CHAPTER II

EXPERIMENTAL

Materials and Methods

The fruit used for the investigation is commonly known as lime (limbu in Gujarati). It was identified by the Department of Botany of this University as Citrus medica, Linn Var-acida. The fruit has been described as follows in "The Flora of British India" (Hooker, 1875) :

"Citrus medica, Linn; Var 3. acida; leaflet elliptic-oblong, petiole many-times shorter than the leaflet, linear or obovate, racemes short, flowers small, petals usually 4, fruit usually small, globose or ovoid, with a thick or thin rind, pulp pale, sharply acid. Brandis, l.c. 52. C. acida, Roxb. Fl. Ind. iii. 390 (who includes under this the varieties, if not species, of Sour Limes and Lemons found in India). The large fruited states of this appear to assume the form of the Citron, and the small to approach the W. Indian Lime, which is, however, described as a bush with white flowers.- The Sour Lime of India."

Collection of the fruits for the analysis

The fruits used in the investigation were obtained from the University gardens. They were collected in the same season and at the same period of the day from a single plot of trees of almost of the same age. Fruits of different

sizes were collected and brought immediately to the laboratory under ice. Their diameter was measured equatorially with vernier callipers and were divided into four groups according to their diameter, namely 0.8 to 1.0 cm, 1.4 to 1.6 cm, 2.4 to 2.6 cm or 4.0 cm diameter.

The number of fruits used in an experiment is shown in Table 1. The same varied depending upon the size of the fruit and the estimation made.

Chemicals used

The chemicals used were of research grade purity.

Adenosine triphosphate (Na salt), nicotinamide-adenine dinucleotide, nicotinamide-adenine dinucleotide (reduced), nicotinamide-adenine dinucleotide phosphate, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, fructose-1, 6-diphosphate, DL-glyceraldehyde-3-phosphate, sodium pyruvate, D-glucuronolactone, glucose-6-phosphate dehydrogenase, aldolase and bovine albumin were obtained from Sigma Chemical Co. (USA).

Estimation of dry weights of different parts of citrus fruit

In order to get quantitative data on the weight of different parts of the fruit, a known number of fruits of the same size were taken and the green skin first removed with a scalpel. Next the white skin was removed carefully

Table 1. Number of fruits taken for each trial for chemical analysis.

Name of the estimation	Number taken in the use of the fruit with diameter (cm)			
	0.8-1.0	1.4-1.6	2.4-2.6	4.0
Dry weight	60	40	10	5
Total sugar Free sugar	10	5	2	2
Free glucose	70	40	10	5
Glucose-1-phosphate Glucose-6-phosphate Fructose-6-phosphate Fructose-1,6-diphosphate	400	250	100	50
Lactic acid	50	30	25	5
Citric acid	20	10	5	2
Free ascorbic acid Dehydro ascorbic acid	50	30	25	5

without removing the vesicles. The remaining fruit was cut into two halves. Each half was expressed with a lime squeezer (local mechanical model) and the juice coming out was allowed to pass through a cheese cloth placed on a beaker. The volume of the juice obtained was noted. The seeds if present were removed from the residual pulp and the vesicles were separated from the septa. Any vesicles adhering to the cheese cloth were also picked up and added. They were both washed quickly with cold water to remove any adhering juice and pressed between filter papers to remove extraneous moisture, weighed and known weights of the same dried in an oven at 60° till constant weights were obtained.

As indicated in Table 1 the determinations were made on samples pooled from a number of fruits, and the values calculated per cent fresh weight.

Estimation of total and free sugar

A known amount of fresh tissue was ground for 15 minutes with 10 volumes of glass distilled water in a mortar at 1-2° and the homogenate obtained was suitably diluted and used for the estimation of total and free sugar by Hanes's method (1954).

Estimation of free glucose

For the estimation of free glucose the fresh tissue was

ground with four volumes of 75% alcohol in a mortar at 1-2° for about 15 minutes. The juice was diluted with four volumes of 75% alcohol. In both the cases the pH was adjusted to 7.0 with 1N potassium hydroxide and centrifuged at 6400 x g at 0° for 15 minutes in a refrigerated centrifuge. The supernatant was evaporated to dryness in a boiling water bath. The dried residue was taken up in 8 volumes of distilled water and again centrifuged at 6400 x g at 0° for 30 minutes. From the supernatant an aliquot containing 10 mg of free sugar was taken and this was used for the quantitative separation of glucose by the column chromatography method described by Khym and Zill (1952). The glucose separated was estimated by the method described by Montgomery (1957).

Estimation of sugar phosphates

A known weight of the tissue was chilled in the deep-freeze at -15° for 12 hours and then extracted with equal volume of 10% trichloroacetic acid in a chilled mortar by grinding for 15 minutes. In the case of juice an equal volume of 10% trichloroacetic acid was added. The homogenate was centrifuged at 6400 x g at 1° for 15 minutes and the supernatant removed. The residue was re-extracted with equal volume of 5% trichloroacetic acid and centrifuged again. The supernatants were combined and made upto a definite volume. The trichloroacetic acid added was removed by extracting the

supernatant thrice with equal volume of chilled ether. To precipitate the polysaccharides two volumes of absolute alcohol were added, the solution kept at 1-2° for 4 hours, and centrifuged at 2500 x g at 0° for 15 minutes. To the supernatant 0.05 volume of 25% barium acetate was added first, followed by the addition of 3 volumes of absolute alcohol. The pH of the solution was adjusted to 8.2 with 18% ammonium hydroxide and the extract kept for about 12 hours at 1-2°. The supernatant was centrifuged off and the precipitate was dissolved in five volumes of water. This was passed through Dowex-50W-X4 (H^+). The column was washed twice with 20 ml of water. The washings and the elute were collected and the pH adjusted to 8.2 with 18% ammonium hydroxide and used for the separation of sugar phosphates by the column chromatography method described by Khym and Cohn (1953). In the case of larger fruits with a greater acid content barium acetate was added first to remove the acids and the polysaccharides were then precipitated by alcohol.

The sugar phosphates which separated were estimated by the method of Montgomery (1957).

Estimation of lactic acid

A known amount of the fruit tissue was ground with 10 volumes of 10% trichloroacetic acid in a mortar at 0° for about 15 minutes. The suspension was centrifuged and

the supernatant was suitably diluted and used for the estimation of lactic acid according to the method of Barker and Summerson (1941).

Estimation of citric acid

A known amount of the fruit tissue was ground with 10 volumes of 10% trichloroacetic acid in a mortar at 0° for about 15 minutes. The addition was made as such to the juice. The suspension was centrifuged and the clear supernatant obtained was used for the estimation of citric acid after suitable dilution. The method used was essentially that of Natelson et al (1948) with the modification that the samples were directly used for the estimation without heating in an oil bath and five ml of petroleum ether (60-80° B.P.) were used for extraction in place of 2 ml of heptane.

Estimation of different forms of ascorbic acid

Free ascorbic acid and dehydroascorbic acid were estimated by the method of Roe et al (1948).

Preparation of enzyme extract

All the operations described were carried out in the cold room maintained at 0-4°, unless otherwise specified. After separating the fruits and grouping them together,

the different parts namely, green skin (flavedo), white skin (albedo), septa, vesicles and the juice, where possible, were removed very carefully. The tissues were kept in a mortar for about 12 hours at -15°. The tissue was then homogenized in the cold room with three volumes of 0.1M tris (tris-hydroxymethyl aminomethane) buffer, pH 7.0 for about 10 to 15 minutes adjusting the pH to 7.0 with ammonium hydroxide (0.5% v/v) when necessary. The extract was filtered through two fold cheese cloth and centrifuged at 6500 x g at 0° for 15 minutes in a refrigerated centrifuge. The supernatant obtained was used for the estimation of the activities of hexokinase, glyceraldehyde-3-PO_4 dehydrogenase and lactate dehydrogenase.

The crude extract was used without centrifugation for the estimation of phosphohexose isomerase, fructose-diphosphate aldolase and phosphoglucomutase.

A 33% phosphate extract (0.02M PO_4 buffer, pH 7.0) was used without centrifugation for the estimation of phospho-fructokinase.

A 25% crude phosphate extract (0.02M PO_4 buffer, pH 7.0) was used for the assay of ascorbic acid oxidase.

For studying the enzymic synthesis of ascorbic acid a 33% extract was prepared using 0.88M sucrose as a grinding

medium. The homogenate was centrifuged at 500 x g at 0° for 10 minutes and the supernatant obtained was used for enzyme assay. For detecting the enzymes in the juice, the fresh juice was neutralized to pH 7.0 using 0,1N ammonium hydroxide and used.

The protein content of the enzyme extract was estimated according to the method of Lowry et al (1951). In the case of crude extract the protein was precipitated with 10% TCA and the precipitate formed was dissolved in 0.1N NaOH and used for the estimation.

For the preliminary detection of the enzymes and the intermediates edible portion of the fruit (1.4-1.6 cm diameter) was used. The outer green skin and the inner white skin were removed and the remaining tissue containing the fruit segments was used after removing the seeds.

Details of the assay system, procedure used in the estimation of enzyme activity and enzyme unit are summarized in Table 2.

The specific activity of the enzyme is defined as enzyme units per mg protein of enzyme preparation.

Colorimetric estimations were carried out in Klett-Summerson Colorimeter. Beckman Spectrophotometer model DU was used with quartz cells having 1.0 cm light path for recording changes in the optical density.

Table 2. Details of enzyme assays.

Details of assay system and procedure	Hexokinase (E.C., 2.7.1.1)	Phosphohexose isomerase (E.C., 5.3.1.9)
Reference	Salas et al (1963)	Black and Humphreys (1962)
Buffer	Tris, pH 7.5, 100 micromoles	Tris, pH 9.0, 20 micromoles
Substrate	Glucose, 1.5 micromoles	Glucose-6-phosphate, 5 micromoles
Amount of enzyme extract	0.2 ml	0.1 ml
Other components	ATP (Na salt), 15 micromoles; glutathione (reduced), 1.5 micromoles, $MgCl_2$, 15 micromoles, NADP, 0.1 micromole, glucose-6-phosphate dehydrogenase, 0.1 ml (0.2 unit*), final volume 3 ml	-
Temperature and period of incubation	30°, 5 minutes	37°, 30 minutes
Initiation of reaction	ATP added	Enzyme added
Termination of reaction	-	3.0 ml of 10N HCl added
Modification for blank	ATP and glucose omitted	Enzyme added after incubation
Parameter measured	Formation of $NADPH_2$ measured by changes in optical density at 340 mμ at 1 minute interval	Formation of ketosugar in the supernatant measured by the method of Roe et al (1949)
Enzyme unit	Amount of enzyme required to reduce 1 micromole of NADP per hour under the assay conditions	Amount of enzyme required to form 1 micromole of fructose-6-phosphate per hour under the assay conditions

* One unit will cause the reduction of 1 micromole of NADP per minute.

Table 2 contd..

Details of assay system and procedure	Phosphofructokinase (E.C., 2.7.1.11)	Fructose diphosphate aldolase (E.C., 4.1.2.13)
Reference	Buell et al (1958)	Sibley and Lehninger (1949)
Buffer	Tris, pH 9.0, 20 micromoles	Tris, pH 8.0, 50 micromoles
Substrate	Fructose-6-phosphate, 5 micromoles	Fructose-1,6-diphosphate (Na salt), 2 micromoles
Amount of enzyme extract	0.5 ml	0.1 ml
Other components	ATP (Na salt), 15 micromoles, hydrazine sulphate, 150 micromoles, $MgCl_2$, 5 micromoles, $(NH_4)_2 HPO_4$, 10 micromoles, aldolase, 0.3 ml (0.6 units*), bovine albumin, 200 micrograms	Hydrazine sulphate, 150 micromoles, iodoacetate, 0.5 micromole, volume made to 1.0 ml
Temperature and period of incubation	37°, 30 minutes	37°, 30 minutes
Start of reaction	Substrate added	Enzyme added
Termination of reaction	1.0 ml of 20% TCA added	2.0 ml of 10% TCA added
Modification for blank	Substrate omitted	Substrate added after incubation
Parameter measured	Formation of triose phosphate measured as 2,4-dinitrophenyl hydrazine by the method of Sibley and Lehninger (1949)	Formation of triose phosphate measured by the method of Sibley and Lehninger (1949)
Enzyme unit	Amount of enzyme required to form 1 micromole of fructose-1,6-di PO_4 per hour under the assay conditions	Amount of enzyme required to form 1 micromole of triose phosphate per hour under the assay conditions

* One unit will breakdown one micromole of fructose-1,6-diphosphate per minute.

Table 2 contd...

Details of assay system and procedure	Glyceraldehyde-3-phosphate dehydrogenase (E.C., 1.2.1.12)	Lactate dehydrogenase (E.C., 1.1.1.27)
Reference	Velick (1955)	Kornberg (1955)
Buffer	Tris, pH 8.5, 300 micromoles	Sodium phosphate, pH 7.0, 100 micromoles
Substrate	DL-Glyceraldehyde-3-phosphate, 1 micromole	Sodium pyruvate, 1.0 micromole
Amount of enzyme extract	0.1 ml	0.1 ml
Other components	NAD, 1 micromole, disodium arsenate, 50 micromoles, l-cysteine hydrochloride (neutralized), 10 micromoles, sodium fluoride, 60 micromoles	$NADH_2$, 0.1 micromole
Temperature and period of incubation	30°, 3 minutes	30°, 5 minutes
Initiation of reaction	Glyceraldehyde-3-phosphate added	Sodium pyruvate added
Termination of reaction	-	-
Modification for blank	Glyceraldehyde-3-phosphate omitted	Sodium pyruvate omitted
Parameter measured	Formation of $NADH_2$ measured by changes in optical density at 340 mμ at 30 seconds interval	Oxidation of $NADH_2$ measured by changes in optical density at 340 mμ at 1 minute interval
Enzyme unit	Amount of enzyme required to reduce 1 micromole of NAD per hour under the assay conditions	Amount of enzyme required to oxidize 1 micromole of $NADH_{12}$ per hour under the assay conditions

Table 2 contd....

Details of assay system and procedure	Phosphoglucomutase (E.C., 2.7.5.1)	Ascorbic acid synthesizing enzyme
Reference	Najjar (1955)	Chatterjee et al (1957)
Buffer	Tris, pH 7.5, 20 micromoles	Phosphate, pH 7.0, 700 micromoles
Substrate	Glucose-1-phosphate, 5 micromoles	D-glucuronolactone, 125 micromoles
Amount of enzyme extract	0.2 ml	2.0 ml
Other components	Neutralized cysteine hydrochloride, 10 micromoles, $MgSO_4$, 1 micromole, final volume, 1 ml.	Potassium cyanide, 250 micromoles
Temperature and period of incubation	37°, 15 minutes	37°, 2 hours
Initiation of reaction	Enzyme added	Enzyme added
Termination of reaction	2.0 ml of 5N H_2SO_4 and 2.0 ml of water added and mixture boiled for 3 minutes and supernatant centrifuged off	2.0 ml of 10% TCA added and supernatant centrifuged off
Modification for blank	Enzyme added after incubation	TCA added before incubation
Parameter measured	Disappearance of glucose-1-phosphate	Ascorbic acid estimated by titrating supernatant against 2,6-dichlorophenol indophenol
Enzyme unit	Amount of enzyme required for the formation of 1 micromole of acid stable phosphate per hour under the assay conditions	Amount of enzyme required to form 1 micromole of ascorbic acid per hour under the conditions of the assay

Table 2 contd..

Details of assay system and procedure	Ascorbic acid oxidase (E.C., 1.10.3.3)
Reference	Parekh *et al* (1969)
Buffer	Sodium phosphate, pH 7.0, 100 micromoles
Substrate	Ascorbic acid, 60 micromoles
Amount of enzyme extract	2.0 ml
Other components	-
Temperature and period of incubation	37°, 1 hour
Start of reaction	Substrate tilted from the side-arm
Termination of reaction	-
Modification for blank	Boiled enzyme used
Parameter measured	O_2 uptake measured at 15 minutes interval
Enzyme unit	Amount of enzyme required to utilize 1 micromole of oxygen per hour under the assay conditions