CHAPTER II

MATERIALS AND METHODS

As mentioned earlier fruits identified as Citrus acida* (Limbu in Gujarati) or more precisely as Citrus medica, Linn, Var-acida were used in the investigation.

The fruit has been described as follows in the 'Flora of British India' (Hooker, 1875): 'Citrus medica, Linn; Var 3.acida; leaflet elliptioblong, petiole many times shorter than the leaflet, linear or obovate, recemes short, flowers small, petals usually 4, fruit usually small globose or ovoid, with a thick or thin rind, pulp pale, sharply acid. Brandis, i.c. 52.c.acida, Hoxb. Fl. Ind. ili. 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 fruits for analysis

The fruits used in the investigation were obtained from the University gardens. They were collected in the

---

* The identification was done by the Botany Department, M.S. University of Baroda.

same season and at the same period of the day from a single plot of trees of almost of same age. Fruits of different sizes were collected and brought immediately to the laboratory under ice. Their diameter was measured equatorially with the vernier callipers and were divided into two groups according to their diameter, namely I.4 to I.6 cm (stage prior to the accumulation of citric acid) and 4.0 cm diameter (stage of accumulation of citric acid). The outer green skin and the inner white skin were removed with a scalpel. The remaining fruit segments after removal of seeds were used for the experiments.

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

Chemicals used:

The chemicals used were of research grade purity. They were obtained from the sources indicated below:

Chemicals	Sources from which obtained
Adenosine-5'-triphosphate (Na-salt)	Sigma Chemical Co.
Nicotinamide adenine dinucleotide phosphate	
Bovine albumin	

Table i: Number of fruits taken for each trial for chemical analysis

	diameter of fruit (cm)	
	1.4-1.6	4.0
	Number of fruits used for each estimation	
Dry weight	40	5
Totl sugar Free sugar	5	2
Free glucose	40	5
Glucose-1-phosphate Glucose-6-phosphate Fructose-6-phosphate Fructose-1.6-diphosphate	250	50
Lactic acid	30	5
Citric acid	10	2
Amino nitrogen	10	2
Free amino acids	10	2

Glucose-6-phosphate dehydrogenase	Sigma Chemical Co.
Aldolase	
Glucose-1-phosphoric acid	
Fructose-1,6-diphosphoric acid	
DL-glyceraldehyde-3-phosphate	
Nicotinamide adenine dinucleotide (reduced)	
Nicotinamide adenine dinucleotide	
2-oxoglutaric acid	
oxaloacetic acid	
L-glutamic acid	British Drug House, Ltd.
L-alanine	
L-aspartic acid	
L-glutamine	
Ninhydrin	
Napthoquinone sulfonic acid	
Magnesium sulfate	
Hydroxylamine hydrochloride	
Ferric chloride	
Gamma aminobutyric acid	L. Light and Co.
Trichloroacetic acid	E. Merck
L-asparagine	
Tris (Hydroxymethyl) amino methane	Nutritional Biochemicals Corporation.

Dowex 50-4X	Calbiochem
DEAE-cellulose	Carl Scheichel and Schuell
Sephadex-G-200	Pharmacia Ltd.

Calcium phosphate gel (dry wt. 10 mg/ml) was prepared by the method described by Colowick (1955).

Estimation of dry weight:

A known amount of fruit tissue was weighed after removal of the skin and the seeds. It was then dried in an oven at 60° till constant weights were obtained.

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' method (1954).

Estimation of free glucose:

For the estimation of free glucose the fresh tissue was ground with four volumes of 75 per cent alcohol in a mortar at 1-2° for about 15 minutes. The pH was adjusted to 7

with one normal potassium hydroxide and centrifuged at 6400 x g at 0^{o} for 15 minutes in a refrigerated centrifuge. The supernatant was evaported 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^{o} 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).

A known amount of tissue was ground with an appropriate volume of 10 per cent TCA (equal volume for sugar phosphates, ten volumes for citric acid and lactic acid and two volumes for amino nitrogen) at 0^{o} in a chilled mortar and this extract used for the estimations of sugar phosphates, citric acid, lactic acid and amino nitrogen.

Estimation of sugar phosphates:

The TCA extract was centrifuged at 6400 x g for 15 minutes and the supernatant removed. The residue was reextracted with equal volume of 5 per cent trichloroacetic acid and centrifuged again. The supernatants were combined and made up to a definite volume. The trichloroacetic acid was removed by extracting the supernatant thrice with equal

volume of chilled ether. The polysaccharides were precipitated by adding two volumes of absolute alcohol and then keeping the solution at 1-2^{o} for 4 hours. The precipitated polysaccharides were removed by centrifugation at 2500 x g at 0^{o} for 15 minutes. To the supernatant was added 0.05 volume of 25 per cent barium acetate followed by the addition of 3 volumes of absolute alcohol. The pH of the solution was adjusted to 8.2 with 18 per cent ammonium hydroxide and the same kept for about 12 hours at 1-2^{o} for the complete precipitation of sugar phosphates. The supernatant was centrifuged off and discarded. The precipitate was dissolved in five volumes of water. This was passed through Dowex-50-4 x (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 per cent 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 extracts were first treated with barium acetate to remove the acids and the polysaccharides were then precipitated by alcohol.

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

Estimation of Citric acid:

The TCA extract 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 used directly 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 lactic acid:

The TCA extract 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 amino nitrogen:

The TCA extract was centrifuged at 2500 x g for 15 minutes and the residue was re-extracted with 10 per cent TCA and centrifuged. The supernatants were combined and diluted 10 times with water and used for the estimation of amino nitrogen by the method of Frame et al (1943). The residue obtained was used for the estimation of protein nitrogen by the micro-kjeldhal method.

Estimation of free glutamic acid, aspartic acid, alanine, gamma-aminobutyric acid, asparagine and glutamine contents of tissue:

The tissue was ground with twice its volume of 75 per cent alcohol in a mortar for 15 minutes. The extract was centrifuged at 2500 x g for 15 minutes and the supernatant collected. The residue was re-extracted, centrifuged and the supernatants were combined. The supernatant was evaported to dryness under vacuum. The residue left after evaportion was taken up in 10 per cent isopropanol in water at pH 5.5, centrifuged ant supernatant made up to a definite volume. This extract was used for the quantitative estimation of free amino acids. 0.1 ml of this solution was spotted on a Whatman No.1 filter paper and the separation and estimation carried out by two dimensional paper chromatography. The method used was the same as described by Hakkinen and Kulonen (1961) except that, for the estimation of amino acids other than GABA, the papers were run three times in the first solvent system. The solvent was allowed to run in the paper to the same distance during each run. After every run the paper was air dried. It was possible to get 90 per cent recovery of the amino acids estimated by this method.

Preparation of tissue slices for studies on respiration:

Freshly detached fruit were brought to the laboratory under ice. The outer green skin and the inner white skin were removed. The edible portion was cut into two and the seeds were removed. The remaining fruit segments were cut into slices of about 0.5 mm thickness using a stainless steel blade. The slices were weighed and used.

Manometric experiments:

Manometric measurements were carried out at 37^{o} using conventional Warburg appartus. Each Warburg flask contained in its main compartment 50 micromoles of substrate, 100 micromoles of potassium phosphate buffer, pH 7.0, four tissue slices, each weighing 100-120 mg. and water to make up the volume to 3.0 ml. The central well contained 0.1 ml of 6N KOH. A blank was run which contained all the components except the substrate. The gas phase was air. After adding the components the flasks were attached to the manometers and secured by rubber bands. The flasks were kept open to the atmosphere by opening the taps and were allowed to equilibrate in the bath for 15 minutes. At the end of this period, the taps were closed. Readings were taken every 30 minutes for two hours, the manometers being shaken all the

time. The endogenous respiration of tissue was expressed in terms of microlitres of oxygen utilized.

Estimation of ammonia

The tissue was ground with two volumes of 70 per cent alcohol in a mortar for 15 minutes. The extract was centrifuged at 2500 x g for 15 minutes and the residue re-extracted with 70 per cent alcohol and centrifuged. The supernatants were combined and made up to a definite volume. Ammonia was estimated from this solution by the nesslerization method described by Koch and McMeekin (1924).

Estimation of protein:

The protein content of the enzyme extract was estimated according to the method of Lowry et al (1951).

Preparation of enzyme extract for detecting glycolytic enzymes:

For the preparation of enzyme extracts the fruits were peeled and kept in a mortar for about 12 hours at -15°. All the operations described were carried out in a cold room maintained at $0-4^{\circ}C$ unless otherwise specified.

The frozen tissue was homogenized with three volumes of 0.1 M tris (tris-hydroxymethyl aminomethane) buffer,

pH 7.5, for about 10 to 15 minutes adjusting the pH to 7.0 with ammonium hydroxide (0.5 per cent v/v) when necessary. The extract was filtered through a double layer of cheese cloth and centrifuged at 6500 x g at 0^{o} for fifteen 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 fructose-diphosphate aldolase, phosphoglucomutase and phosphohexose-isomerase.

A 33 per cent phosphate extract (0.02M PO_4 buffer, pH 7.0) was used without centrifugation for the estimation of phosphofructokinase.

Preparation of enzyme extract for determining enzymes involved in the breakdown of protein and utilization of glutamate:

A 25 per cent tissue extract was prepared by grinding the frozen fruit tissue for 10 minutes using 0.05M potassium phosphate buffer, pH 7.5 as grinding medium. The crude homogenate was filtered through chesse cloth. The filtrate obtained was treated with Triton X-100 (1 mg/ml) for 30' and centrifuged at 10,000 x g for 20 minutes. The supernatant was used for

activities of glutamate dehydrogenase, alanine aminotransferase, aspartate aminotransferase and glutamate decarboxylase.

For the detection and partial purification of glutamine synthetase the enzyme extract was prepared using 0.02M sodium bicarbonate buffer, pH 8.0 as grinding medium. The crude homogenate was centrifuged at 10,000 x g and 0^{o} for 20 minutes. The supernatant was used for estimation of the activity of glutamine synthetase.

For the estimation of the activities of asparagine synthetase and protease the extract was prepared as described above using 0.05M tris buffer, pH 7.5 as grinding medium. The crude homogenate was centrifuged at 10,000 x g and 0^{o} for 20 minutes and the supernatant obtained was used for the enzyme assays.

The details of the assay systems and procedures used for the estimation of enzyme activity are summarized in Table 2. Except in the case of hexokinase, glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase and glutamate dehydrogenase which involved spectrophotometric determinations the assay components were added to test tubes kept on ice.

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 umole; glucose-6-phosphate dehydrogenase, 0.1 ml (0.2 unit*), water to a 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 1ON HC1 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 mu at 1 minute interval	formation of ketosugar in the supertant measured by the method of Roe *et al* (1949).
enzyme unit	amount of enzyme required to reduce 1 umole of NADP per minute	amount of enzyme required to form 1 umole of fructose-6-phosphate per minute.

* One unit being the amount necessary for the reduction of 1 umole 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 unit*) bovine albumin, 200 micrograms	hydrazine sulphate, 150 micromoles, iodoacetate, 0.5 micromoles, volume made to 1.0 ml. with water
temperature and period of incubation	37^o, 30 minutes	37^o, 30 minutes
start of reaction	substrate added	enzyme added
termination of reaction	1.0 ml of 20 per cent TCA added	2.0 ml of 10 per cent 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 Legninger (1949)
enzyme unit	amount of enzyme required to form 1 µmole of fructose 1:6 di PO_4 per minute	amount of enzyme required to form 1 µmole of triose phosphate per minute

* One unit being the amount necessary for the breakdown of 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^{o}, 3 minutes	30^{o}, 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 mu at 30 second interval	oxidation of $NADH_2$ measured by change in optical density at 340 mu at 1 minute interval
enzyme unit	amount of enzyme required to reduce 1 umole of NAD per minute	amount of enzyme required to oxidize 1 umole of $NADH_2$ per minute.

Table 2 contd...

Details of assay system and procedure	Phosphoglucomutase (E.C.,2.7.5.1)	Protease
reference	Najjar (1955)	Greenberg (1955)
buffer	tris, pH 7.5, 20 micromoles	phosphate buffer, pH 6.0, 50 micromoles
substrate	glucose-1-phosphate, 5 micromoles	0.5 ml casein (containing 2 mg/ml casein adjusted to pH 6.5)
amount of enzyme extract	0.2 ml	0.5 ml
other components	Neutralized cysteine hydrochloride, 10 micromoles; $MgSO_4$, 1 micromole; final volume, 1 ml.	final volume made to 2 ml with water
temperature and period of incubation	37°, 15 minutes	37°C, 30 minutes
initiation of reaction	enzyme added	enzyme added
termination of the 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 ml of 10 percent TCA added and the supernatant centrifuged off
modification for blank	enzyme added after incubation	TCA added before incubation
parameter measured	Disappearance of glucose-1-phosphate	formation of tyrosine in the supernatant (Folin and Ciocalteau, 1939).
enzyme unit	amount of enzyme required for the formation of 1 umole of acid stable phosphate per minute	amount of enzyme required to form 1 micromole of tyrosine per 30 minutes.

Table 2 contd...

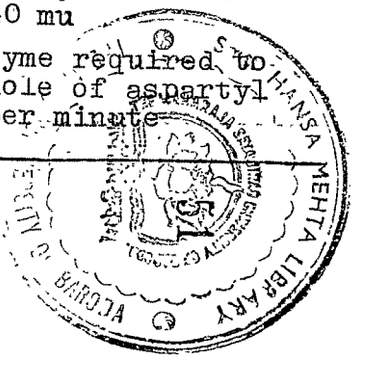
Details of assay system and procedure	Glutamate dehydrogenase (E.C., 1.4.1.2)	Alanine aminotransferase (E.C., 2.6.1.2)
reference	Bulen (1956)	Rietman and Frankel (1957)
buffer	tris, pH 8.0, 100 micromoles	phosphate, pH 7.5, 50 micromoles
substrate	2-oxoglutarate, 3 micromoles	L-alanine, 50 micromoles
enzyme extract	0.2 ml	0.1 ml
other components	$NADH_2$, 0.1 micromole; ammonium sulfate, 300 micromoles; final volume 3.0 ml	2-oxoglutarate, 10 micromoles; final volume 1.0 ml.
temperature and period of incubation	30°; 2 minutes	37°; 30 minutes
initiation of the reaction	2-oxoglutarate added	enzyme added
termination of reaction	-	1 ml of 0.02 per cent DNPH added
modification for blank	2-oxoglutarate omitted	enzyme added after incubation
parameter measured	oxidation of $NADH_2$ measured by changes in optical density at 340 mu at 30 seconds intervals	formation of pyruvate measured by colour intensity of the DNPH derivative at 540 mu
enzyme unit	amount of enzyme required to oxidise 1 micromole of $NADH_2$ per minute	amount of enzyme required to form 1 micromole of pyruvic acid per minute.

Table 2 contd...

Details of assay system and procedure	Aspartate aminotransferase (E.C., 2.6.1.1)	Glutamate decarboxylase (E.C.,4.1.1.15)
reference	Shah and Ramakrishnan (1963)	Rajalakshmi et al (1965)
buffer	phosphate, pH 7.5, 50 micromoles	phosphate, pH 6.0, 50 micromoles
substrate	oxaloacetate, 10 micromoles	L-glutamate, 10 micromoles
enzyme extract	0.4 ml	0.3 ml
other components	L-glutamate, 20 micromoles; final volume 2.0 ml	pyridoxal phosphate, 0.015 micromoles; final volume 1.0 ml
temperature and period of incubation	37^{o}; 30 minutes	37^{o}; 30 minutes
initiation of the reaction	enzyme added	enzyme added
termination of reaction	3.0 ml of absolute alcohol added	1.0 ml of absolute alcohol added
modification for blank	enzyme added after incubation	enzyme added after incubation
parameter measured	aspartate formed assayed chromatographically (Giri et al, 1952) using butanol : water : acetic acid (40:7:5) as solvent system	formation of GABA assayed chromatographically (Giri et al, 1952) using butanol: water: acetic acid (40:7:5) as solvent system
enzyme unit	amount of enzyme required to form 1 micromole of aspartic acid per minute	amount of enzyme required to form 1 micromole of GABA per minute

Table 2 contd...

Details of assay system and procedure	Glutamine synthetase (E.C.,6.3.1.2)	Asparagine synthetase
reference	Elliott (1955)	Elliott (1955)
buffer	tris, pH 7.5, 50 micromoles	tris, pH 7.0, 50 micromoles
substrate	L-glutamate, 30 micromoles	L-aspartate, 30 micromoles
enzyme	0.15 ml	0.1 ml
other components	ATP (Na salt), 10 micromoles; $MgSO_4$, 10 micromoles; neutralized hydroxylamine hydrochloride, 200 micromoles, final volume, 2.0 ml	ATP (Na salt), 10 micromoles; $MgSO_4$, 10 micromoles; neutralized hydroxylamine hydrochloride, 200 micromoles; final volume 2.0 ml
temperature and period of incubation	$37^{o}C$; 30 minutes	$37^{o}C$, 30 minutes
initiation of reaction	enzyme added	enzyme added
termination of reaction	0.8 ml of ferric chloride reagent (the reagent contained equal volumes of 10 percent $FeCl_3 \cdot 6H_2O$ in 0.2N HCl, 24 percent TCA and 50 percent v/v HCl) added	0.8 ml of ferric chloride reagent (the reagent contained equal volumes of 10 percent $FeCl_3 \cdot 6H_2O$ in 0.2 NHCl, 24 percent TCA and 50 percent v/v HCl) added
modification for blank	glutamate omitted	aspartate omitted
parameter measured	formation of micromoles of glutamyl hydroxamic acid measured by colour intensity of ferric-hydroxamate complex at 540 mu	formation aspartyl hydroxamic acid measured by colour intensity of ferric-hydroxamate complex at 540 mu
enzyme unit	amount of enzyme required to form 1 micromole of glutamyl hydroxamate per minute	amount of enzyme required to form 1 micromole of aspartyl hydroxamate per minute



Except where otherwise specified Klett-summerson colorimeter was used for the colour estimation.

Specific activity of the enzyme is defined as enzyme units per milligram protein.

Partial purification of glutamate dehydrogenase:

All the operations to be described below were carried out at 0 to 4°.

40 ml of the crude extract (A) were brought to 30 per cent saturation with solid ammonium sulfate (21 g/100 ml) and allowed to stand at 0° for 10 minutes with gentle stirring. The precipitate was removed by centrifugation at 2000x g at 0° for 10 minutes and the supernatant brought to 50 per cent saturation by adding a further 14g of ammonium sulfate per 100 ml of solution. The resulting precipitate was removed by centrifugation at 7000 x g at 0° for 15 minutes and dissolved in 0.02M potassium phosphate buffer, pH 7.5 to a final volume of 15 ml. This solution was dialysed for 3 hours against 2 litres of 0.02M potassium phosphate buffer, pH 7.5 (Fraction B).

Fourteen ml of fraction B were added to the residue obtained after the centrifugation of 10 ml of calcium phosphate gel (10 mg/ml). The mixture was allowed to stand at 0° for

10 minutes with gentle stirring and the resulting solution centrifuged at 2500 x g at 0^{o} for 15 minutes and the supernatant collected. The residue was eluted with 14 ml of 0.2M potassium phosphate buffer, pH 7.5. The eluate was dialysed against 2 litres of 0.02M potassium phosphate buffer, pH 7.5 for 12 hours (Fraction C).

Fourteen ml of fraction C was further purified using DEAE-cellulose column which was activated as follows: 12 g of DEAE-cellulose were shaken gently with 200 ml of 1N HC1 for 5', allowed to settle and the supernatant decanted. This was repeated twice. The acid washed resin was then activated with successive amounts of 1N NaOH by shaking with 500 ml of alkali each time for 5 minutes. A total volume of 2 litres of 1N NaOH was used for the activation of 15 g of the resin. Finally the resin was washed free of excess of alkali with distilled water. To the activated DEAE-cellulose a sufficient quantity of 0.02M potassium phosphate buffer, pH 7.5 was added so as to form a thin slurry. The slurry was added to a 50 ml burette of 0.8 cm diameter in which the bottom was plugged with glass-wool so as to form a column of 20 cm in length when settled. The column was equilibrated with 50 ml of 0.02M potassium phosphate buffer, pH 7.5. Twelve ml of fraction C were introduced in the column. The solution was allowed to pass through the column till 0.5 ml of the solution was

left on the surface of resin bed. 100 ml portions of potassium phosphate buffer, pH 7.5 at concentrations of 0.025M, 0.05M and 0.1M were then passed through the column successively. The rate of flow of the buffer was adjusted to 1 ml per minute. Fractions of 12 ml each were collected manualy. The glutamate dehydrogenase activity of the fractions was estimated. The third fraction obtained with 0.1M potassium phosphate buffer was found to show enzyme activity (Fraction D).

For studying the balance of reaction of glutamate dehydrogenase the assay system was the same as given in Table 2, except that 1 ml of enzyme and 0.5 umole of $NADH_2$ were used instead of 0.2 ml of enzyme and 0.1 umole of $NADH_2$. Four tubes containing the assay system were incubated at $37^{o}C$ for 2 minutes. After incubation 2 ml of absolute alcohol and 2 ml of 10 per cent TCA were added to the first two tubes for the estimation respectively of glutamic acid and keto acid. The additions were made before incubation in the case of the blanks. Keto acid content was estimated by the method of Friedemann (1957) and glutamic acid content by the paper chromatographic method described by Giri et al (1952).

Partial purification of alanine aminotransferase and aspartate aminotransferase

Fortyfive ml of the crude extract (A) were subjected

to ammonium sulfate fractionation and calcium gel adsorption in the same manner as in the case of glutamate dehydrogenase except, that 10 ml of 0.02M potassium phosphate buffer, pH 7.5 was used for dissolving the ammonium sulphate fraction (Fraction B) and 9 ml of fraction B was used for adsorption on calcium gel. The supernatant obtained after calcium gel adsorption (Fraction C) was found to show alanine aminotransferase and aspartate aminotransferase activities and this was used for further purification using DEAE-cellulose column chromotography.

Eight ml of calcium phosphate gel supernatant (Fraction C), were added to DEAE column. The elution was carried out in the same manner as in the case of glutamate dehydrogenase. Eight ml fractions were collected manualy. The fourth and the fifth fraction were found to contain alanine aminotransferase and aspartate aminotransferase. They were combined and used for characteristic studies.

For studying the balance of reaction of alanine aminotransferase and aspartate aminotransferase the assay systems were the same as given in Table 2 except that in the case of alanine aminotransferase 0.4 ml of enzyme was used instead of 0.1 ml of enzyme. Four tubes containing the assay system for alanine aminotransferase were incubated at 37^{o} for 30 minutes. For the estimations of

alanine and pyruvic acid respectively 2 ml of absolute alcohol and 2 ml of 10 per cent TCA were added after incubation. The additions were made before incubation in the case of the blanks. Pyruvic acid was estimated by the method of Friedemann (1957) and alanine by the paper chromatographic method described by Giri et al (1952).

In the case of aspartate aminotransferase the assay tubes were incubated at 37° for 30 minutes. The enzyme was added after incubation in the case of the blank. Two ml of absolute alcohol were added after incubation. Glutamic acid and aspartic acid were estimated by the paper chromotographic method of Giri et al (1952).

Partial Purification of glutamate decarboxylase:

Thirty ml of the crude extract (A) were subjected to ammonium sulfate fractionation and calcium gel adsorption in the same manner as in the case of glutamate dehydrogenase, except, that 30 ml of 0.02M potassium phosphate buffer, pH 7.5, was used for dissolving the ammonium sulfate fraction (Fraction B) and twentyfive ml of fraction B was used for adsorption on calcium gel. After adsorption the calcium gel was eluted with 25 ml portions of potassium phosphate buffer, pH 7.5, at concentrations of 0.10M and 0.15M. The eluate obtained with

0.15M potassium phosphate buffer, pH 7.5 which was found to show the enzyme activity was dialysed against 2 litres of 0.01M potassium phosphate buffer, pH 7.5 for 12 hours (Fraction C) and used for further purification using sephadex G-200 column.

The sephadex column was set according to the procedure described by Andrews (1964). Sephadex G-200 was suspended in 0.01M potassium phosphate buffer, pH 7.5, for 2 days and small particles floating at the top were removed by decantation. The gel was deaerated under reduced pressure and poured into a chromatography glass column (60 cm x 2.5 cm). The excess liquid was allowed to percolate through the disc at the bottom taking care not to allow the gel to get dried up. When the gel height was 20 cm a reservoir containing 200 ml of 0.01M potassium phosphate buffer, pH 7.5 was set at the top of the glass column and the flow of the buffer maintained at 30 ml/hr. After washing the column with the above buffer, Fraction C was introduced into the column. The solution was allowed to pass through the column till 0.5 ml of the solution was left on the surface of the gel bed. Two hundred ml of 0.02M potassium phosphate buffer, pH 7.5 were then introduced into the column and allowed to pass through at a flow rate of 30 ml/hour. Fractions of 10 ml each were collected manualy. The glutamate decarboxylase activity of the

fractions was estimated. The third and fourth fractions which were found to show enzyme activity were combined together and used.

Glutamine and asparagine synthetases:

The crude extracts prepared for these two enzymes were subjected to ammonium sulfate fractionation and calcium gel adsorption as described for alanine aminotransferase and aspartate aminotransferase except that 0.02M sodium bicarbonate, pH 8.0 was used for dissolving the precipitate obtained during ammonium sulfate fractionation in the case of glutamine synthetase and 0.05M tris buffer, pH 7.5, in the case of asparagine synthetase. The calcium phosphate gel supernatant (containing 1.2 mg protein/ml) was used as enzyme source for studies on optimum conditions for enzyme activity.

Protease:

Fruits just detached from the plant were peeled and finely cut in a cold room maintained at 0-4^{o}. They were then weighed and blended with 10 volumes of cold acetone (-10^{o}) for one minute in a waring blendor. After filtration in a buchner funnel with suction, the residue obtained was spread over a large surface and allowed to dry at room temperature. It was stored in air tight tubes at 4^{o} till use.

A three per cent extract of the acetone powder was prepared by stirring the powder in 0.05M tris buffer, pH 7.5 for 30 minutes at 0^{o}. The suspension was centrifuged at 10,000 x g for 20 minutes. The supernatant (containing 0.8 mg protein/ml) was used for studies on optimum conditions for protease activity.