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STIMULATION OF THE GLYOXYLATE SHUNT IN GAMMA-IRRADIATED BANANA

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Abstract—Succinic dehydrogenase was the most susceptible among the TCA cycle enzymes to gamma irradiation in preclimacteric banana. Maximum inhibition occurred at the 3rd day after irradiation. Impairment of this activity did not affect operation of the TCA cycle, assessed from the incorporation pattern of acetate [2-^{14}C] into the organic acids such as citric, malic and succinic. Nevertheless, incorporation into keto acids like glyoxylate, α-ketoglutarate and oxaloacetate showed a difference. The rate of labelling into α-ketoglutarate and oxaloacetate was reduced on the 3rd day while incorporation into glyoxylate was increased indicating the operation of glyoxylate shunt pathway. Studies on the individual enzymes of this pathway, isocitrate lyase and malate synthetase confirmed its operation. The reduction in oxalo-acetate has been attributed to the increased gluconeogenesis.

INTRODUCTION

The energy required for the ripening process in fruits is provided from the oxidation of substrates derived from the breakdown of starch through the TCA cycle. A gradual shift in the metabolic pathways to provide more fructose phosphate esters is also essential during ripening. Barker and Solomos [1] and recently Salminen and Young [2] have reported an increase in phosphofructokinase activity which closely corresponded with the rise in climacteric respiration in bananas. Our earlier studies [3] on the utilization of carbohydrate by gamma-irradiated banana showed that there is a shift in the metabolism of carbohydrate from the glycolytic to the pentose phosphate pathway. This shift, coupled with activation of fructose-1,6-diphosphatase [4] and fructose-6-phosphatase, facilitated the accumulation of fructose, thereby reducing energy production through degradation of starch mediated by (starch) phosphorylase [3]. There was a reduction in the utilization of free sugars accompanied by the inhibition of hexokinase caused by gamma-irradiation. After establishing the shift in carbohydrate metabolism we have ascertained in the present investigation the effect of gamma irradiation on TCA cycle activity.

RESULTS AND DISCUSSION

Massey [5] has suggested that succinic dehydrogenase is the most vulnerable to gamma irradiation among the Kreb's cycle enzymes, although a clear demonstration of the inhibition of this activity by irradiation has hitherto not been demonstrated. In our studies, the activity of succinic dehydrogenase was determined in the mitochondrial fraction isolated from unirradiated and irradiated banana at various periods after exposure to gamma rays. A marked decrease in succinic dehydrogenase activity was observed in bananas as a result of gamma irradiation. The maximum difference was observed on the 3rd day after irradiation. At this time there was about a 50% decrease in activity of this enzyme. As the fruit began to ripen the succinic dehydrogenase increased rapidly in the unirradiated fruit, while the activity in the irradiated fruit remained at almost the same level until the 6th day. As the fruit reached the climacteric the succinic dehydrogenase activity also showed a corresponding increase.

Next, we have determined whether the decrease in succinic dehydrogenase activity had a direct effect on the incorporation of acetate [2-^{14}C] into intermediates like citrate, malate, and succinate (Table 1). There was a significant increase in the incorporation of acetate into citric acid and succinic acid, whereas the incorporation into malate did not show much variation. These results indicated that the normal functioning of the Kreb's cycle was not affected, even though succinic dehydrogenase activity was impaired.

Alternatively, when succinic dehydrogenase was impaired the intermediates might be utilized through the glyoxylate bypass. In order to ascertain this possibility, the incorporation of acetate [2-^{14}C] into keto acids was determined (Table 1). On the first day the incorporation into glyoxylate showed a 52% increase in the irradiated sample while oxaloacetate showed only a 25% increase, and no difference was found in the rate of incorporation into α-ketoglutarate. On the 3rd day, however, when succinic dehydrogenase was also decreased, the difference in the incorporation of radioactivity into glyoxylate increased to 80% while there was a marked reduction to about 75% in the labelling of α-ketoglutarate as well as oxaloacetate. The reduction in the labelling of oxaloacetate could be attributed to an increase in gluconeogenesis resulting from the activation of fructose-1,6-diphosphatase [4]. To test this hypothesis, incorporation of acetate [2-^{14}C] into sucrose, glucose and fructose was

Table 1. Incorporation of acetate [2-^{14}C] into various metabolic products of banana

Metabolites	Days of storage: 1st day Unirradiated (cpm)	1st day Irradiated (cpm)	3rd day Unirradiated (cpm)	3rd day Irradiated (cpm)
Organic acids				
Citric acid	7080 ± 150	8970 ± 120	8220 ± 100	9120 ± 70
Malic acid	9730 ± 110	9830 ± 130	9960 ± 244	10910 ± 300
Succinic acid	5630 ± 190	5910 ± 200	7180 ± 70	8670 ± 470
Keto acids				
Glyoxylate	870 ± 50	1330 ± 90	2060 ± 140	3800 ± 190
Oxaloacetate	860 ± 10	1080 ± 50	1070 ± 70	850 ± 60
α-Keto glutarate	2260 ± 40	2170 ± 100	2690 ± 250	750 ± 140
Sugars				
Sucrose	492 ± 40	930 ± 30	550 ± 10	1040 ± 40
Glucose	200 ± 10	270 ± 10	300 ± 40	470 ± 30
Fructose	210 ± 10	310 ± 30	300 ± 40	520 ± 40

Values given are mean ± SE. Each values represent average of 6 to 10 independent experiments.

determined (Table 1). On the 3rd day, the extent of labelling in these sugars showed a definite increase in the irradiated banana; the increase being 92, 53, and 75% respectively over the controls.

The increased incorporation of acetate [2-^{14}C] into glyoxylate indicated an enhanced operation of the glyoxylate pathway, which was further confirmed by the studies on the activities of isocitrate lyase and malate synthetase. In the unirradiated banana isocitrate lyase activity was very low and remained steady during storage up to 9 days. However, after irradiation a 2·5 fold increase in this activity was observed on the first day. On further storage, this activity showed a rapid increase to a maximum of about 3·2 fold on the 3rd day and a gradual fall thereafter to normal levels on the 9th day. Malate synthetase, the next enzyme in this pathway, also exhibited a corresponding increase in activity although the maximum was attained in 5 days. In unirradiated banana activity present initially decreased as ripening started. On irradiation a 20% increase was observed on the first day, followed by a further increase to about 50% over the control on the 3rd day. At the fifth day the difference in the activities was about 2·5 fold.

The immobilization of the sugar phosphate esters by the activation of the corresponding phosphatases and the shift towards pentose phosphate pathway could hinder energy production to some extent. Further reduction in the energy production is affected by the inhibition of succinic dehydrogenase and this block in the utilization of substrate through the TCA cycle is by-passed by the enhanced operation of the glyoxylate shunt. The existence of this pathway in irradiated banana is supported by the increase in the labelling of glyoxylate and a large decrease in the labelling of α-keto glutarate, from acetate [2-^{14}C] on the 3rd day. The confirmatory evidence is provided by the demonstration of the activation of individual enzymes, isocitrate-lyase and malate synthetase on the 3rd day as a result of irradiation. At the preclimacteric stage both these enzymes are low in activity and as the unirradiated fruit reached climacteric the activity decreased further. Irradiation thus has a specific effect on the activation of these enzymes. Except for a recent report on the presence of isocitrate-lyase in mature mangoes [6] there is no other evidence for the operation of the glyoxylate pathway in fruits.

The existence of this pathway in plants is observed mainly in fatty seedlings, where it is necessary for the conversion of fat to carbohydrate [7–10]. Beevers [10] reported a rapid conversion of fat to carbohydrate through this pathway in ripe caster bean seedlings during germination. Our observation on the reduction of label in oxaloacetate, from acetate [2-^{14}C] on the 3rd day when the glyoxylate pathway is functioning efficiently suggests that gluconeogenesis is occurring in irradiated banana. This contention is well supported by the observation (Table 1) that a gluconeogenic substrate viz acetate [2-^{14}C] could be readily incorporated into fructose, sucrose and glucose. This study does not imply that sugars are converted to acetate and then channelled through gluconeogenic pathway to sugars. However, there is a strong possibility that gluconeogenic substrates are derived from fat or other sources like amino acids. Thus, gamma irradiation of banana alters their normal metabolic pathways for the oxidation of sugars and thereby reduces the energy production from them.

EXPERIMENTAL

Cavendish bananas (*Musa cavendishii*) were used for these studies. Experimental procedure for irradiation and storage are described in ref. [3]. To study the incorporation of acetate [2-^{14}C] into various metabolic products, 20 μCi of 2-^{14}C NaOAc (23·4 mCi/mmol) was injected into two holes of 2 mm dia. and 5 mm depth made by a stainless steel cork borer at equidistant points from both ends of control and 35 krad irradiated bananas. The fruits were incubated for 3 hr at room temp. After incubation the bananas were peeled and used for isolation of different metabolites.

Isolation of organic acids and sugars. Banana pulp (30g) was cut into small pieces and homogenised in hot 80% EtOH. Supernatant obtained on centrifugation was collected and the residue homogenised with 80% EtOH. The extracts were pooled and EtOH was removed under vacuum. The EtOH free extract was made to a known vol and passed through Dowex-50 (H^+) column to remove amino acids. The eluate was then passed through Dowex-1 (formate) column to separate organic acids from sugars. The organic acids were eluted

with 4N-formic acid. The eluates were concentrated *in vacuo* and made up to a known vol.

Isolation of keto acids. Keto acids were converted to their 2,4-dinitrophenyl hydrazones [11]. Acetate [2-^{14}C] administered fruits after the incubation were extracted with 5% metaphosphoric acid. To 20 ml aliquot of the supernatant 20 ml 1% 2,4-dinitrophenylhydrazine in 5N H_2SO_4 was added. The mixture was incubated for 30 min at 37°. The DNP hydrazones formed were then extracted 3× with 1/3rd vol of peroxide-free Et_2O. The Et_2O layers were pooled and shaken with saturated $NaHCO_3$ soln. The $NaHCO_3$ extract was acidified to pH 2 with 3N H_2SO_4 and extracted 3× with 1/3 vol of $CHCl_3$ containing 15% Et_2O. The $CHCl_3$ layer was evaporated to dryness under vacuum and the derivative was dissolved in a known vol. of EtOAc. The sugars and organic acids were separated using BuOH–HOAc–H_2O (12 : 3 : 5) and HCOOH: *n*-PrOH and eucalyptus oil (2 : 5 : 5) systems respectively on Whatman No. 3 paper. The spots were identified using aniline diphenylamine phosphate for sugars and bromocresol green for organic acids. The keto acid hydrazones were separated by TLC on Si gel. The solvent system used was C_6H_6–Me_2CO. HOAc (17 : 2 : 1 : 10 : 5) and 10% NaOH in 90% EtOH was used as detecting reagent. The spots were identified by comparing the R_f values with authentic samples. The spots were cut out from PC or scraped from TLC plates and scintillation counted. Cabosil was used for uniform suspension of Si gel.

Determination of isocitrate lyase, malate synthetase, and succinic dehydrogenase. Preparation of enzymes. Me_2CO powders were prepared from unirradiated and irradiated banana pulp for different periods of storage up to 9 days. Frozen banana tissue was homogenized with 5× its wt of cold Me_2CO (−30°) for 1 min at 0–4° and the slurry filtered. This process was repeated twice with half the original amount of Me_2CO. The powder was dried at 0–4° and stored at −30°. Me_2CO powder (10g) was mixed with 25 ml of 0·1 M KPi buffer pH 7·6 in a pre-cooled pestle and mortar. The mass was frozen with liquid N_2 and was thawed out by grinding. During this process the temp. was not allowed to rise above 2°. The well ground slurry was filtered through cheese cloth and the filtrate was centrifuged at 15000 rpm for 25 min. The supernatant was used as the enzyme for these studies. A mitochondrial preparation [12] was used for succinic dehydrogenase assay. 20 g of banana pulp was cut into small pieces and ground in a precooled pestle and mortar with 20 ml grinding medium consisting of 0·3 M mannitol, 1 mM EDTA and 10 mM β-mercaptoethanol in 0·1M KPi buffer pH 7·2. After grinding, the slurry was passed through a double layer of cheese cloth and the filtrate was centrifuged at 1000 *g* for 15 min. The pellet obtained was uniformly suspended in the grinding medium and again centrifuged at 6000 *g* for 15 min. The supernatant was discarded and the pellet was again dispersed in the grinding medium and used for assay of succinic dehydrogenase.

Assay of enzyme activity. Isocitrate lyase was determined according to the method of ref. [13]. The reaction mixture consisted of 200 μmol of KPi buffer pH 6, 6 μmol of cystein (HCl) 15 μmol of $MgSO_4$, 67 μmol of NaOH and 60 μmol of semicarbazide (HCl) and 400–600 μg of enzyme protein in 2·9 ml. The reaction was started by the addition of 0·1 ml of D,L-Na isocitrate which was excluded in the blank. The change in A at 252 mm was determined at room temp. The sp. act. of the enzyme was expressed as nmol of semicarbazone (HCl) formed/mg protein/min.

Malate synthetase activity was measured according to the method of ref. [7]. In this case the reaction mixture contained 100 μmol of KPi buffer, pH 7·6, 10 μmol of $Mg Cl_2$ 10 μmol GSH, 10 μmol of ATP, 5 μmol NaOAc, 2 μmol containing μCi of NaOAc [2-^{14}C] (sp. act. 23·4 mCi/mmol) 0·08 μmol of coenzyme A and 400–600 μg of enzyme protein in 1 ml. This mixture was incubated for 10 min at room temp. then 10 μmol of glyoxylate was added to the tubes and incubated for 30 min more at 30°. The blank did not contain glyoxylate. After incubation, the reaction was stopped by adding 3 ml hot EtOH and the ppt. removed by centrifugation. The filtrate was evaporated to dryness at 60°. The residue was dissolved and made up to a known vol with 20% EtOH. The organic acids were separated by chromatography as described earlier and the spots corresponding to malate were cut out and counted. The difference in count between experimental and blank samples was taken as a measure of malate synthetase which was expressed as cpm per mg protein.

Succinic dehydrogenase activity was determined according to the method of ref. [14]. The reaction mixture consisted of 300 μmol of KPi buffer pH 7·2, 30 μmol of NaCN, 3 μmol of K_4 Fe (CN_6) and 40 μmol of Na succinate in a total vol of 2·8 ml in a cuvette. The reference cell contained buffer alone. At the beginning of the reaction 0·2 ml of enzyme (0·8–1·1 mg) was added to both the cells and the decrease in A at 400 nm was measured as a function of time. The enzyme activity was expressed as the decrease in A/min/mg of protein.

Protein was determined by the biuret method [15].

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