C H A P T E R - V

E N Z Y M E - III

C H A P T E R - V

ENZYME III

The kinetics of Enzyme I (with 3.7 - pH optimum) and Enzyme II (with 4.4 - pH optimum) within the available laboratory facilities have been studied and described in chapters III and IV respectively.

This chapter is devoted to express the kinetic studies of Enzyme III (with pH optimum 5.00).

pH Optimum :

The enzyme sample was prepared in 0.2 M acetate buffer of pH 5.00. The enzyme activities were confirmed by using varied pH of 0.2 M acetate buffer (3.7, 3.8, 4, 4.2, 4.4, 4.6, 4.8, 5, 5.2, 5.4, 5.6 as per Dawson et.al., 1978).

The results were expressed in Table 21 as enzyme activity in units per mg proteins. The alterations are illustrated in graphic form in Figure 19.

Optimum temperature :

Enzyme III was further studied to determine the optimal temperature for its activity.

TABLE - 21

(Effect of pH)

Sr.No.	pH	AcPase activity (Units/mg protein)
1.	3.7	0.022 ± 0.00088
2.	3.8	0.020 ± 0.00081
3.	4.0	0.019 ± 0.00076
4.	4.2	0.020 ± 0.00082
5.	4.4	0.021 ± 0.00084
6.	4.6	0.020 ± 0.00083
7.	4.8	0.018 ± 0.00072
8.	5.0	0.045 ± 0.00180
9.	5.2	0.020 ± 0.00085
10.	5.4	0.020 ± 0.00087
11.	5.6	0.020 ± 0.00080

Values are mean ± SE of five experiments.

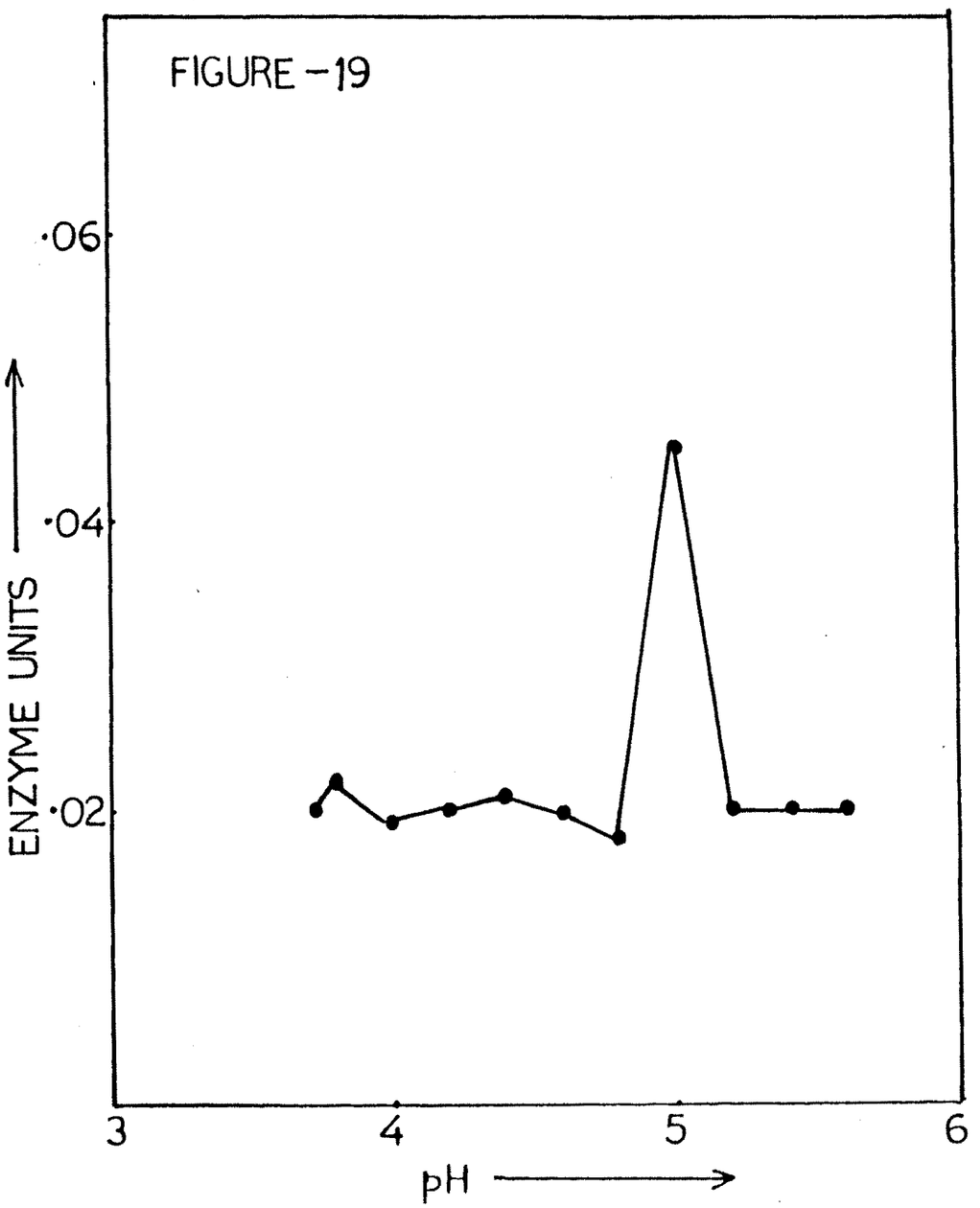


FIGURE -19
·06
·04
·02
ENZYME UNITS
3
4
5
6
pH

The enzyme activity was studied in 0.2 M acetate buffer at pH 5.00 with varying temperatures of incubation ($15^{o}C$, $20^{o}C$, $25^{o}C$, $30^{o}C$, $33^{o}C$, $37^{o}C$, $39^{o}C$, $40^{o}C$).

The results were obtained as enzyme activities per mg protein. They are given in Table 22. The graphic expressions are in Figure 20.

The optimum temperature for activity of Enzyme III was reported as $30^{o}C$ just similar to Enzyme I and II.

At further temperatures enzyme activity showed plateau.

The incubation time for the optimum enzyme activities was also determined using optimum temperature constant and varying the incubation period (5', 10', 15', 20', 25', 30', 40').

The units in terms of per mg protein are exhibited in Table 23 and same are depicted graphically in Figure 21.

The observations indicated the increase in enzyme activity along with the extension in incubation period. This was true upto 20 minutes of incubation. But with further enhancement in the incubation period, the maximum activity that was attained at 20 minutes was practically maintained.

TABLE - 22

(Effect of Temperature)

Sr.No.	Temperature (^{o}C)	AcPase activity (Units/mg protein)
1.	15	0.0090 ± 0.00036
2.	20	0.0185 ± 0.00074
3.	25	0.0500 ± 0.00200
4.	30	0.0650 ± 0.00260
5.	33	0.0650 ± 0.00240
6.	37	0.0630 ± 0.00252
7.	39	0.0660 ± 0.00264
8.	40	0.0640 ± 0.00256

Values are mean ± SE of five experiments.

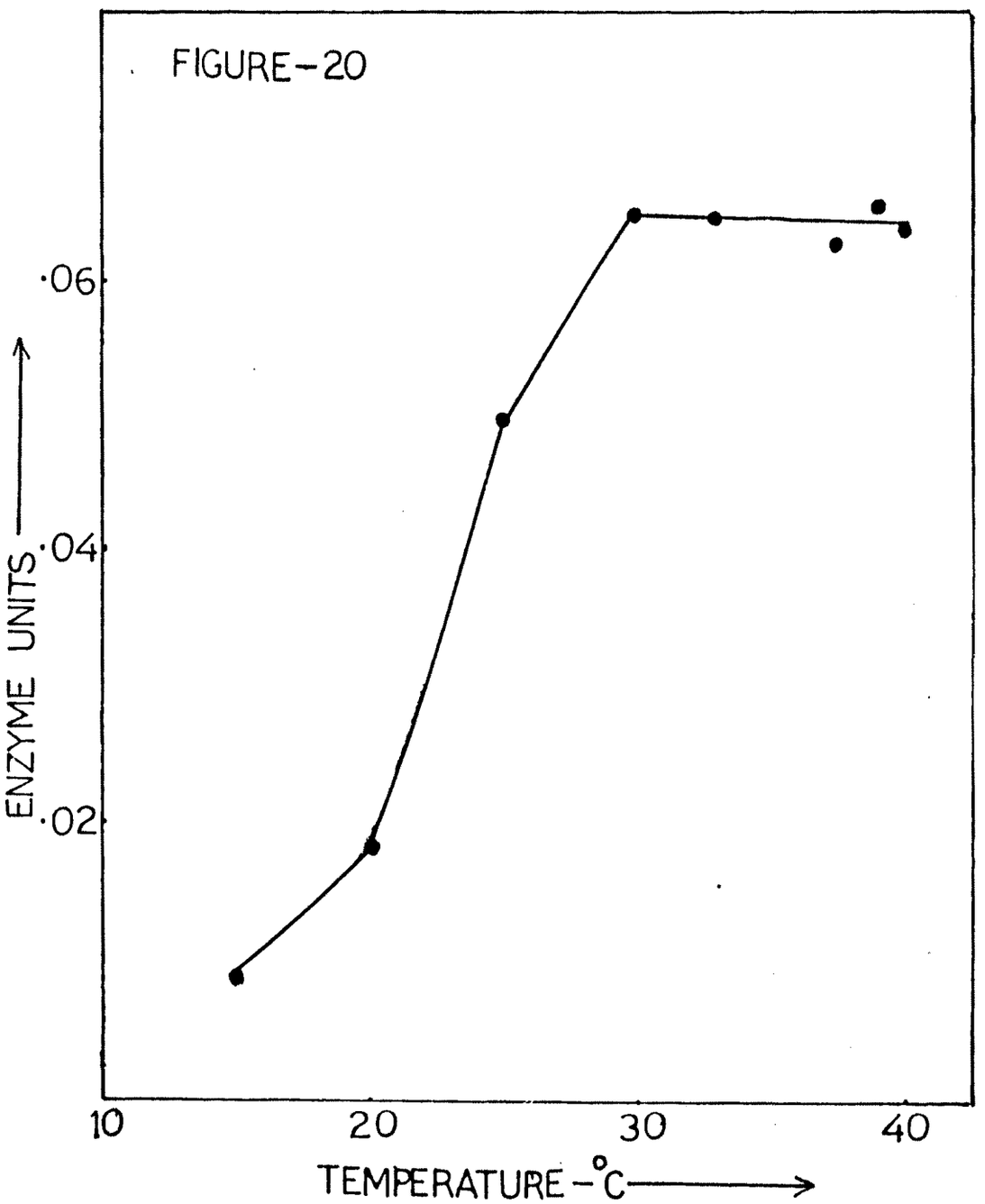


FIGURE-20
ENZYME UNITS
·06
·04
·02
10
20
30
40
TEMPERATURE - °C

TABLE - 23

(Effect of Incubation Time)

Sr.No.	Time (Minutes)	AcPase activity (Units/mg protein)
1.	5	0.40 ± 0.0160
2.	10	0.70 ± 0.0280
3.	15	1.04 ± 0.0420
4.	20	1.38 ± 0.0550
5.	25	1.38 ± 0.0552
6.	30	1.38 ± 0.0555
7.	35	1.37 ± 0.0548
8.	40	1.39 ± 0.0556

Values are mean ± SE of five experiments.

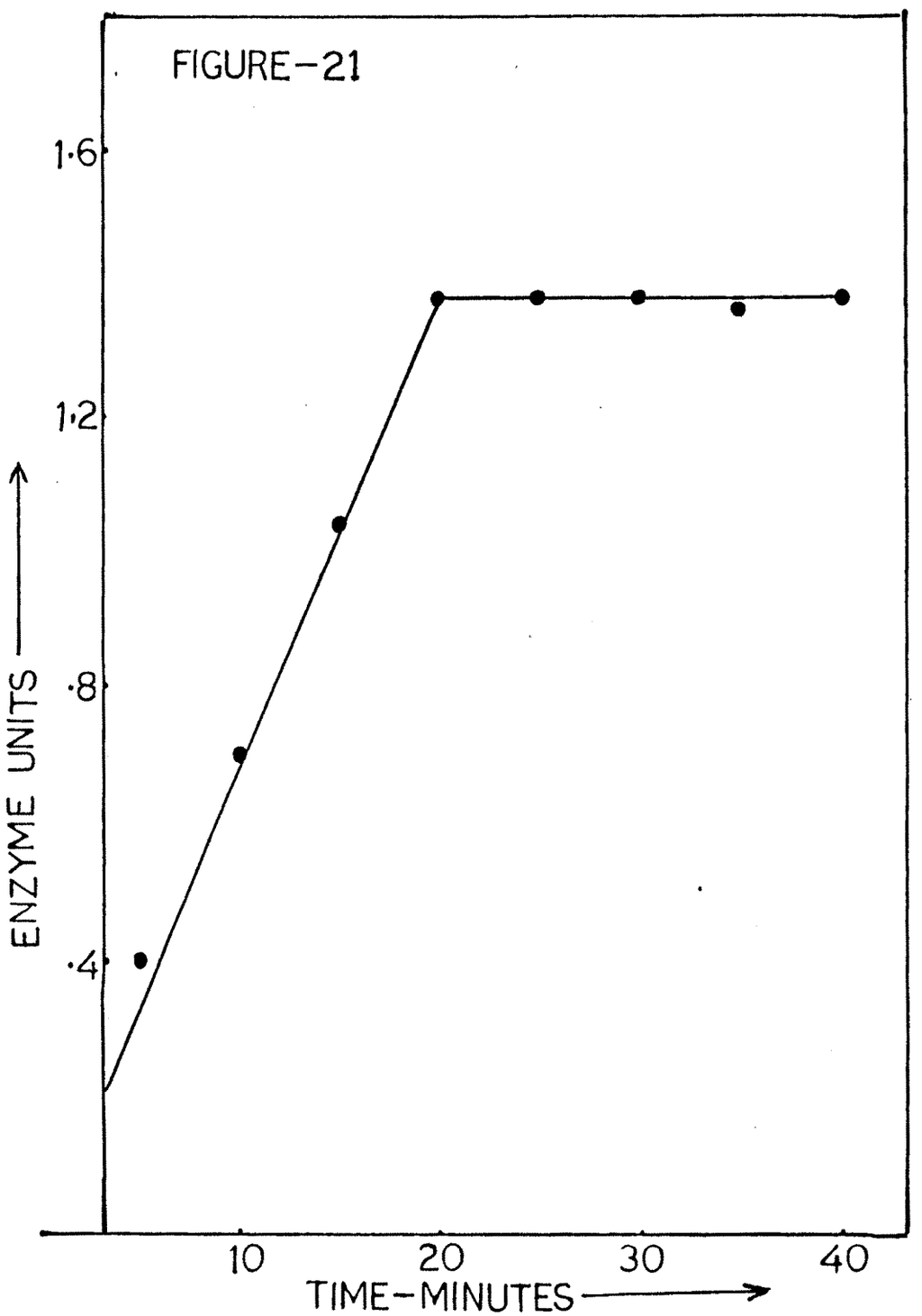


FIGURE-21
1·6
1·2
·8
·4
ENZYME UNITS
10
20
30
40
TIME-MINUTES

Ten minutes of incubation period was used for the further Kinetic studies.

Km determination :

To determine the Michealis constant the activities of Enzyme III were studied using various concentrations of p-nitrophenyl-phosphate (5mM,, 10mM, 15mM, 20mM, 25mM, 30mM, 35mM, 40mM, 45mM, 50mM) buffered at pH 5.0 in 0.2M acetate buffer.

The enzyme activities were as usual estimated as units per mg protein. They are denoted in Table 24; and are exhibited graphically in Figure 22.

The enzyme activities as observed in their alterations indicated slow expressions in low concentrations of p-nitrophenyl-phosphate but were suddenly incresed in further concentrations. Steady elevation in enzyme activities was depicted from 20mM concentration to 30mM concentration achieving the maximum activity at 35mM concentration. At further increasing concentrations of the substrate the activity was more or less steady.

From the figure 22, the Km values were 30.4mM of p-

TABLE - 24

(Effect of Substrate Concentration)

Sr.No.	Concentration (mM)	AcPase activity (Units/mg protein)
1.	5	0.000 ± 0.0000
2.	10	0.000 ± 0.0000
3.	15	0.000 ± 0.0000
4.	20	0.033 ± 0.0013
5.	25	0.133 ± 0.0053
6.	30	0.350 ± 0.0140
7.	35	0.470 ± 0.0188
8.	40	0.480 ± 0.0192
9.	45	0.480 ± 0.0190
10.	50	0.490 ± 0.0196

Values are mean ± SE of five experiments.

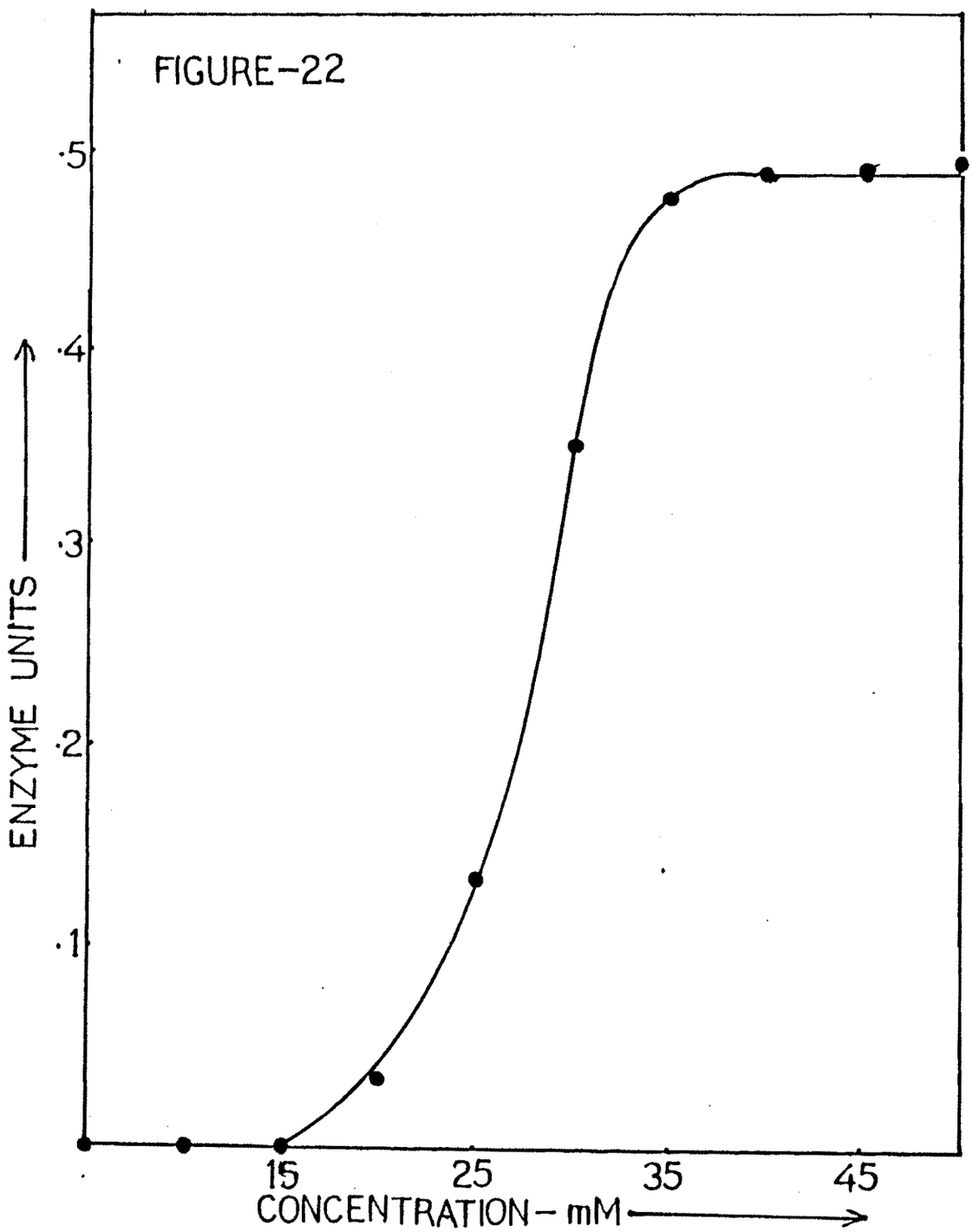


FIGURE-22
·5
·4
·3
·2
·1
ENZYME UNITS
15
25
35
45
CONCENTRATION – mM

nitrophenyl phosphate. This concentration of the substrate was used for the kinetic studies of Enzyme III.

Effect of divalent ions on the enzyme activity :

The alterations in the enzyme activities as a result of influence of various divalent ions were studied using following sources of ions :

1) $MgSO_4$ - Magnesium sulfate
2) $MnSO_4$ - Manganese sulfate
3) $CuSO_4$ - Copper sulfate
4) $CaCl_2$ - Calcium chloride

1) Effect of $MgSo_4$:

To study the alterations in presence of magnesium sulfate, varied concentrations of the salts (5 mM, 10mM, 15 mM, 20mM, 25 mM, 30mM, 35 mM, 40mM, 45 mM, 50mM, 55 mM) were used to assay the enzyme activities in their presence.

The enzyme activity was not influenced positively or negatively by the presence of magnesium sulfate in assay.

2) Effect of $MnSO_4$:

Effect of manganese sulfate was also studied by using various concentrations of the salt (5 mM, 10mM, 15 mM, 20mM, 25 mM, 30mM, 35 mM, 40mM, 45 mM, 50mM, 55 mM).

The results of assay were given as the units per mg protein.

To express the results the alterations occurring in enzyme activities are described in Table 25. The figure 23 shows the graphical exhibition of the same.

The obserevations indicated that the enzyme activity continued to increase with increase in manganese sulfate concentrations upto 40mM. But the further high concentrations showed practically maintenance of the high levels of enzyme activities expressed at 40mM concentration.

3) Effect of $CuSO_4$

Various concentrations of Copper sulfate (5mM, 10mM, 15mM, 20mM, 25mM, 30mM, 35mM, 40mM, 45mM, 50mM) were used for the study of effect of copper ions on the Enzyme III assay.

The results of enzyme assays expressed in units per mg protein were estimated by usual procedures.

Table 26 shows the changes occurring in enzyme activities while Figure 24 shows the graphic pattern of alterations.

The results indicated that pattern of alteration was linearly enhancing along with the increase in concentration of

TABLE - 25

(Effect of $MnSO_4$)

Sr.No.	Concentration (mM)	AcPase activity (Units/mg protein)
1.	5	0.20 ± 0.0080
2.	10	0.50 ± 0.0200
3.	15	0.80 ± 0.0320
4.	20	1.00 ± 0.0400
5.	25	1.30 ± 0.0520
6.	30	1.50 ± 0.0600
7.	35	1.70 ± 0.0680
8.	40	2.10 ± 0.0840
9.	45	2.00 ± 0.0800
10.	50	2.00 ± 0.0810
11.	55	1.98 ± 0.0792

Values are mean ± SE of five experiments.

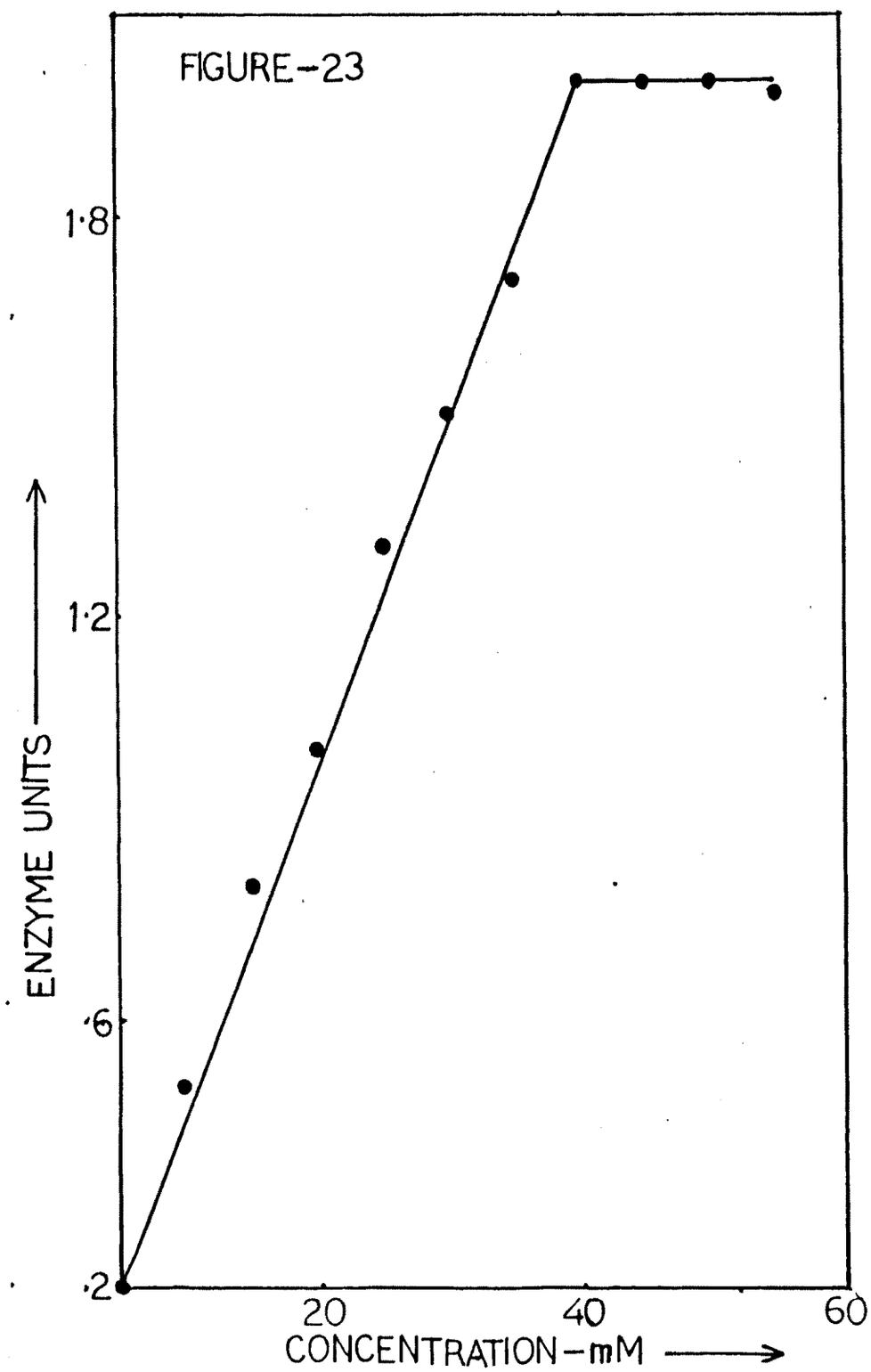


FIGURE-23
1·8
1·2
·6
·2
ENZYME UNITS
20
40
60
CONCENTRATION-mM

TABLE - 26

(Effect of $CuSo_4$)

Sr.No.	Concentration (mM)	AcPase activity (Units/mg protein)
1.	5	0.50 ± 0.0200
2.	10	1.00 ± 0.0400
3.	15	1.38 ± 0.0552
4.	20	1.75 ± 0.0700
5.	25	2.38 ± 0.0952
6.	30	2.88 ± 0.1152
7	35	3.31 ± 0.1324
8.	40	3.63 ± 0.1452
9.	45	3.65 ± 0.1460
10	50	3.63 ± 0.1450

Values are mean ± SE of five experiments.

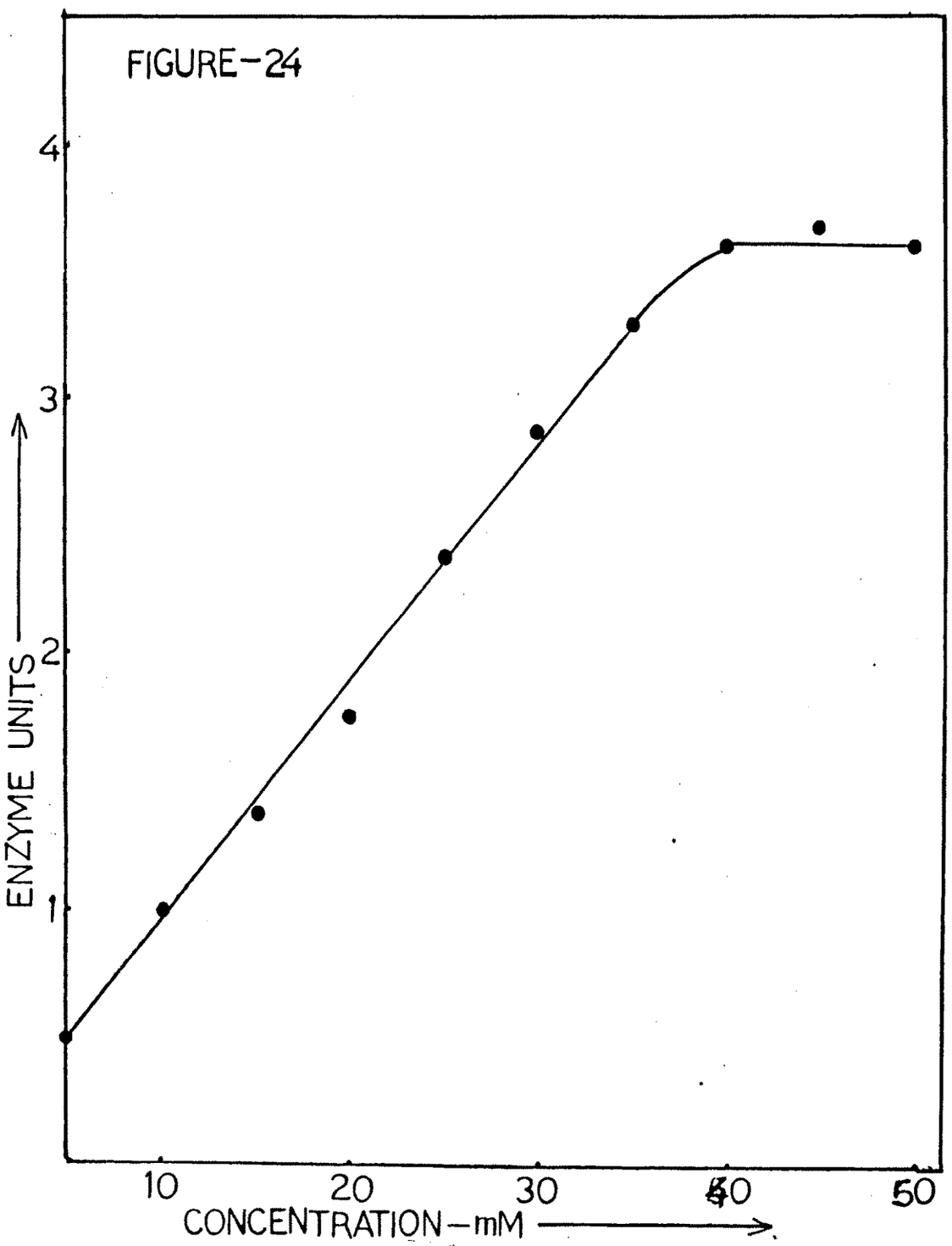


FIGURE-24
4
3
2
1
ENZYME UNITS
10
20
30
40
50
CONCENTRATION – mM

Copper sulfate upto 40mM strength of Copper sulfate.

At further increasing strengths of Copper sulfate the enzyme activities were practically at the same levels maintaining a plateau at maxima.

4) Effect of $CaCl_2$:

Calcium chloride was also analysed with the various concentrations for its influence on enzyme assay.

Any strength of Calcium chloride resulted in the inhibition of the enzyme activity.

Effect of EDTA and EGTA :

EDTA did not interfere with the enzyme activities same was true for EGTA. EGTA along with Magnesium sulfate did not alter the enzyme activity..

Effects of other chemicals :

To study the influence of the other chemicals on Enzyme III activity following chemicals were used :

1) Formalin
2) Triton X-100
3) Methanol

4) Ethanol

5) Tartarate

6) acetone

7) Citrate

8) Glycerol

9) NaF.

The results are given in Table 27.

From the observations it was evident that the enzyme activities were totally inhibited by 1% formalin, 1% Triton X-100, 1% methanol, 1% ethanol, 1% acetone, 0.05M citrate and upto 20mM NaF.

Tartarate (< 20mM) and glycerol (< 1%) did not affect the enzyme activity.

Effect of preincubation of Enzyme III on enzyme activity :

For the preliminary work the enzyme sample was preincubated at various temperatures ($40^{o}C$, $45^{o}C$, $50^{o}C$, $55^{o}C$, $60^{o}C$, $65^{o}C$, $70^{o}C$, $75^{o}C$, $80^{o}C$) and was used for the estimation of enzyme activities.

Enzyme III retained the enzyme activity on preincubation upto $60^{o}C$.

TABLE - 27

(Effect of Other Chemicals)

Sr.No.	Name of the Chemical	Inhibition of Enzyme-III	No Influence
1.	1 % Formaline	Total	-
2.	1 % Triton X-100	Total	-
3.	1 % Methanol	Total	-
4.	1 % Ethanol	Total	-
5.	20mM Tartarate	-	No influence
6.	1 % Acetone	Total	-
7.	0.05 mM Citrate	Total	-
8.	1 % Glycerol	-	No influence
9.	20 mM NaF	Total	-

The sample of Enzyme III was preincubated at 60°C for various time intervals. This preincubated Enzyme III at 60°C (3', 5', 7', 10', 15', 20', 20', 25', 30', 35', 40',) was further used for the study of enzyme activity.

The alterations were studied as per mg protein expression of units.

The results are included in Table 28. The graphic form is depicted in Figure 25.

The results indicated that the enzyme activity was increased slowly in early intervals of preincubation (3', 5', 7', 10'). At 15' of preincubation period of sample the enzyme activity showed sudden expression of the enzyme activity. This was retained practically with the following increasing intervals till 40 minutes.

From the above studies it can be concluded that Enzyme III isolated from Rana cyanophlyctus ovary during prebreeding conditions showed the following characters:

1. pH optimum - 5.00
2) Temperature optimum 30°C
3) Km for p-nitrophenyl-phosphate- 30.4mM.
4) Activated linearly by

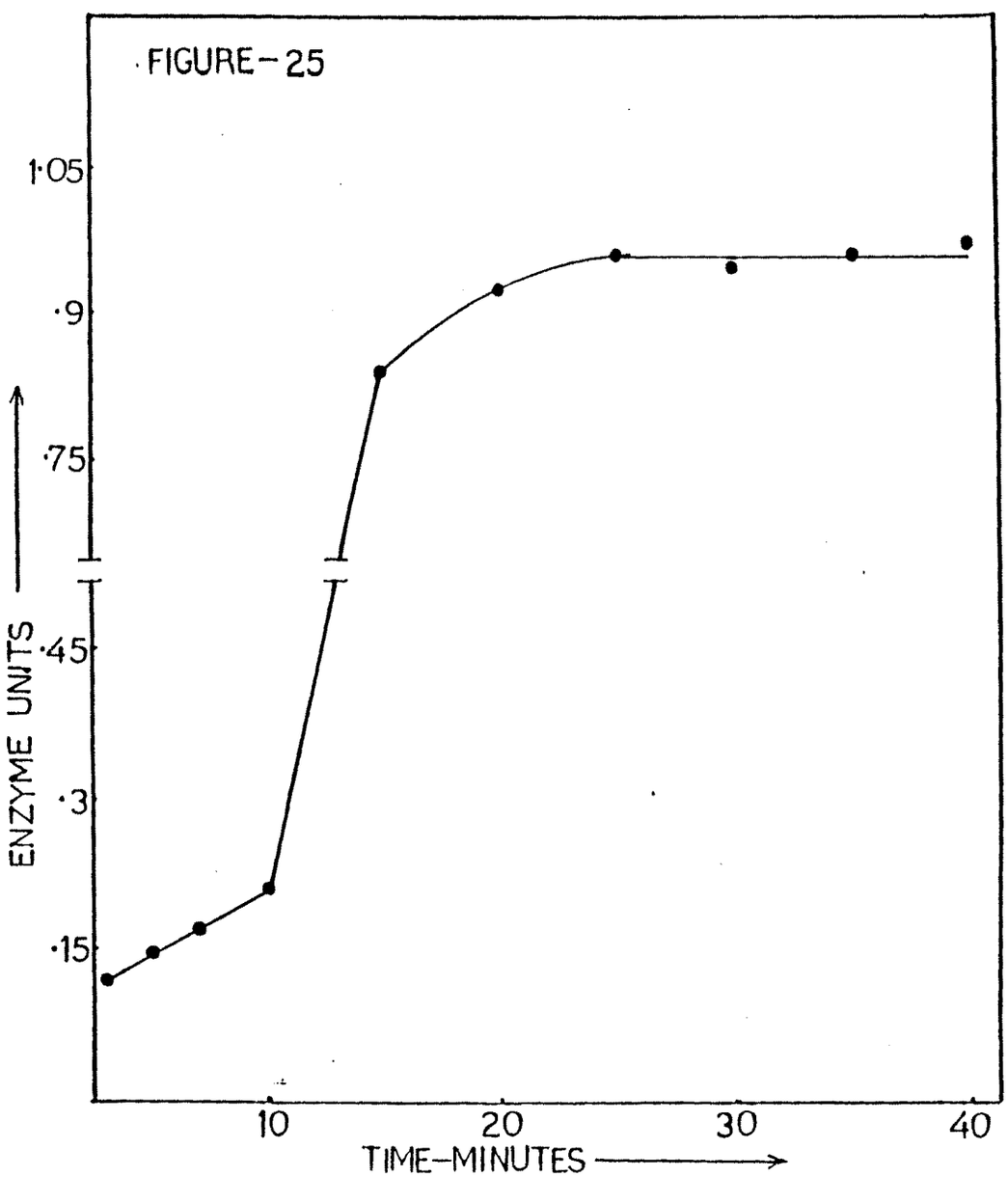
TABLE - 28

(Effect of Preincubation at $60^{o}C$)

Sr.No.	Time (Minutes)	AcPase activity (Units/mg protein)
1.	3	0.12 ± 0.0048
2.	5	0.15 ± 0.0060
3.	7	0.17 ± 0.0068
4.	10	0.21 ± 0.0084
5.	15	0.85 ± 0.0340
6.	20	0.92 ± 0.0368
7.	25	0.96 ± 0.0384
8.	30	0.95 ± 0.0380
9.	35	0.96 ± 0.0382
10.	40	0.97 ± 0.0388

Values are mean ± SE of five experiments.

FIGURE – 25



$MnSO_4$ (Upto 60mM)

$CuSO_4$ (Upto 40mM)

5) Not influenced by $MgSO_4$

6) Inhibited by $CaCl_2$

7) Not influenced by EDTA, EDTA, EFTA + $MgSO_4$

8) Totally inhibited by

1% formalin

1% Triton X-100

1% Methanol

1% Ethanol

1% Acetone

0.05 M Citrate

NaF (0.1mM)

9) Not influenced by tartarate ($<$ 20mM) and glycerol ($<$ 1%).

10) The effect of preincubation of enzyme III on enzyme activity also resulted in the burst of the enzyme activity on 15' of preincubation which was retained upto 40' of preincubation.

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