CHAPTER III

RESULTS AND DISCUSSION

As stated earlier, these studies were concerned with the chemical and biochemical make up of the fruit tissues of *Citrus acida* with special reference to the intermediates and enzymes of the glycolytic cycle and glutamate metabolism.

Table 3 shows the composition of the fruit tissue with regard to total sugar, free sugar, glucose, sugar phosphates, lactic acid and citric acid. These results are graphically represented in Fig. 2. The increase in citric acid content in the mature fruit is associated with decreases in total sugar, free sugar, glucose, sugar phosphates and lactic acid. It is interesting to note that glucose-1-phosphate could not be detected in mature fruit.

Data on the specific activities of the glycolytic enzymes as well as pyruvate dehydrogenase and citrate synthase in young and mature fruits are presented in Table 4 and Fig. 3. Although the activity of hexokinase shows some increase in the mature fruit, the rate of

Table 3: Comparative data on the chemical composition of young and mature fruits of Citrus acida

(values expressed per 100 g dry weight)

	I	II	$\frac{II}{I}$ X 100*
diameter of fruit (cm)	young fruit (1.4-1.6)	mature fruit (4.0)	
Total sugar (µmoles)	$1.4x10^5$ ($1.25x10^5$, $1.5x10^5$)	$0.7x10^5$ ($0.6x10^5$, $0.8x10^5$)	50
Free sugar (µmoles)	$0.8x10^5$ ($0.7x10^5$, $0.9x10^5$)	$0.3x10^5$ ($0.2x10^5$, $0.4x10^5$)	40
Glucose (µmolse)	4610 (4150,5070)	3200 (3100,3300)	70
Glucose-1-phosphate (µmolss)	120 (115,125)	0	0
Glucose-6-phosphate (µmoles)	175 (165,185)	44 (40,48)	25
Fructose-6-phosphate (µmoles)	350 (340,360)	38 (35,41)	10
Fructose-1,6-diphosphate (µmoles)	49 (42,56)	18 (16,20)	35
Lactic acid (µmoles)	2640 (2600,2680)	1000 (910,1090)	40
Citric acid (µmoles)	$1.8x10^4$ ($1.6x10^4$, $2.0x10^4$)	$24x10^4$ ($22x10^4$, $26x10^4$)	1300

Values based on two trials with individual values shown in parentheses.

* Corrected to round figure.

Fig. 2: Chemical composition of young and mature fruits of Citrus acida

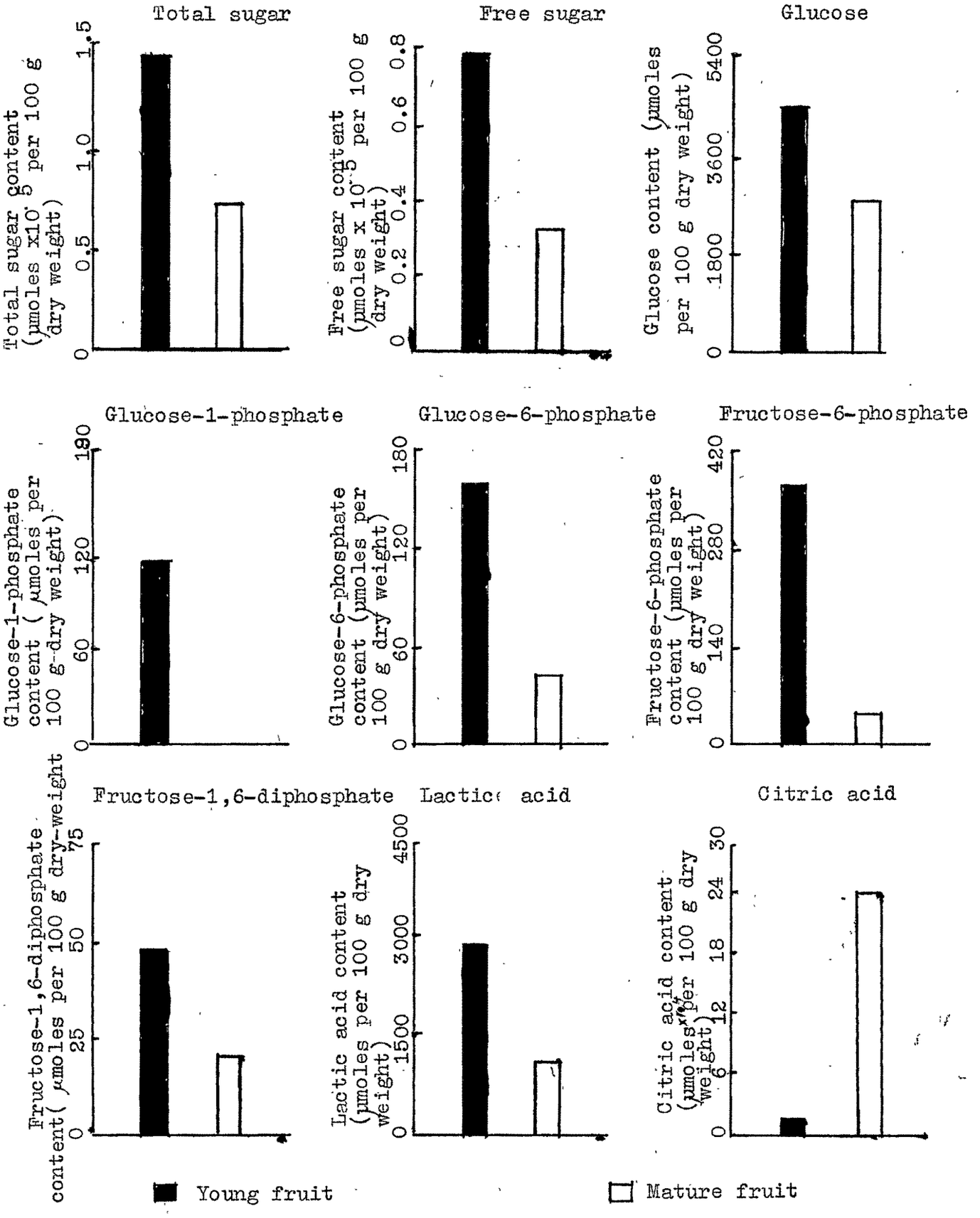
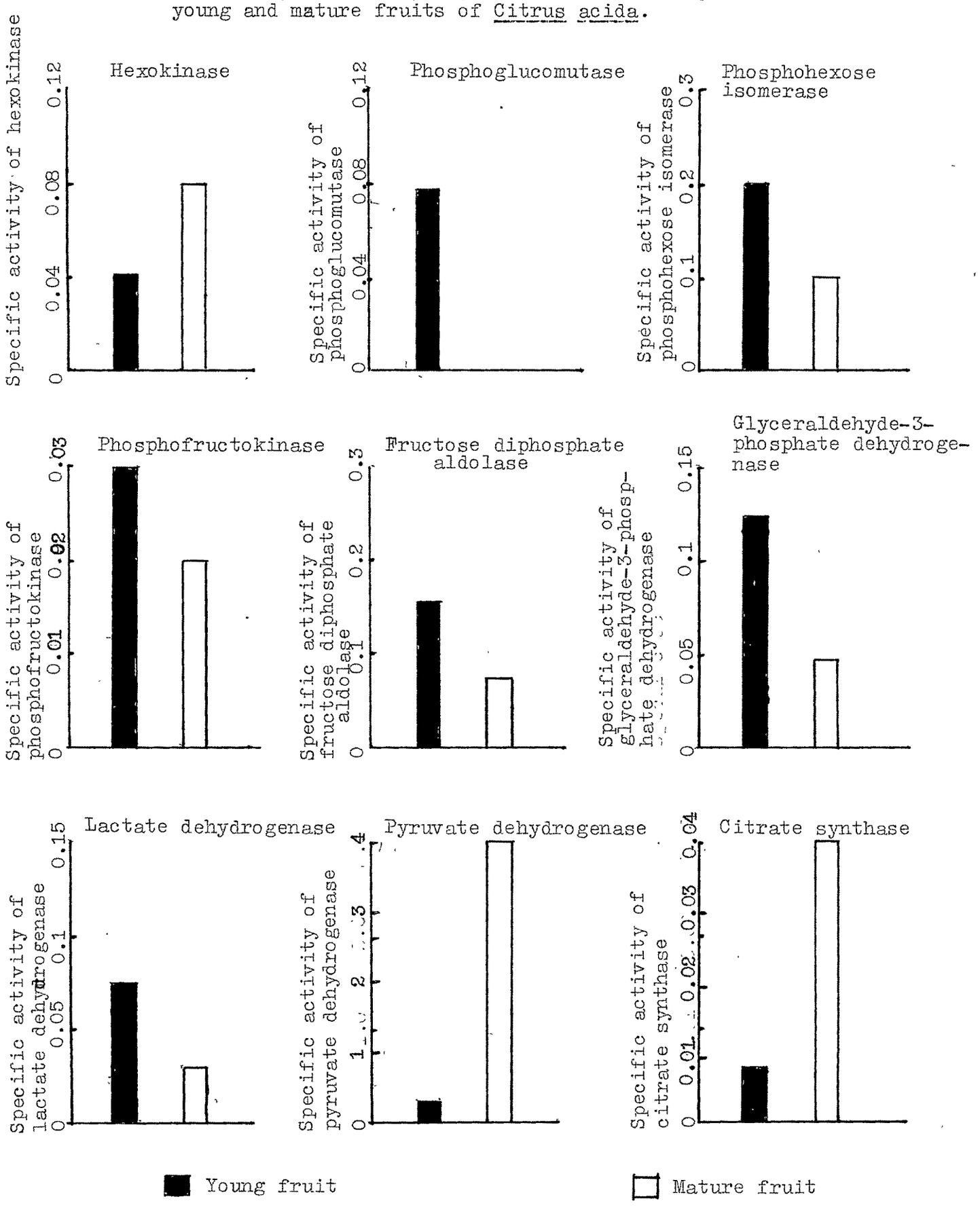


Table 4: Comparative data on the specific activities of the enzymes of glycolytic cycle in the young and mature fruits of Citrus acida

	I	II	$\frac{II}{I}$ X 100
diameter of fruit (cm)	youmg fruit (1.4-1.6)	mature fruit (4.0)	
Present studies			
Hexokinase	0.04 (0.035,0.045)	0.08 (0.07,0.09)	200
Phosphoglucomutase	0.07 (0.065,0.075)	0.00 (0.00,0.00)	0
Phosphohexose isomerase	0.20 (0.18,0.22)	0.10 (0.09,0.11)	50
Phosphofructokinase	0.03 (0.025,0.035)	0.02 (0.015,0.025)	70
Fructose diphosphate aldolase	0.16 (0.15,0.17)	0.07 (0.06,0.08)	44
Glyceraldehyde-3-phosphate dehydrogenase	0.12 (0.10,0.14)	0.04 (0.03,0.05)	33
Lactate dehydrogenase	0.07 (0.06,0.08)	0.03 (0.025,0.035)	43
Ramakrishnan and Varma (1959)			
Pyruvate dehydrogenase	0.33	4.0	1200
Citrate synthase	0.007	0.04	570

Values based on two trials with individual values shown in parentheses.

Fig.3: Specific activities of the enzymes of glycolytic cycle as well as pyruvate dehydrogenase and citrate synthase in the young and mature fruits of Citrus acida.



glycolysis is less in the mature fruit. On the other hand, previous studies in this laboratory (Ramakrishnan and Varma, 1959) showed enormous increases in pyruvate dehydrogenase and citrate synthase which are involved in the formation of citric acid and its precursor. This pattern is consistent with the data on chemical composition presented earlier.

Thus the operation of the glycolytic cycle is these fruits is consistent with expectation as earlier studies confirmed the operation in the same of the TCA cycle.

In a separate study in this department it is found that enzymes involved in the formation of ascorbic acid from glucose via glucose-1-phosphate and D-glucuronolactone are very active in young fruit tissues (Parekh et al., 1969). It is possible that glycolytic activity in young fruit tissues helps in generating more glucose-1-phosphate required for ascorbic acid formation.

As mentioned earlier one of the enzymes of the TCA cycle, aconitase, is absent in the mature fruit raising the question as to how respiration takes place in the same.

Other reports suggest that amino acids formed from protein breakdown can be utilized for respiration. In orde r to investigate whether this occurs in the present case estimations were made of protein, amino nitrogen and selected amino acids, namely, glutamic acid, aspartic acid, alanine glutamine, asparagine and gamma aminobutyric acid in the young and mature fruit. Estimations were also made of the specific activities of the enzymes suspected to be involved in such breakdown and utilization, namely, protease, glutamate dehydrogenase, alanine aminotransferase, aspartate aminotransferase, glutamate decarboxylase, glutamine synthetase and asparagine synthetase.

The data on amino acids and protein and amino nitrogen presented in Table 5 and Fig 4 show the expected increase in the mature fruit in amino acids and amino nitrogen and decrease in protein nitrogen.

Since there is a breakdown of protein and increase in free amino nitrogen as well as asparagine and glutamine in the mature fruit tissues studies were made to find out whether the mature fruit tissues can utilize free amino acids for respiration. Preliminary studies carried out showed that only glutamic acid can be used for respiration by fruit tissue slices. Further studies were conducted to

Table 5: Comparative data on protein and amino nitrogen as well as free amino acid contents of the young and mature fruits of Citrus acida

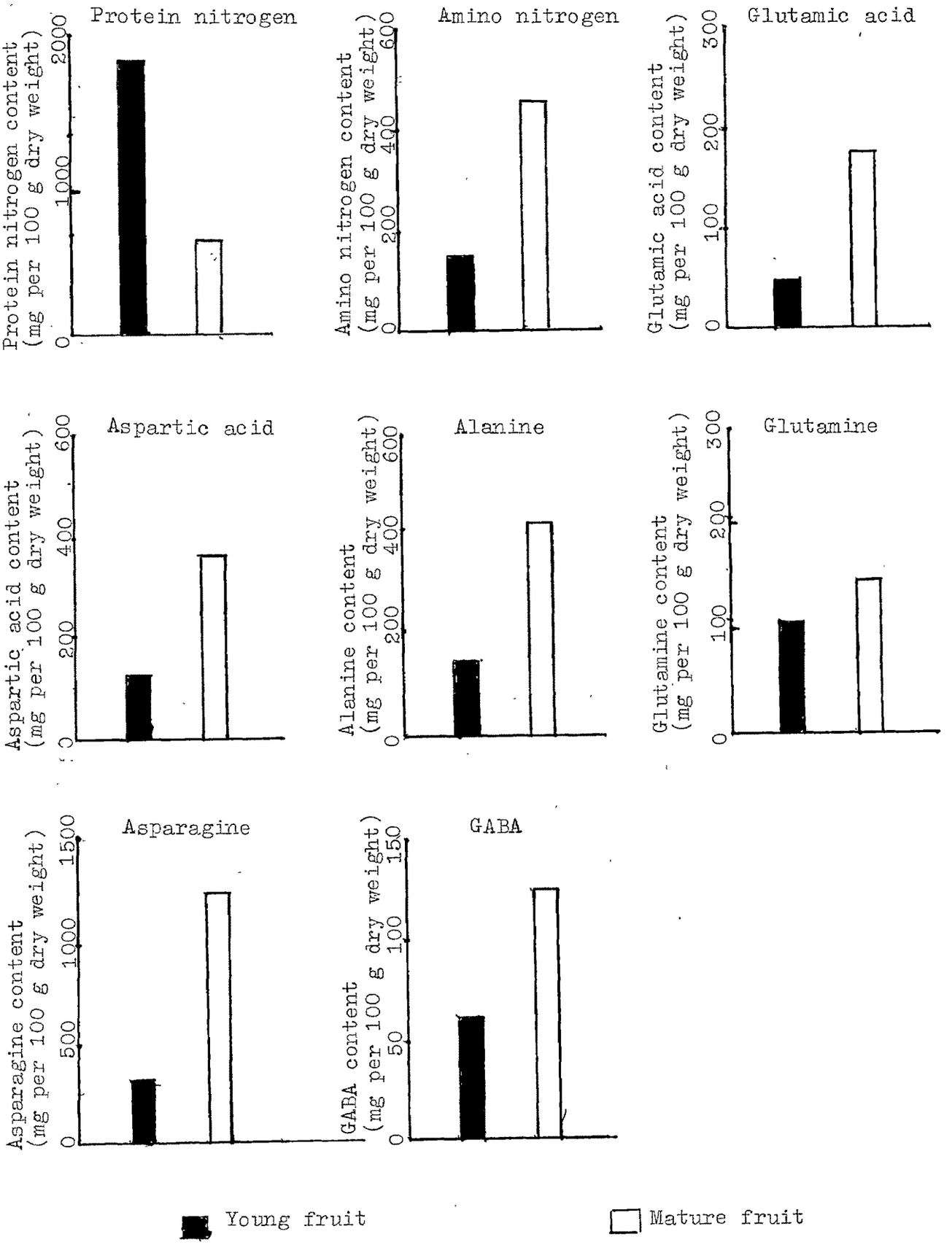
(Values expressed as mg per 100 g dry weight)

diameter of fruit (cm)	I young fruit (1.4–1.6)	II mature fruit (4.0)	$\frac{II}{I}$ X 100*
Protein nitrogen	1800 (1750,1850)	600 (580,620)	30
Amino nitrogen	150 (140,160)	450 (440,460)	300
Glutamic acid	55 (52,58)	170 (165,175)	300
Aspartic acid	130 (120,140)	360 (352,368)	280
Alanine	162 (158,166)	435 (430,440)	270
Glutamine	110 (105,115)	150 (142,158)	140
Aspargine	320 (310,330)	1260 (1200,1320)	400
GABA	60 (58,62)	125 (120,130)	200

Values based on two trials with individual values shown in parentheses.

* Corrected to round figure.

Fig.4: Protein and aminonitrogen as well as free amino acid contents of young and mature fruits of Citrus acida



determine to what extent glutamate is utilized in the presence and absence of aspartate. The results of these studies are shown in Tables 6a and 6b. It can be seen that the uptake of glutamate and aspartate from medium is twice as much in the mature fruit as in the young fruit. Further, the addition of glutamate has no effect on oxygen uptake in the young fruits whereas it increases the same by 50 per cent in mature fruit. The further addition of aspartate has no effect on oxygen uptake. Incidentally the endogenous respiration is greater in the young fruit suggesting a higher rate of respiration in these tissues. This is consistent with the greater activity in the young fruit of glycolytic enzymes and the complete operation of the TCA cycle. Addition of glutamic acid increases the formation of ammonia, but when aspartate is also added, this increase is reversed accompanied by an increase in asparagine content. This suggests that the increase in asparagine in the mature fruit is of metabolic significance. Such an increase is also found in the mature tissue slices when both glutamate and aspartate are added to the medium. But no such increase is found in the young tissue. Nor is there any increase in the concentration of glutamine.

Table 6a: Studies on the utilization of glutarate for respiration by tissue slices of fruit tissues of Citrus acida (period of experiment - 2 hours).

Substrate added		O_2 uptake (µl)	Tissues							Medium						
			NH_3 content (µmole)	Glu (µmole)	Asp (µmole)	Ala (µmole)	Glu (NH_2) (µmole)	Asp (NH_2) (µmole)	GABA (µmole)	NH_3 content (µmole)	Glu (µmole)	Asp (µmole)	Ala (µmole)	Glu (NH_2) (µmole)	Asp (NH_2) (µmole)	GABA (µmol
None	young fruit	75 (72,78)	0.3 (0.25, 0.35)	0.35 (0.33, 0.37)	1.1 (1.0, 1.2)	1.8 (1.7, 1.9)	0.8 (0.7, 0.9)	3.0 (2.8, 3.2)	0.3 (0.28, 0.32)	-	-	-	-	-	-	-
	mature fruit	40 (38,42)	0.5 (0.45, 0.55)	0.9 (0.85, 0.95)	1.8 (1.7, 1.9)	3.0 (2.9, 3.1)	0.6 (0.55, 0.65)	6.5 (6.4, 6.6)	0.7 (0.65, 0.75)	-	-	-	-	-	-	-
50 umoles glutamate	young fruit	74 (70,78)	0.3 (0.25, 0.35	2.85 (2.8, 2.9	1.7 (1.6, 1.8	2.5 (2.4, 2.6	0.85 (0.8, 0.9	3.1 (3.0, 3.2	0.8 (0.75, 0.85)	-	45 (44,46)	-	-	-	-	-
	mature	60 (58,62)	1.6 (1.5, 1.7)	6.9 (6.8, 7.0)	2.4 (2.3, 2.5)	4.0 (3.8, 4.2)	0.65 (0.6, 0.7)	6.7 (6.6, 6.8)	1.2 (1.1, 1.3)	-	40 (38,42)	-	-	-	-	-
50 umoles glutamate + 50 umoles aspartate	young fruit	75 (70,80)	0.3 (0.25, 0.35)	2.9 (2.8, 3.0)	4.4 (4.3, 4.5)	2.6 (2.5, 2.7)	0.85 (0.75, 0.85)	3.0 (2.8, 3.2)	0.85 (0.8, 0.9)	-	45 (44,46)	47 (46,48)	-	-	-	-
	mature fruit	60 (56,64)	0.65 (0.6, 0.7)	7.0 (6.9, 7.1)	5.9 (5.8, 6.0)	4.1 (4.0, 4.2)	0.65 (0.6, 0.7)	7.8 (7.6, 8.0)	1.15 (1.1, 1.2)	-	40 (39,41)	44 (43,45)	-	-	-	-

Table 6b: Studies on the utilization of glutamate for respiration by tissue slices of fruit tissues of Citrus acida (period of experiment - 2 hours)

Substrate added	Amount added (µmoles)		Amount absorbed into the tissue (µmoles)		Net O_2 µptake (ul)	Net tissue NH_3 (µmoles)	Change in free amino acid content of the tissue (µmoles)					
			Glu	Asp			Glu	Asp	Ala	Glu (NH_2)	Asp (NH_2)	GABA
		young fruit	5	-	-	-	2.5	0.6	0.7	0.05	0.1	0.5
Glutamate	50											
		mature fruit	10	-	20	1.1	6	0.6	1.0	0.05	0.2	0.5
Glutamate	50	young fruit	5	3	-	-	2.6	3.3	0.8	0.05	-	0.55
+												
Aspartate	50	mature fruit	10	6	20	0.15	6.1	4.1	1.1	0.05	1.3	0.45

Data on the specific activities of the enzymes concerned with the breakdown of protein and the utilization of glutamate are given in Table 7 and Fig. 5. All the enzymes are found to increase the maximum increase being found in the case of asparagine synthetase and aspartate aminotransferase and the minimum increase in the case of glutamine synthetase.

Thus, the results of these studies suggest that the mode of respiration of mature fruit is by the conversion of glutamate to 2-oxo-glutarate which gets further oxidized by the Krebs cycle. The formation of asparagine from aspartic acid and ammonia suggests itself the mechanism of ammonia detoxification in these tissues.

As mentioned earlier glycolytic enzymes were found to be active in both the young and mature fruit. The optimum conditions for the activities of these enzymes were sought to be identified.

The extracts used for these studies and the assay conditions are already detailed in Chapter 2.

The effect of varying the enzyme and substrate concentrations, period of incubation and pH on the activities of different enzymes of glycolysis are shown in Tables 8-14.

Table 7: Comparative data on the specific activities of the enzymes involved in protein breakdown and utilization of glutamate in the young and mature fruits of Citrus acida

	I	II	$\frac{II}{I}$ X 100
diameter of fruit (cm)	young fruit (1.4 - 1.6)	mature fruit (4.0)	
Protease	0.10 (0.09,0.11)	0.3 (0.25,0.35)	300
Glutamate dehydrogenase	0.11 (0.1,0.12)	0.35 (0.31,0.39)	320
Asparagine synthetase	0.50 (0.48,0.52)	2.5 (2.3,2.7)	500
Alanine aminotransferase	0.44 (0.39,0.49)	1.3 (1.2,1.4)	300
Aspartate aminotransferase	0.24 (0.22,0.26)	1.2 (1.1,1.3)	500
Glutamate decarboxylase	0.26 (0.23,0.29)	0.70 (0.6,0.8)	300
Glutamine synthetase	0.3 (0.25,0.35)	0.6 (0.5,0.7)	200

Values based on two trials with individual values shown in parentheses.

Fig.5: Specific activity of enzymes involved in protein breakdown and utilization of glutamate in young and mature fruits of Citrus acida

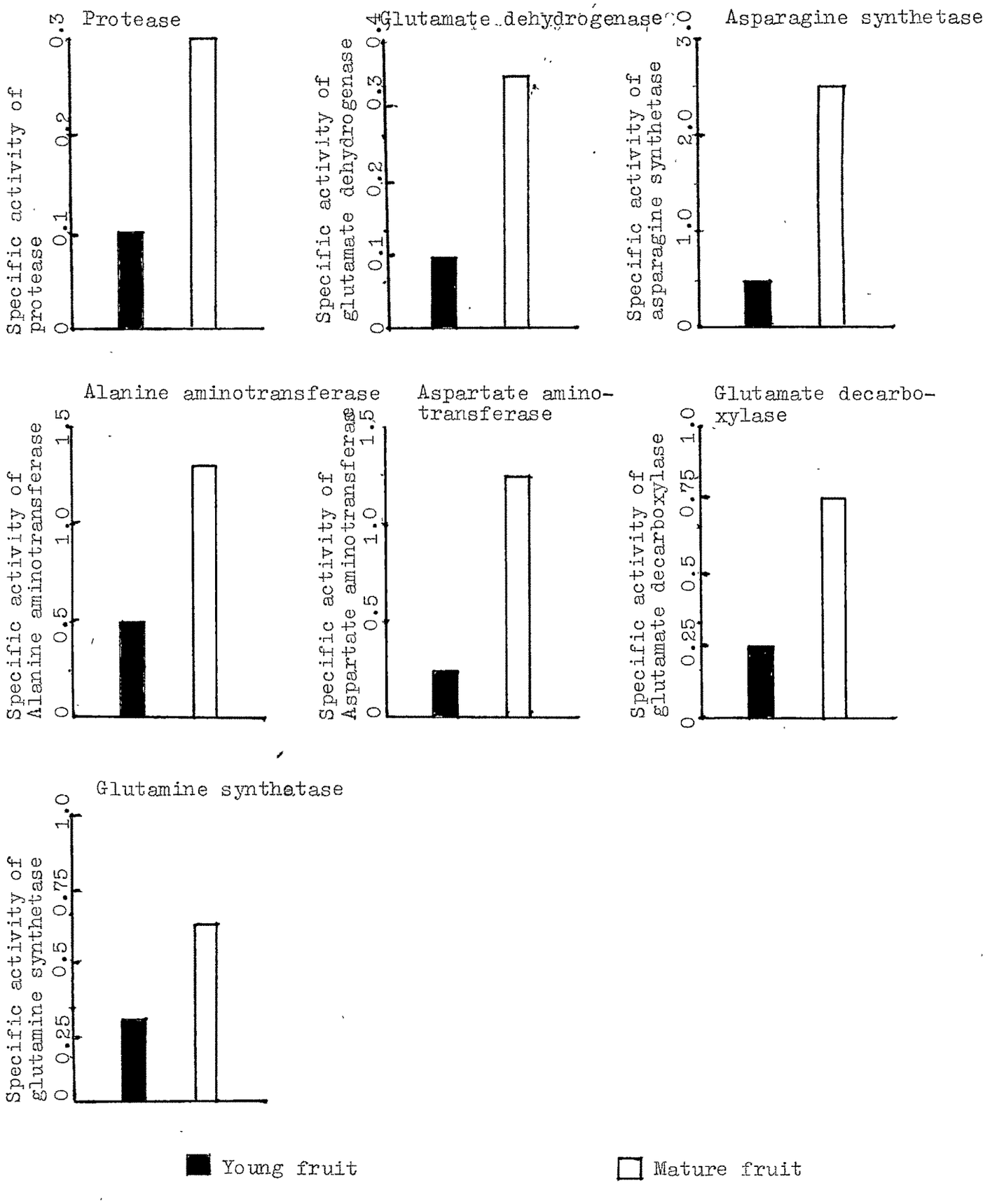


Table 8: Effect of enzyme concentration, period of incubation, glucose concentration and pH on hexokinase, activity in the fruit tissues of Citrus acida*+

Variable	$NADPH_2$ formed (micromoles)
Amount (ml) of enzyme preparation**	
0.00	0.000
0.05	0.012
0.10	0.028
0.15	0.040
0.20	0.051
0.25	0.054
0.30	0.054
Period of incubation (minutes)	
0	0.000
1	0.008
2	0.019
3	0.028
4.	0.041
5	0.051
6	0.056
7	0.056
Micromoles of glucose	
0.0	0.000
0.5	0.015
1.0	0.033
1.5	0.051
2.0	0.058
pH (phosphate buffer was used up to pH 7.0 and tris-HCl buffer was used for pH 7.0 - 9.0)	
Phosphate buffer 6.0	0.032
Phosphate buffer 7.0	0.042
Tris-HCl buffer 7.0	0.044
Tris-HCl buffer 7.5	0.051
Tris-HCl buffer 8.0	0.051
Tris-HCl buffer 9.0	0.047

* Assay system was the same as given in Table 2 except for the variables mentioned.

\+ A 33 per cent tris homogenate was prepared from edible portion of the fruit (1.4-1.6 cm diameter), centrifuged and the supernatant used as enzyme source.

** Protein content, 1.4 mg/ml.

Table 9: Effect of enzyme concentration, period of incubation, glucose-6-phosphate concentration and pH on phosphohexose isomerase activity in the fruit tissues of Citrus acida* +

Variable		Fructose-6-phosphate formed (micromoles)
Amount (ml) of enzyme preparation**		
	0.000	0.00
	0.025	0.18
	0.050	0.42
	0.075	0.65
	0.100	0.88
	0.125	0.92
	0.150	0.95
Period of incubation (minutes)		
	0	0.00
	10	0.30
	20	0.57
	30	0.88
	40	0.96
	50	1.12
Micromoles of glucose-6-phosphate		
	0	0.00
	1	0.15
	2	0.33
	3	0.50
	4	0.66
	5	0.88
	6	0.93
pH (phosphate buffer was used up to pH 7.0 and tris HCl buffer for pH 7.0-9.0 and glycine-NaOH buffer for pH 9-10)		
Phosphate buffer	6.0	0.65
	7.0	0.80
Tris-HCl buffer	7.0	0.81
	8.0	0.82
	9.0	0.88
Glycine-NaOH buffer	9.0	0.86
	10.0	0.76

* Assay system was the same as given in Table 2 except for the variables mentioned.

\+ A 33 per cent tris homogenate prepared from edible portion of the fruit (1.4-1.6 cm diameter) was used.

** Protein content, 1.5 mg/ml.

Table 10: Effect of enzyme concentration, period of incubation, fructose-6-phosphate concentration and pH on phosphofructokinase activity in the fruit tissues of *Citrus acida** +

Variable	Fructose diphosphate formed (micromoles)
Amount (ml) of enzyme preparation**	
0.2	0.20
0.4	0.38
0.5	0.56
0.6	0.60
0.7	0.64
Period of incubation (minutes)	
0	0.00
10	0.17
20	0.36
30	0.56
40	0.64
50	0.64
Micromoles of fructose-6-phosphate	
0	0.00
2	0.22
4	0.43
5	0.56
6	0.56
8	0.60
pH (tris. HCl buffer was used for pH 7.0-9.0 and glycine-NaOH buffer for pH 9.0-10.0).	
Tris-HCl buffer 7.0	0.38
Tris-HCl buffer 8.0	0.45
Tris-HCl buffer 9.0	0.56
Glycine NaOH buffer 9.0	0.56
Glycine NaOH buffer 10.0	0.48

* Assay system was the same as that given in Table 2 except for the variables mentioned.

\+ Crude 33 per cent phosphate homogenate prepared for edible portion of the fruit (1.4-1.6 cm diameter) was used.

** Protein content, 1.6 mg/ml.

Table 11: Effect of enzyme concentration, period of incubation, fructose-1,6-diphosphate concentration and pH on fructose diphosphate aldolase activity in the fruit tissues of Citrus acida*+

Variable	Triosephosphate formed (micromoles)
Amount (ml) of enzyme preparation**	
0.000	0.00
0.025	0.17
0.050	0.36
0.075	0.55
0.100	0.70
0.125	0.78
Period of incubation (minutes)	
0	0.00
10	0.25
20	0.48
30	0.70
40	0.81
50	0.88
Micromoles of fructose-1,6-diphosphate	
0.00	0.00
0.50	0.15
1.00	0.32
1.50	0.51
2.00	0.70
2.50	0.78
pH (tris HCl buffer used)	
7.0	0.59
7.5	0.63
8.0	0.70
8.5	0.66
9.0	0.62

* Assay system was the same as that given in Table 2 except for the variables mentioned.
\+ A 33 per cent tris homogenate prepared from the edible portion of fruit (1.4-1.6 cm diameter), was used.
** Protein content, 1.5 mg/ml.

Table 12: Effect of enzyme concentration, period of incubation, glyceraldehyde-3-phosphate concentration and pH on glyceraldehyde-3-phosphate dehydrogenase activity in the fruit tissues*+

Variable	$NADH_2$ formed (micromoles)
Amount (ml) of enzyme preparation**	
0.000	0.000
0.025	0.012
0.050	0.025
0.075	0.037
0.100	0.050
0.125	0.056
Period of incubation (minutes)	
0	0.000
1	0.017
2	0.035
3	0.050
4	0.058
5	0.058
Micromoles of glyceraldehyde-3-phosphate	
0.00	0.000
0.25	0.014
0.50	0.026
0.75	0.039
1.00	0.050
1.25	0.050
pH (tris-HCl buffer used)	
7.0	0.032
7.5	0.036
8.0	0.042
8.5	0.050
9.0	0.050

* Assay system was the same as that given in Table 2 except for the variables mentioned.

\+ A 33 per cent tris homogenate was prepared from edible portion of the fruit (1.4-1.6 cm diameter), centrifuged at 6500 x g and the supernatant used as enzyme source.

** Protein content, 1.4 mg/ml.

Table 13: Effect of enzyme concentration, period of incubation, pyruvate concentration and pH on lactate dehydrogenase activity in the fruit tissues of Citrus acida*+

Variable	$NADH_2$ oxidized (micromoles)
Amount (ml) of enzyme preparation**	
0.000	0.000
0.025	0.012
0.050	0.025
0.075	0.038
0.100	0.050
0.125	0.054
Period of incubation (minutes)	
0	0.000
1	0.008
2	0.018
3	0.030
4	0.041
5	0.050
6	0.055
7	0.055
Micromoles of sodium pyruvate	
0.00	0.000
0.25	0.013
0.50	0.024
0.75	0.037
1.00	0.050
1.25	0.056
pH (phosphate buffer used)	
6.0	0.032
6.5	0.043
7.0	0.050
7.5	0.050
8.0	0.047

* Assay system was the same as that given in Table 2 except for the variables mentioned.

\+ A 33 per cent tris homogenate was prepared from edible portion of the fruit (1.4-1.6 cm diameter), centrifuged at 6500 x g and the supernatant used as enzyme source.

** Protein content, 1.4 mg/ml.

Table 14: Effect of enzyme concentration, period of incubation, glucose-1-phosphate concentration and pH on phosphoglucomutase activity in the fruit tissues of Citrus acida*+

Variable	Glucose-1-phosphate disappeared (micromoles)
Amount (ml) of enzyme preparation**	
0.00	0.00
0.05	0.08
0.10	0.15
0.15	0.23
0.20	0.30
0.25	0.34
Period of incubation (minutes)	
0	0.00
5	0.09
10	0.17
15	0.30
20	0.35
25	0.39
Micromoles of glucose-1-phosphate	
0	0.00
2	0.12
4	0.22
5	0.30
6	0.32
8	0.32
pH (tris-HCl buffer used)	
7.0	0.22
7.5	0.30
8.0	0.26
8.5	0.24
9.0	0.24

* Assay system was the same as that given in Table 2 except for the variable mentioned.
\+ A 33 per cent tris homogenate prepared from edible portion of the fruit (1.4-1.6 cm diameter) was used.
** Protein content, 1.5 mg/ml.

The activity of each enzyme increased linearly with enzyme and substrate concentrations and period of incubation within a certain range.

The pH optima found in the present studies generally compare with those found for enzymes in other sources as can be seen from the comparisons made in Table 15. However, the pH optimum (9.0) for phosphofructokinase was found to be higher than the value of 8.0 reported for rabbit brain. That for lactate dehydrogenase was somewhat less than the value of 7.5 reported for other sources.

Data on the change in specific activities of the enzymes involved in the breakdown of protein and the utilization of glutamate in mature fruit tissues are given earlier. Further studies were made on the purification and characterization of these enzymes.

The yield of glutamate dehydrogenase and the degree of purification achieved in the different fractions are shown in Table 16. The specific activity of glutamate dehydrogenase in the DEAE-cellulose eluate is seen to increase more than 60 fold over that of the crude extract, but this increase is accompanied by a loss of 66 per cent in total activity.

Table 15: pH optima for the glycolytic enzymes obtained from different sources

Enzyme	Optimum pH			
	Citrus acida (present study)	Other sources		
		material used	pH	reference
Hexokinase	7.5	Excised roots of *Phaseolus mungo* L.	7.5	Parekh (1965)
		Excised roots of *Trigonella foenum grecum* L.	8.0	Antony *et al* (1965)
Phosphohexose isomerase	9.0	Etiolated corn seedlings	9.0	Black and Humphreys (1962)
		Muscle	9.0	Slein (1955)
		Phaseolus radiatus	7.8	Ramasarma and Giri (1956)
Phosphofructokinase	9.0	Rabbit brain	8.0	Buell *et al* (1958)
Fructose diphosphate aldolase	8.0	Rabbit muscle	7.5-8.5	Neilands and Stumpf (1955)
		Rat tissues	8.6	Sibley and Lehninger (1949)
		Yeast	7.4	Christian (1955)
Glyceraldehyde-3-phosphate dehydrogenase	8.5	Yeast	8.3-8.5	Krebs (1955)
		Rabbit muscle	8.5	Neilands and Stumpf (1955)
Lactate dehydrogenase	7.0	Rabbit muscle	7.4	Kornberg (1955)
		Excised roots of *Phaseolus mungo* L.	7.5	Parekh (1965)

Table 16: Purification of glutamate dehydrogenase

Purification step	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific-activity (units/mg protein)	Activity yield (per cent)	Protein recovery (per cent)
A. Crude extract	40	48	5.7	0.12	100	100
B. Amm. sulfate fraction (30-50 per cent saturation)	15	22	4.5	0.21	80	45
C. Calcium gel eluate	14	9.6	3.5	0.40	62	20
D. DEAE eluate	12	0.22	1.8	8.0	34	0.45

The effect of varying the enzyme and substrate concentrations, period of incubation and pH on the activity of glutamate dehydrogenase is shown in Table 17. Enzyme activity was found to be proportional to enzyme concentration and period of incubation within a certain range. The pH optimum was found to be 8.0. The enzyme activity was found to be proportional to substrate concentration up to 3 micromoles. The Km for 2-oxoglutarate was found to be 1.3×10^{-3}M (Fig. 6). The concentrations of ammonium sulfate and $NADH_2$ required for optimum enzyme activity were found to be 300 micromoles and 0.1 micromole respectively (Tables 18 and 19).

Studies were made of the effects of varying the concentrations of glutamate and NAD on the reverse reaction catalysed by glutamate dehydrogenase. In these studies the assay system used was the same as given in Table 2, except for the substitution of glutamate and NAD for 2-oxoglutarate and $NADH_2$. The results are shown in Tables 20 and 21 respectively. The rate of reverse reaction was found to increase proportionately up to 40 micromoles of glutamate and 0.2 micromole of NAD. The Km for glutamate was found to be 16×10^{-3}M (Fig. 7).

Table 17: Effect of enzyme concentration, periodoof incubation, pH and 2-oxoglutarate concentration on glutamate dehydrogenase activity in the fruit tissues of Citrus acida*

Variable	$NADH_2$ oxidized (micromoles)
Amount of enzyme (ml)	
0.00	0.000
0.05	0.016
0.10	0.030
0.15	0.050
0.20	0.065
0.30	0.090
Period of incubation (seconds)	
0	0.000
30	0.015
60	0.030
90	0.045
120	0.060
150	0.068
pH (potassium phosphate buffer, pH 6-7 and tris buffer pH 7.5 - 9.0 were used).	
6.0	0.015
7.0	0.040
7.5	0.060
8.0	0.065
8.5	0.040
9.0	0.030
2-oxoglutarate (micromoles)	
0.0	0.000
1.0	0.020
2.0	0.037
3.0	0.060
4.0	0.070
6.0	0.075

* Assay system was the same as given in Table 2 except for the variables mentioned.

Fig. 6: Lineweaver-Burk plot for glutamate dehydrogenase with 2-oxoglutarate

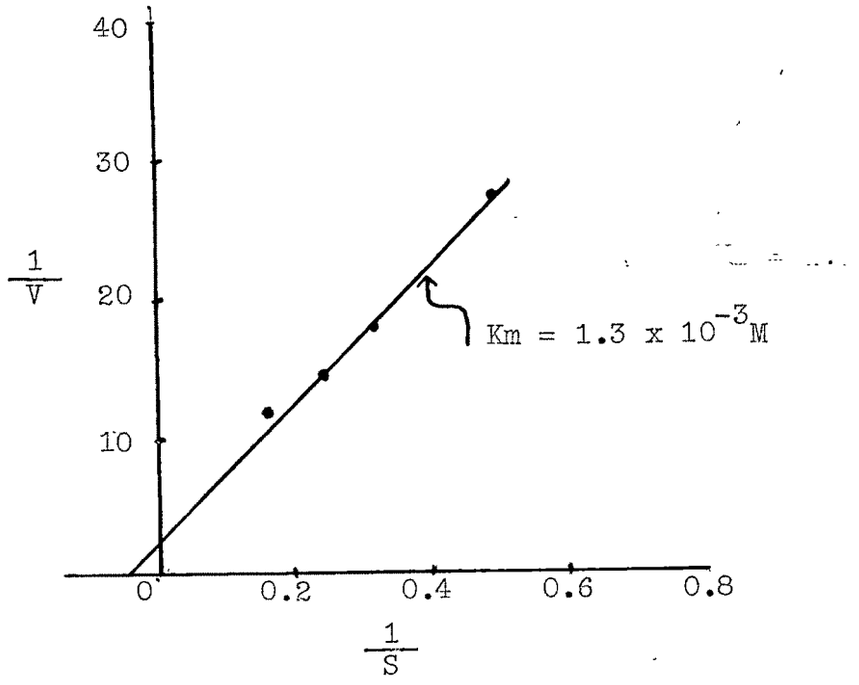


Fig. 7: Lineweaver-Burk plot for glutamate dehydrogenase with glutamate.

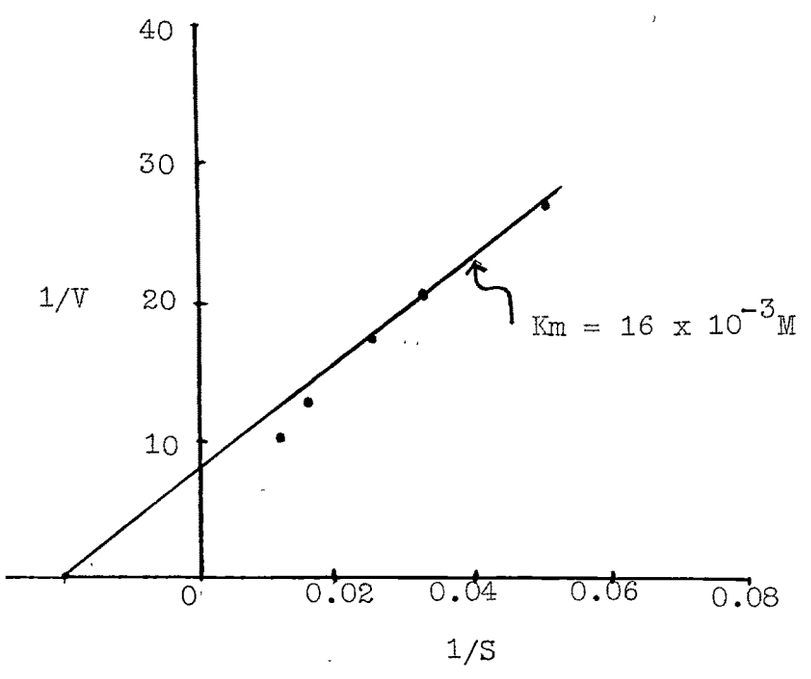


Table 18: Effect of $(NH_4)_2SO_4$ concentration on glutamate dehydrogenase activity

$(NH_4)_2SO_4$ added (micromoles)	$NADH_2$ oxidized (micromoles)
0	0.000
50	0.012
100	0.025
200	0.050
300	0.060
500	0.060
600	0.065

Table 19: Effect of $NADH_2$ concentration on glutamate dehydrogenase activity

$NADH_2$ added (micromoles)	$NADH_2$ oxidized (micromoles)
0.000	0.000
0.025	0.016
0.050	0.030
0.100	0.060
0.150	0.065
0.200	0.070

Table 20: Effect of glutamate concentration on the reverse reaction catalysed by glutamate dehydrogenase*

Glutamate added (micromoles)	$NADH_2$ formed (micromoles)
0	0.000
10	0.020
20	0.035
30	0.050
40	0.065
60	0.085
80	0.090

* 0.2 umole of NAD was added to the assay system.

Table 21: Effect of NAD concentration on the reverse reaction catalysed by glutamate dehydrogenase **

NAD added (micromoles)	$NADH_2$ formed (micromoles)
0.00	0.000
0.05	0.015
0.1	0.030
0.2	0.062
0.3	0.085
0.4	0.100
0.6	0.110

** 40 umoles of glutamate was added to the assay system.

Many metal ions namely, Zn, Cu, Fe, Hg and Ag as well as PCMB were found to inhibit the enzyme whereas citrate has no effect even at concentration of 50 micromoles. The inhibitory effect of PCMB was found to be reversed by cysteine (Table 22).

The effect of period of storage on the enzyme activity of both crude extract and the purified enzyme was studied by storing them in test tubes at -10°. Different batches were removed at regular intervals and assayed for enzyme activity. The crude extract retained 50 per cent of the activity after 7 days of storage whereas the purified enzyme lost all its activity during this period (Table 23).

The results of the balance studies carried out on glutamate dehydrogenase are represented in Table 24. It can be seen that 0.36 micromole of 2-oxoglutarate was utilized in 2 minutes resulting in the formation of 0.34 micromole of glutamate.

It was not possible to separate alanine aminotransferase and aspartate aminotransferase from one another. However, it was possible to get a fraction in which both enzymes were found to be highly active.

The data on the purification of these two enzymes is given in Table 25 which shows the yield of both the enzymes

Table 22: Effect of metal ions and PCMB on glutamate dehydrogenase activity

Addition	Concentration (micromoles)	$NADH_2$ oxidized (micromoles)	Inhibition (per cent)
None	-	0.060	0
Ag^{+}	0.05	0.000	100
Zn^{++}	0.02	0.030	50
Hg^{++}	0.10	0.030	50
Fe^{+++}	0.10	0.040	30
PCMB	0.10	0.025	60
PCMB+Cysteine	0.10 + 3.0	0.055	9
Citrate	50.0	0.060	0

Table 23: Effect of period of storage* on glutamate dehydrogenase activity

	Percentage enzyme activity on different days			
	0 day	3rd day	5th day	7th day
Crude extract	100	83	70	50
Purified enzyme	100	38	15	0

* Stored at -10°C

Table 24: Reaction balance for glutamate dehydrogenase

2-oxoglutarate added (µmoles)	2-oxoglutarate utilized (µmoles)	Glutamate formed (µmoles)
3.0	0.36	0.34

Table 25: Purification of alanine aminotransferase and aspartate aminotransferase

Purification step	Total volume (ml)	Total protein (mg)	Total activity (units)		Specific activity (units/mg protein)		Yield (per cent)		Protein recovery (per cent)	
			Alanine aminotransferase	aspartate aminotransferase	alanine aminotransferase	aspartate aminotransferase	alanine aminotransferase	aspartate aminotransferase	alanine aminotransferase	aspartate aminotransferase
A. Crude extract	45	52	24	13	0.46	0.25	100	100	100	100
B. Amm. sulfate fraction 30-50 per cent saturation)	10	23	16	9	0.75	0.40	66	64	44	44
C. Calcium gel supernatant	9	12	15	7.5	1.25	0.62	62	56	23	23
D. DEAE-eluate	16	0.35	10	4.6	28	13	41	34	0.70	0.70

and the degree of purification achieved in the different fractions. It can be seen that alanine aminotransferase could be purified more than 60 fold and aspartate aminotransferase more than 50 fold. But this was achieved by a loss respectively of 59 per cent and 66 per cent in total activity.

The effects of varying the enzyme and substrate concentrations, period of incubation and pH on the activities of alanine aminotransferase and aspartate aminotransferase are shown in Tables 26 and 27 respectively. In the case of both enzymes the activities were found to increase proportionately with enzyme concentration and period of incubation upto the optima shown. In the case of both enzymes optimum pH was found to be 7.5. The optimum substrate concentration for enzyme activity was determined in the case of both alanine and 2-oxoglutarate for the activity of alanine aminotransferase. It was found to be 50 micromoles in the former case and 10 micromoles in the latter case. The Km of the enzyme was found to be 5×10^{-2}M for alanine (Fig.8). Similar studies on aspartate aminotransferase showed the optimum concentration of glutamate and oxaloacetate to be 20 micromoles and 10 micromoles respectively. The Km of

Table 26: Effect of enzyme concentration, period of incubation, pH and alanine and 2-oxoglutarate concentrations on alanine aminotransferase activity in the fruit tissues of Citrus acida*

Variable	Pyruvate formed (micromoles)
Amount of enzyme (ml)	
0.00	0.0
0.05	0.85
0.10	1.80
0.15	2.50
0.20	3.00
0.25	3.50
Period of incubation (minutes)	
0	0.0
10	0.7
20	1.5
30	2.0
40	2.4
60	3.0
pH (Citrate-NaOH buffer, pH 4.0 - 5.5, phosphate buffer, pH 6.0 - 7.0 and tris buffer, pH 7.5 - 9.0 were used)	
4.0	0.20
5.0	0.55
5.5	0.70
6.0	1.20
7.0	1.55
7.5	1.80
8.0	1.40
9.0	0.80
Alanine (micromoles)	
0	0.00
10	0.38
20	0.77
40	1.50
50	1.90
60	2.10
80	2.30
2-oxoglutarate (micromoles)	
2.5	0.48
5.0	0.95
7.5	1.42
10.0	1.80
15.0	1.85

* Assay system was the same as given in Table 2 except for the variables mentioned.

Table 27: Effect of enzyme concentration, period of incubation pH and glutamate and oxaloacetate concentrations on aspartate aminotransferase activity in the fruit tissues of Citrus acida*

Variable	Aspartate formed (micromoles)
Amount of enzyme (ml)	
0.00	0.00
0.10	0.85
0.20	1.75
0.30	2.70
0.40	3.45
0.50	3.60
Period of incubation (minutes)	
0	0.0
15	1.7
30	3.6
45	4.5
60	5.5
pH (Citrate-NaOH buffer, pH 4.0-5.5, Phosphate buffer, pH 6.0-7.0 and tris buffer, pH 7.5-9.0 were used)	
4.0	0.40
5.0	0.70
5.5	1.20
6.0	1.70
7.0	3.10
7.5	3.40
8.0	2.80
9.0	2.10
Glutamate (micromoles)	
0	0.0
5	0.9
10	1.9
20	3.5
40	4.2
Oxaloacetate (micromoles)	
2.5	0.8
5.0	1.6
7.5	2.5
10.0	3.2
12.5	3.5
15.0	3.7

* Assay system was the same as given in Table 2 except for the variables mentioned.

FIG. 8: Lineweaver-Burk plot for Alanine aminotransferase

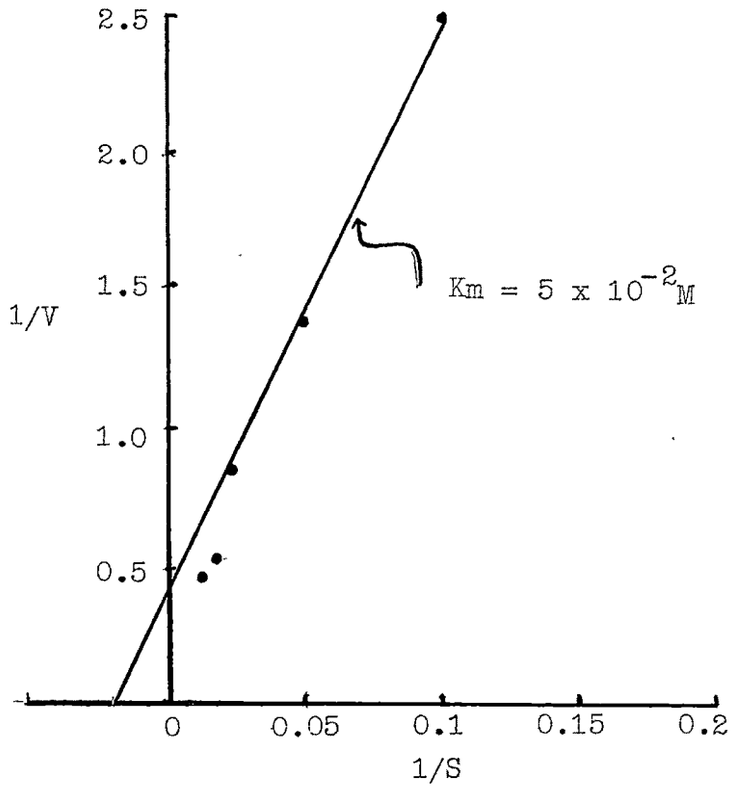
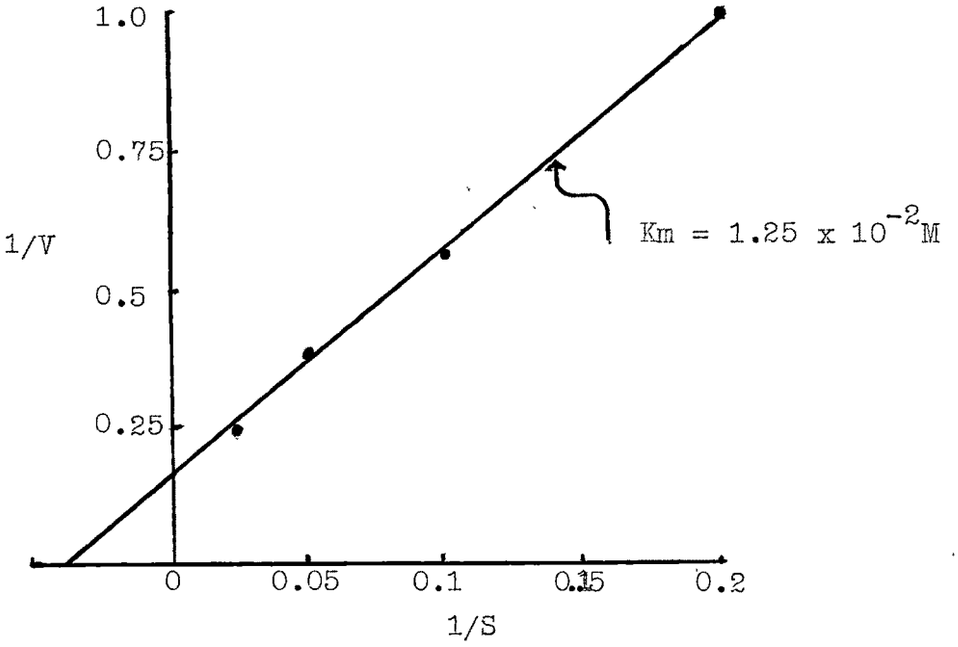


Fig. 9: Lineweaver-Burk plot for Aspartate aminotransferase



the enzyme for glutamate was found to be 1.25 x 10^{-2}M (Fig.9). In the case of both enzymes the optimum temperature was found to be 37°C (Table 28).

Both the enzymes showed a considerable degree of stability at high temperature. They were found to be stable when heated at 60° for 2 minutes. Heating at a higher temperature of 70° was found to result only in about 50 per cent loss of activity (Table 29). Studies were made of the effect of dialysis on the fresh enzyme and heat treated enzyme the results of which are shown in Table 30. No activity was lost on dialysis for 12 hours against 0.02 M potassium phosphate buffer, pH 7.5 in the case of the fresh enzyme. But dialysis of the enzyme heated at 60° for 2 minutes was found to result in about 50 per cent loss of activity. This loss was fully restored on addition of pyridoxal phosphate.

The effects of citrate, ascorbate and PCMB on the activities of alanine aminotransferase and aspartate aminotransferase were studied. Neither citrate nor ascorbate was found to affect the activity of these enzymes, but PCMB was found to inhibit the same. This effect was reversed by cysteine suggesting that these enzymes contain sulphydryl group (Table 31).

Table 28: Effect of incubation temperature on alanine aminotransferase and aspartate aminotransferase

Incubation temperature (°C)	Enzyme activity	
	Alanine aminotransferase (umoles of pyruvate formed)	Aspartate aminotransferase (umoles of aspartate formed)
20	0.6	1.8
30	1.1	3.1
37	1.8	3.5
45	1.6	3.2
50	1.4	3.0
55	1.1	2.7

Table 29: Effect of heat treatment on alanine amino-transferase and aspartate aminotransferase

Temperature (^{o}C)	Enzyme activity	
	Alanine amino-transferase (umoles of pyruvate formed)	Aspartate amino-transferase (umoles of aspartate formed)
Control (37^{o})	1.90	3.3
45	1.85	3.3
55	1.80	3.2
60	1.80	3.2
70	0.82	1.6
80	0.00	0.5

Enzyme was kept for 2 minutes at different temperatures, cooled and assayed.

Table 30: Effect of heat treatment and dialysis on alanine aminotransferase and aspartate aminotransferase

Treatment	Enzyme activity (per cent)	
	Alanine amino-transferase	Aspartate amino-transferase
No heat treatment, no dialysis	100	100
Only dialysis*	100	100
Only heat treatment**	100	100
Heat treatment and dialysis***	50	53
Addition of pyridoxal phosphate after heat treatment and dialysis****	100	100

* Dialysis was done for 12 hrs against 3 litres of 0.02M potassium phosphate buffer, pH 7.5.

** Kept at 60°C for 2 minutes, cooled and used.

*** Kept at 60°C for 2 minutes, cooled and dialysed for 12 hrs against 3 litres of 0.02M potassium phosphate buffer, pH 7.5.

**** 0.01 micromole of pyridoxal phosphate was added.

Table 31: Effect of citrate, ascorbate and PCMB on alanine aminotransferase and aspartate aminotransferase

Addition	Concentration (micromoles)	Enzyme activity		Inhibition (per cent)	
		Alanine amino-transferase (micromoles of pyruvic acid formed)	Aspartate amino-transferase (micromoles of aspartic acid formed)	Alanine amino-transferase	Aspartate amino-transferase
None	-	1.9	3.2	0	0
Citrate	100	1.9	3.2	0	0
Ascorbate	40	1.9	3.25	0	0
PCMB	0.4	0.4	0.45	80	85
PCMB+CSH	0.4+4.0	1.8	3.2	0	0

The effect of period of storage on the activities of the crude enzyme and the purified enzyme was studied in the case of alanine aminotransferase and aspartate aminotransferase by storing them in test tubes at -10°. The tubes were removed at regular intervals and the enzyme activity assayed. The crude extract was found to retain 75 per cent of the activity after 15 days of storage, whereas the purified enzyme lost about 70 per cent of the original activity during this period (Table 32).

Balance studies carried out on alanine aminotransferase and aspartate aminotransferase showed that alanine and glutamate are almost stoichiometrically converted to pyruvic acid and aspartic acid respectively.

The yield of glutamate decarboxylase and the degree of purification achieved in the different fractions are shown in Table 33. The specific activity of glutamate decarboxylase in the sephadex eluate is seen to increase 40 fold over that of the crude extract, but this increase is accompanied by a loss of 30 per cent in total activity.

The effect of varying enzyme concentration, period of incubation, pH and glutamate concentration on the activity of glutamate decarboxylase is shown in Table 34. The enzyme

Table 32: Effect of period of storage* on alanine aminotransferase and aspartate aminotransferase

	Enzyme activity on different days (per cent)											
	Alanine aminotransferase						Aspartate aminotransferase					
Days →	0	3	6	9	12	15	0	3	6	9	12	15
Crude extract	100	100	91	85	80	75	100	100	90	82	75	72
Purified enzyme	100	95	80	60	41	25	100	91	82	60	42	30

* Stored at $-10^{\circ}C$

Table 33: Purification of glutamate decarboxylase

Purification step	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Activity yield (per cent)	Protein recovery (per cent)
A. Crude extract	30	34	10	0.30	100	100
B. Amm. sulfate fraction (30-50 per cent saturation)	29	16	8.6	0.54	86	47
C. Calcium gel eluate	25	3.0	7.3	2.4	73	9
D. Sephadex eluate	20	0.6	7.0	12	70	1.7

Table 34: Effect of enzyme concentration, period of incubation, pH and glutamate concentration on glutamate decarboxylase activity in the fruit tissue of Citrus acida*

Variable	GABA Formed (micromoles)
Amount of enzyme (ml)	
0.00	0.0
0.10	1.2
0.20	2.5
0.30	3.6
0.40	4.1
Period of incubation (minutes)	
15	1.8
30	3.5
45	4.1
60	4.5
pH (citrate-KOH buffer, pH 5, potassium phosphate buffer pH 6 - 7.0 and tris buffer, pH 7.5 - 8.0 were used)	
5.0	2.1
6.0	3.5
6.5	2.9
7.0	2.0
7.5	1.1
8.0	0.0
Glutamate (micromoles)	
0	0.0
5	1.8
7.5	2.7
10	3.4
20	4.1
30	4.5

* Assay system was the same as given in Table 2, except for the variables mentioned.

activity was seen to be proportional to enzyme concentration and period of incubation within the ranges shown. The pH optimum was found to be 6.0 and the optimum concentration of glutamate to be 10 micromoles. Fig 10 shows the Km value of the enzyme to be 4×10^{-2}M. Table 35 shows the optimum concentration of pyridoxal phosphate to be 0.015 micromoles.

A number of compounds namely, citrate, ascorbate and PCMB were tested for their effect on enzyme activity. None of them had any effect on the enzyme activity (Table 36).

The effect of period of storage on the activities of both the crude extract and the purified enzyme was studied by storing them in test tubes at -10°. The tubes were removed at regular intervals and the enzyme activity assayed. The crude extract was found to retain 45 per cent of the activity after 10 days of storage whereas the purified enzyme lost its complete activity during this period (Table 37).

Balance studies with the enzyme suggested that GABA is formed almost stoichiometrically from glutamate.

The activities of glutamine synthetase, asparagine synthetase and protease increased linearly with enzyme concentration, period of incubation and substrate concentration within the ranges shown (Tables 38-40).

Fig. 10: Lineweaver-Burk plot for glutamate decarboxylase

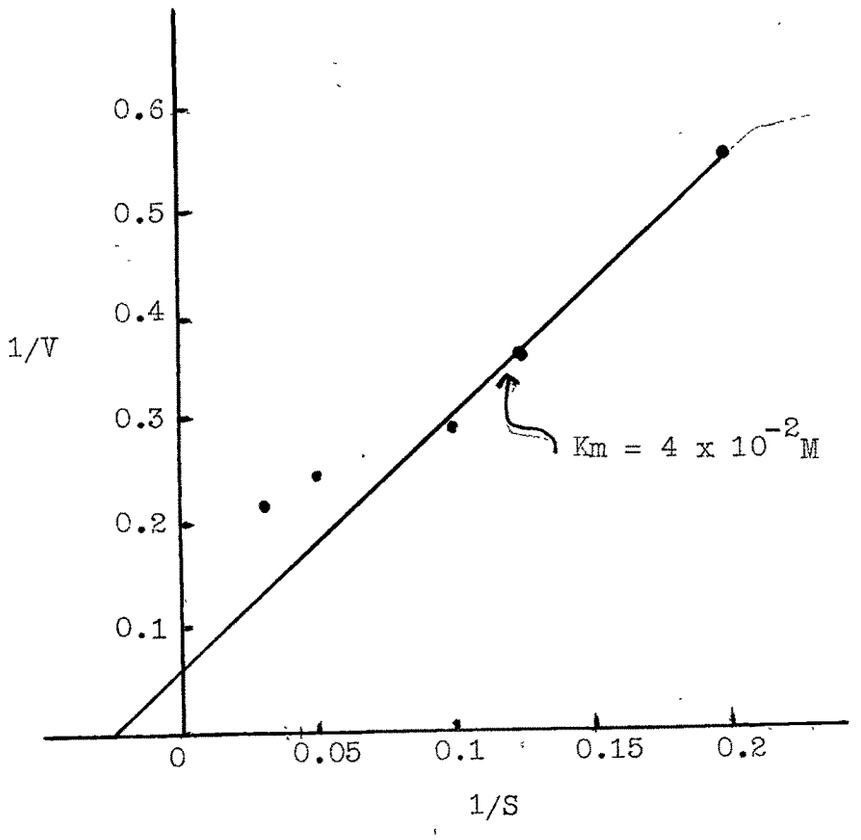


Table 35: Effect of pyridoxal phosphate concentration on glutamate decarboxylase activity

Pyridoxal phosphate added (micromoles)	GABA formed (micromoles)
0.000	0.50
0.005	0.95
0.010	1.80
0.015	3.50
0.020	3.60
0.040	3.60

Table 36: Effect of citrate, ascorbate and PCMB on glutamate decarboxylase activity

Addition	Concentration (micromoles)	GABA formed (micromoles)
None	0.0	3.50
Citrate	50.0	3.40
Ascorbate	30.0	3.45
PCMB	0.20	3.40

Table 37: Effect of period of storage on glutamate decarboxylase activity

Fraction	Enzyme activity on different days (per cent)					
	0 day	2nd day	4th day	6th day	8th day	10th day
Crude extract	100	98	92	70	61	45
Purified enzyme	100	70	45	30	14	0

Table 38: Effect of enzyme concentration, period of incubation, pH and glutamate concentration on glutamine synthetase activity in the fruit tissues of Citrus acida*

Variable	Hydroxamate formed (micromoles)
Amount of enzyme (ml)	
0.000	0.00
0.050	0.29
0.100	0.56
0.150	0.88
0.200	0.92
Period of incubation (minutes)	
0	0.00
10	0.28
20	0.52
30	0.84
40	1.00
60	1.20
pH (phosphate buffer pH 6-7 and tris buffer, pH 7.5-8.5 were used)	
6.0	0.36
7.0	0.68
7.5	0.88
8.0	0.64
8.5	0.36
Glutamate (micromoles)	
0	0.00
10	0.26
20	0.56
30	0.81
40	0.90

* Assay system was the same as given in Table 2, except for the variables mentioned.

Table 39: Effect of enzyme concentration, period of incubation, pH and aspartate concentration on aspargine synthetase activity in the fruit tissues of Citrus acida*

Variable	Hydroxamate formed (micromoles)
Amount of enzyme (ml)	
0.000	0.00
0.050	0.50
0.100	1.00
0.150	1.25
0.200	1.50
Period of incubation (minutes)	
10	0.35
20	0.75
30	1.00
40	1.10
pH (phosphate buffer, pH 6-7 and tris buffer, pH 7.5 - 8.5 were used)	
6.0	0.70
7.0	1.00
7.5	0.80
8.0	0.70
8.5	0.65
Aspartate (micromoles)	
10	0.35
20	0.70
30	1.10
40	1.20
60	1.40

* Assay system was the same as given in Table 2 except for the variables mentioned.

Table 40: Effect of enzyme concentration, period of incubation, pH and casein concentration on protease activity in the fruit tissues of Citrus acida*

Variable	Tyrosine liberated (micromoles)
Amount of enzyme (ml)	
0.25	0.23
0.50	0.46
0.75	0.60
1.00	0.70
Period of incubation (minutes)	
15	0.21
30	0.48
45	0.53
60	0.56
pH (citrate-phosphate buffer, pH 4.0-6.5 and tris buffer, pH 7.5-9.0 were used)	
4.0	0.16
5.0	0.32
6.0	0.48
6.5	0.40
7.5	0.25
8.0	0.15
9.0	0.10
Casein (mg)	
0.25	0.08
0.50	0.17
0.75	0.27
1.00	0.46
2.00	0.56

* Assay system was the same as given in Table 2 except for the variables mentioned.

The characteristics of the partially purified enzymes are compared with those of enzymes in other sources (Tables 41-44).

Glutamate dehydrogenase seems to resemble the enzymes obtained from Phaseolus mungo (Damodaran and Nair, 1938) and corn leaves (Bulen, 1956). It resembles the one obtained from beef liver with regard to inhibition by heavy metals. (Olson and Anfinsen, 1953).

Alanine aminotransferase resembles that in other plant tissues with regard to optimum pH. Aspartate aminotransferase isolated from Citrus acida resembles that from hog and pig hearts in its optimum pH.

Glutamate decarboxylase isolated from Citrus acida resembles the one isolated from squash with regard to the optimum pH which is found to be higher than the optima observed in the case of the enzyme isolated from microbial sources.

Table 41: Comparison of the characteristics of glutamate dehydrogenase obtained from different sources

Source	pH	Km with glutamate	Km with 2-oxoglutarate	Heavy metal inhibitors	References
Citrus acida	8.0	16×10^{-3}M	1.3×10^{-3}M	Ag,Zn,Fe	Present study
Beef liver	7.6	1.1×10^{-3}M	0.7×10^{-3}M	Ag,Zn,Fe	Olson and Anfinsen (1953)
Phaseolus mungo	8.0	45×10^{-3}M	-	-	Demodaran and Nair (1938)
Ox liver	8.5	5.36×10^{-3}M	1.23×10^{-4}M	-	Strecker (1953)
Corn leaves	8.1	-	1.51×10^{-3}M	-	Bulen (1956)
N. crassa	8.4	28.2×10^{-3}M	-	-	Roberts (1966)

Table 42: Comparison of the characteristics of alanine aminotransferase obtained from different sources

Source	pH	Km	Reference
Citrus acida	7.5	5.0×10^{-2} M	Present Study
Rat liver	8.0	1.75×10^{-2} M	Segal et al (1962)
Pig heart	7.5	-	
Mung bean	7.5	-	Wilson et al (1954)
B. subtiles	8.5	-	Curtis et al (1955)
Wheat	7.5	2.5×10^{-2} M	Cruickshank and Isherwood (1958)

Table 43: Comparison of the characteristics of aspartate aminotransferase obtained from different sources

Source	pH	Km	Reference
Citrus acida	7.5	1.25×10^{-2} M	Present study
Cauli flower	7.0	3.6×10^{-2} M	Ellis and Davies (1961)
Pig heart	7.6	50×10^{-2} M	Meister *et al* (1964)
Rat liver	8.0	3.3×10^{-2} M	Goldstone and Adams (1962)
E. coli	8.3	-	Rudman and meister (1953)
Hog heart	7.4	0.6×10^{-2} M	Nisonoff and Barnes (1952)
Wheat	8.0	2.5×10^{-2} M	Cruickshank and Isherwood (1958)

Table 44: Comparison of the characteristics of glutamate decarboxylase obtained from different sources

Source	pH	Km	Reference
Citrus acida	6.0	4.0×10^{-2}M	Present study
Squash	5.7	3.6×10^{-2}M	Hood (1954)
Barley	5.0	9.6×10^{-3}M	Beevers (1951)
Yeast	4.5	2.75×10^{-3}M	Krishnaswamy and Giri (1953)
E.coli	3.8	-	Strausbuch and Fischer (1967).