. J.* **13** (5) 305–9, 1929.
26. Friedemann, T. E., and Hangen, Gladys E., Pyruvic acid; II, The determination of keto acids in blood and urine, *J. Biol. Chem.* **147** 415, 1943.
27. ——, Determination of α-keto acids, *Met. in Enzymology* **3** 414, 1957.
28. Fruton, Joseph S., and Simmonds, Sofia, General Biochemistry, 2nd ed., John Wiley & Sons, New York, N.Y. p. 474, 1960.
29. Gibbs, M., TPN Triosephosphate dehydrogenase from plant tissue, *Met. in Enzymology* **1** 411–15, 1955.

30. Goddard, D. R., and Meeuse, J. D., Respiration of higher plants, *Ann. Rev. Plant Physiol.* **1** 207, 1950.
31. Hamner, K. C., Effects of nitrogen supply on rates of photosynthesis and respiration in plants, *Bot. Gaz.* **97** 744, 1936.
32. Hartt, C. E., Some effects of potassium upon the growth of surgarcane and upon the absorption and migration of ash constituents, *Plant Physiol.* **9** 399–452, 1934.
33. ——, Some effects of potassium upon the amounts of protein and amino forms of nitrogen, sugars, and enzyme activity of sugarcane, *Plant Physiol.* **9** 453–90, 1934.
34. ——, The synthesis of sucrose by excised blades of sugarcane, *Hawaii. Planters Rec.* **41** 33–46, 1937.
35. ——, The synthesis of sucrose by excised blades of sugarcane; Time and temperature, *Hawaii. Planters Rec.* **44** 89–116, 1940.
36. ——, The fluctuation of sugars in the leaf sheaths of the sugarcane plant during the day and the night, *Hawaii. Planters Rec.* **40** 329–54, 1936.
37. Hill, A. V., *Biochem. & Biophys. Acta* **4** 4, 1950.
38. Humbert, R. P., Potash fertilization in the Hawaiian sugar industry; Potassium Symp., Editors Int. Potash Institute, Berne, pp. 319–44, 1958.
39. Kuijper, J., *Archief Suikerind,* Ned.-Indiee, **23** 528–56, 1915. (See reference 20, pp. 103–5).
40. Lehninger, A. L., Fatty acid oxidation and the Krebs tricarboxylic acid cycle, *J. Biol. Chem* **161** 413, 1945; **164** 291, 1946.
41. Leloir, L. F., and Cardini, C. E., The biological synthesis of sucrose phosphate, *J. Biol. Chem.* **214** 157–65, 1955.
42. Lipmann, F., Metabolic generation and utilization of phosphate bond energy, *Adv. in Enzymology* **1** 99, 1941.
43. Liebknecht, W. L., *Biochem. Z.* **303** 96, 1939.
44. Lipmann, F., and Tuttle, L. C., A specific micromethod for the determination of acyl phosphates, *J. Biol. Chem.* **159** 21–8, 1945.
45. Lohmann, K., and Schuster, P., *Biochem. Z.* **294** 188, 1937.
46. Lugo-López, M. A., and Capó, B. G., The effect of weather and climate on the sucrose content of sugarcane, *J. Agr. Univ. P.R.* **38**(4) 149–69, 1954.
47. Macleod, Morna, and Robinson, Robert, The application of the iodimetric method to the estimation of small amounts of aldoses, *Biochem. J.* **23** 517, 1929.
48. Methods of Analysis of the Association of Official Agricultural Chemists, 6th ed., Washington, D. C., pp. 127–8, 1945.
49. Mechanism of Photosynthesis, Hawaii Sugar Planters Assn. Expt. Sta. Rept., p. 10, 1959.
50. Meyerhof, O., Further studies of the Harden-Young effect in alcoholic fermentation of yeast preparations, *J. Biol. Chem.* **180** 575, 1949.
51. Neilands, J. B., and Stumpf, Paul K., Outlines of Enzyme Chemistry, John Wiley & Sons, Inc., New York, N. Y., p. 108, 1955.
52. Normal course of photosynthesis in sugarcane, Hawaii Sugar Planters Assn. Expt. Sta. Rept., p. 13, 1960.
53. Ochoa, Severo, Crystalline condensing enzyme from pig heart, *Methods in Enzymology* **1** 685, 1955.
54. Paladini, A. C., and Leloir, L. F., Studies on uridine-diphosphate-glucose, *Biochem. J.* **51** 426–30, 1952.
55. Perry, S. V., The adenosinetriphosphatase activity of myofibrils isolated from skeletal muscle, *Biochem. J.* **48** 257, 1951.

56. Photosynthesis secrets studied, Hawaii. Sugar Planters Assn. Expt. Sta. Rept., p. 15, 1957.
57. Roe, J. R., A colorimetric method for the determination of fructose in blood and urine, *J. Biol. Chem.* **107** 15, 1934.
58. Samuels, G., and Capó, B. G., Research with sugarcane fertilizers in Puerto Rico, 1910–1054, Agr. Expt. Sta. Univ. of P.R. Tech. Paper 16, March 1956.
59. Samuels, G., Landrau, P., Jr., and Capó, B. G., The influence of fertility levels on the varietal response of sugarcane, *J. Agr. Univ. P.R.* **40**(3) 129–43, 1956.
60. Samuels, G., Influence of fertilizers on the components of available sucrose-per-cent-cane, *J. Agr. Univ. P.R.* **39**(2) 84–91, 1955.
61. Samuels, G., and Cibes, H., Influence of mineral deficiencies on the growth and yield of sugarcane, *J. Agr. Univ. P.R.* **47**(2) 61–75, 1953.
62. Scott, E. M., Estimation and purification of fumarase, *Arch. Biochem.* **18** 131, 1948.
63. Schade, A. L., Levy, H., Bergmann, L., and Harris, S., Studies on the respiration of the white potato; III, Changes in the terminal oxidase pattern of potato tissue associated with time of suspension in water, *Arch. Biochem.* **20** 211, 1949.
64. Speck, J. F., The enzymic synthesis of glutamine, *J. Biol. Chem.* **168** 403, 1947.
65. Stadtman, E. R., Stadtman, T. C., and Barker, H. A., Tracer experiments on the mechanism of synthesis of valeric and caproic acids by *Clostridium kluyveri*, *J. Biol. Chem.*, **178** 677, 1949.
66. Stern, Joseph R., Assay of tricarboxylic acids. Estimation of citric acid, *Methods in Enzymology* **3** 425–31, 1957.
67. Sumner, James B., and Somers, G. Fred, Chemistry and Methods of Enzymes, Academic Press Inc., New York, N. Y., 3d. ed., p. 349, 1953.
68. Sumner, James B., Dinitrosalicylic acid: A reagent for the estimation of sugar in normal and diabetic urine, *J. Biol. Chem.* **47** 5, 1921.
69. Sutherland, E. W., Cori, C. F., Haynes, R., and Olsen, N. S., Purification of the hyperglycemic-glycogenolytic factor from insulin and from gastric mucosa, *J. Biol. Chem.* **180** 825, 1949.
70. Taniguchi, S., Sato, R., and Egami, F., The Enzymatic Mechanism of Nitrate and Nitrite Metabolism in Bacteria, Inorganic Nitrogen Metabolism, ed. by W. D. McElroy and B. Glass, Johns Hopkins Press, Baltimore, Md., 1956.
71. Tonhazy, N. E., White, N. G., and Umbreit, W. W., A rapid method for the estimation of the glutamic-aspartic transaminase in tissues and its application to radiation sickness, *Arch. Biochem.* **28** 36, 1950.
72. Umbreit, W. W., Burris, R. H., and Stauffer, I. F., Manometric Techniques, 3d. ed., Burgess Publishing Co., Minneapolis, Minn., p. 47, 1959.
73. Vickery, H. B., Pucher, G. W., Wakeman, A. J., and Leavenworth, C. S., Conn. Agr. Expt. Sta. B. 442, 1940.
74. Wainio, W. W., Person, P., and Eichel, B., The reaction between cytochrome oxidase and ferrocytochrome C, *J. Biol. Chem.* **192** 349, 1951.
75. Whelan, W. J., Phosphorylase from plants, *Methods in Enzymology* **1** 192, 1955.
76. Weil-Malherbe, H., The enzymatic phosphorylation of vitamine B, *Biochem. J.* **33** 1997, 1939.
77. Wood, G. H., Some factors influencing starch in sugarcane, Proc. S. African Sugar Technol. Assn., p. 123, April 1962.