C H A P T E R – IV

E N Z Y M E – II

CHAPTER - IV

ENZYME - II

From the preliminary results it was revealed that three enzymes acting at different pH were isolated from the ovary of Rana cyanophlyctis in prebreeding condition.

In Chapter III the kinetic studies on Enzyme I with pH optimum of 3.7 are included.

This chapter deals with Enzyme II with pH optimum of 4.4.

pH optimum :

The pH optimum of the enzyme was confirmed using 0.2M acetate buffer pH 4.4 from the isolated enzyme sample dissolving it at pH 4.4.

The pH optimum was confirmed using 0.2 M acetate buffers with various pH (3.7, 3.8, 4, 4.2, 4.4, 4.6, 4.8, 5, 5.2, 5.4, 5.6).

The results were expressed in units per mg proteins and are given in Table 13 and Figure 12.

TABLE - 13

(Effect of pH)

Sr.No.	pH	AcPase activity (Units/mg protein)
1.	3.7	0.020 ± 0.00080
2.	3.8	0.021 ± 0.00084
3.	4.0	0.020 ± 0.00060
4.	4.2	0.020 ± 0.00085
5.	4.4	0.055 ± 0.00220
6.	4.6	0.021 ± 0.00082
7.	4.8	0.020 ± 0.00060
8.	5.0	0.018 ± 0.00070
9.	5.2	0.020 ± 0.00088
10.	5.4	0.019 ± 0.00076
11.	5.6	0.020 ± 0.00086

Values are mean ± SE of five experiments.

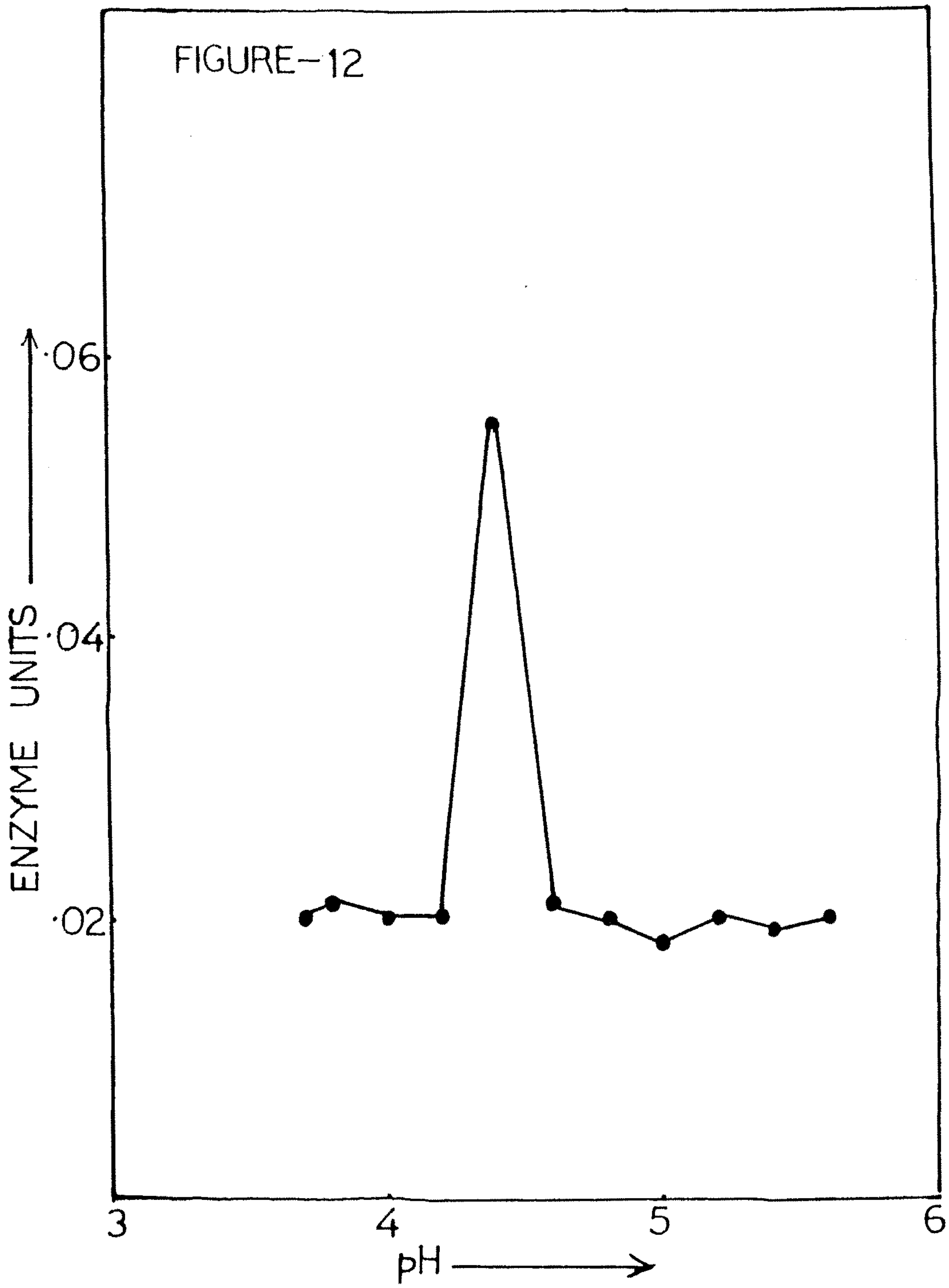
FIGURE-12
ENZYME UNITS
·06
·04
·02
3
4
5
6
pH

The alternations confirmed the optimum pH 4.4 for Enzyme II.

Optimum temperature:

The effect of temperature on enzyme activities was studied using incubation temperature of various degrees ($15^{o}C$, $20^{o}C$, $25^{o}C$, $30^{o}C$, $33^{o}C$, $37^{o}C$, $39.\ ^{o}C$ and $40^{o}C$). The enzyme activities were estimated as units per mg proteins.

The results are given in Table 14 and are graphically expressed in Figure 13.

The observations indicated that the maximum activity of Enzyme II was expressed at $30^{o}C$ incubation. At further temperatures, activities showed plateau.

The time interval of incubation was also decided using various time intervals of incubation (5',10', 15', 20', 30',35' and 40'). The enzyme activity was expressed as units per mg proteins and is given in Table 15 and expressed as graph in Figure 14.

The results indicated that Enzyme II activity was increased linearly upto 30' of interval it continued to maintain plateau of maximum activity.

For the further kinetic studies ten minutes of incubation period was used.

TABLE - 14

(Effect of Temperature)

Sr.No.	Temperature (^{o}C)	AcPase activity (Units/mg protein)
1.	$15^{o}C$	0.0085 ± 0.00034
2.	$20^{o}C$	0.0150 ± 0.00060
3.	$25^{o}C$	0.0460 ± 0.00184
4.	$30^{o}C$	0.0550 ± 0.00230
5.	$33^{o}C$	0.0550 ± 0.00220
6.	$37^{o}C$	0.0550 ± 0.00200
7.	$39^{o}C$	0.0540 ± 0.00216
8.	$40^{o}C$	0.0560 ± 0.00224

Values are mean ± SE of five experiments.

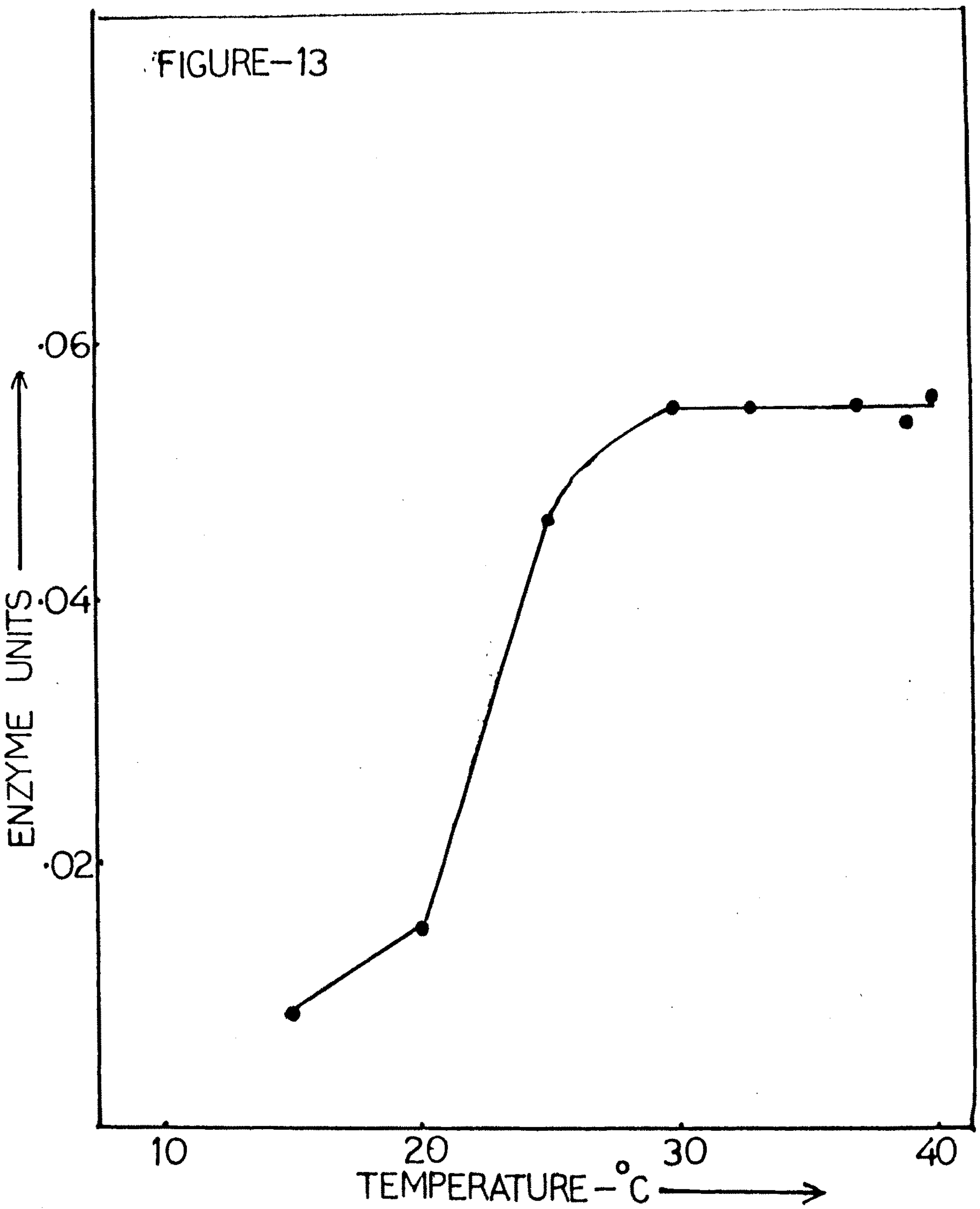
FIGURE-13
ENZYME UNITS
·06
·04
·02
10
20
30
40
TEMPERATURE-°C

TABLE - 15

(Effect of Incubation Time)

Sr.No.	Time (Minutes)	AcPase activity (Units/mg protein)
1.	5	0.17 ± 0.0068
2.	10	0.34 ± 0.0136
3.	15	0.50 ± 0.0200
4.	20	0.67 ± 0.0268
5.	25	0.67 ± 0.0260
6.	30	0.67 ± 0.0264
7.	35	0.68 ± 0.0272
8.	40	0.67 ± 0.0261

Values are mean ± SE of five experiments.

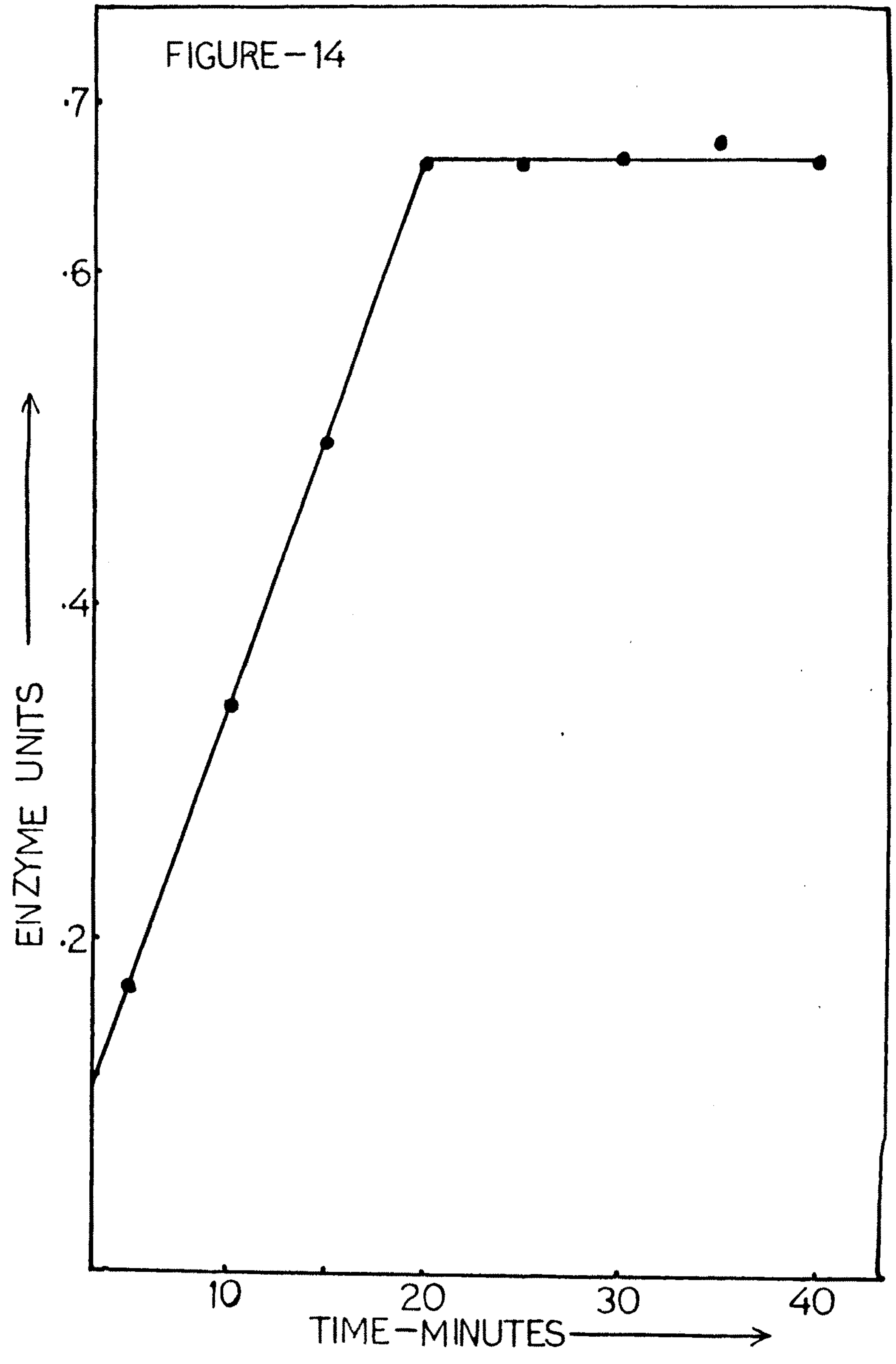
FIGURE-14
·7
·6
·4
·2
ENZYME UNITS
10
20
30
40
TIME-MINUTES

Km determination :

To study the Km of the enzyme, various concentrations of the substrate p-nitrophenyl phosphate were used (5mM, 10mM, 15mM, 20mM, 25mM, 30mM, 35mM, 40mM, 45mM, 50mM) in 0.2 M acetate buffer at pH 4.4.

The values of the enzyme activities were calculated as per mg protein of the purified enzyme sample.

The observations are tabulated in Table 16 and expressed in graphic forms in Figure 15.

From the illustrations very slow expression of enzyme activity upto 20mM could be evident. Then steady increase in the expressions of the enzyme activities were noted which continued upto 35mM concentrations of p-nitrophenyl phosphate. At further concentrations of the p-nitrophenyl phosphate, the enzyme activities remained steady.

The Km value of the substrate determined comes about 29mM of p-nitrophenyl phosphate.

All the kinetic studies were done by using the substrate concentration of 29mM of p-nitrophenyl phosphate. Effect of divalent ions on the enzyme activity following salts were used for the studies.

TABLE - 16

(Effect of Substrate Concentration)

Sr.No.	Concentration (mM)	AcPase activity (Units/mg protein)
1.	5	0.00 ± 0.0000
2.	10	0.00 ± 0.0000
3.	15	0.00 ± 0.0000
4.	20	0.05 ± 0.0020
5.	25	0.16 ± 0.0064
6.	30	0.33 ± 0.0132
7.	35	0.48 ± 0.0192
8.	40	0.48 ± 0.0189
9.	45	0.48 ± 0.0190

Values are mean ± SE of five experiments.

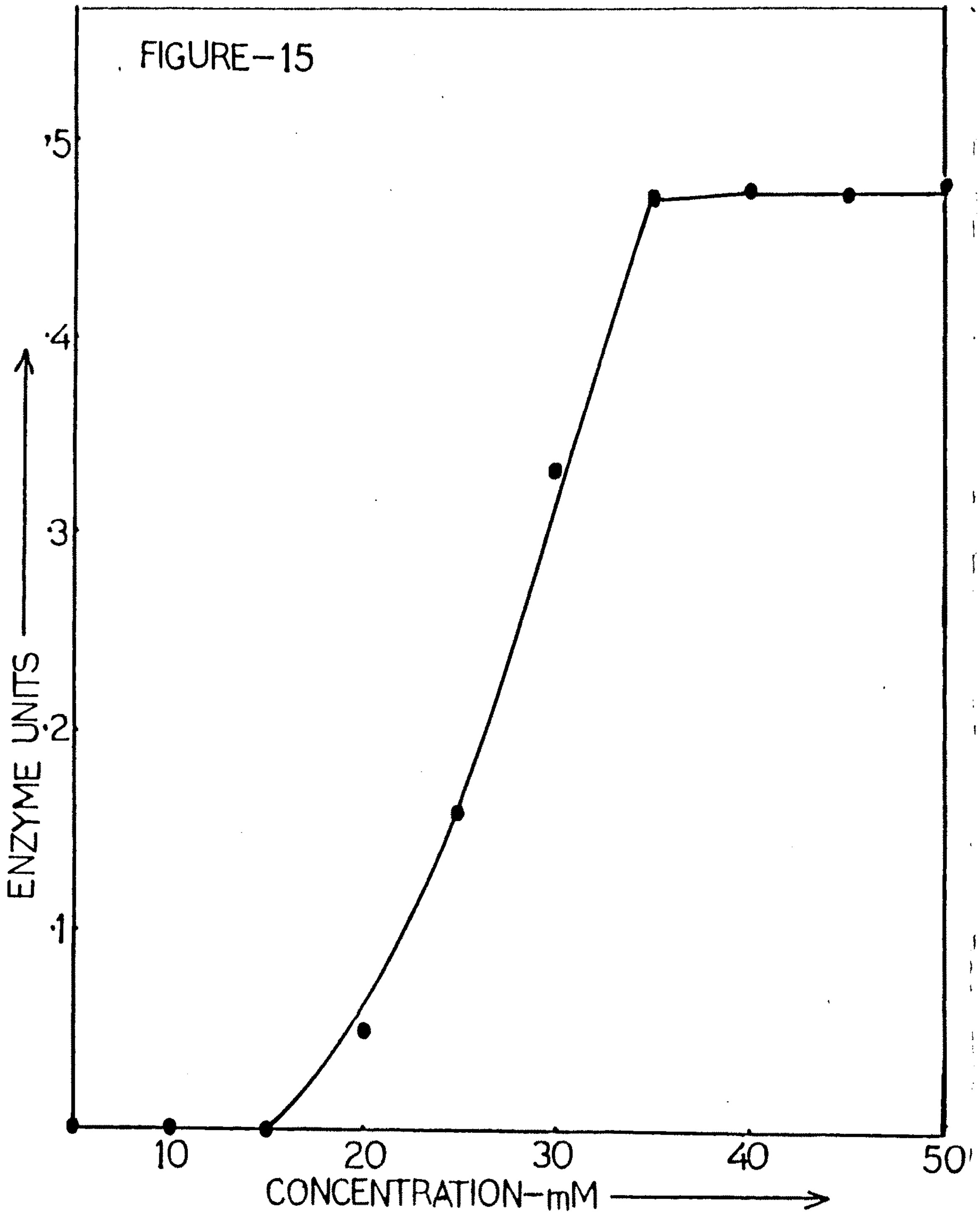
FIGURE-15
ENZYME UNITS
·5
·4
·3
·2
·1
10
20
30
40
50
CONCENTRATION-mM

1) $MgSO_4$ - Magnesium Sulfate
2) $MnSO_4$ - Manganese Sulfate
3) $CuSO_4$ - Copper Sulfate
4) $CaCl_2$ - Calcium Chloride.

1) Effect of $MgSO_4$:

The effect of Magnesium sulfate was studied on enzyme activities using various concentrations (5 mM, 10mM, 15 mM, 20mM, 25 mM, 30mM, 35 mM, 40mM, 45 mM, 50mM).

The results were estimated as units per mg protein of the sample.

The values of the experimental results are given in Table 17 and graphic illustrations are given in Figure 16.

The observations indicated that the enzyme activities increased along with the increase in Magnesium sulfate concentrations.This continued till 35 mM concentration of magnesium sulfate.

At further increasing concentrations of manganese sulfate the activities remained practically constant giving the plateau in graphic illustrations.

TABLE - 17

(Effect of $MgSO_4$)

Sr.No.	Concentration (mM)	AcPase activity (Units/mg protein)
1.	5	0.05 ± 0.0020
2.	10	0.10 ± 0.0040
3.	15	0.15 ± 0.0060
4.	20	0.21 ± 0.0084
5.	25	0.24 ± 0.0096
6.	30	0.30 ± 0.0120
7.	35	0.33 ± 0.0132
8.	40	0.33 ± 0.0130
9.	45	0.34 ± 0.0136
10.	50	0.33 ± 0.0128

Values are mean ± SE of five experiments.

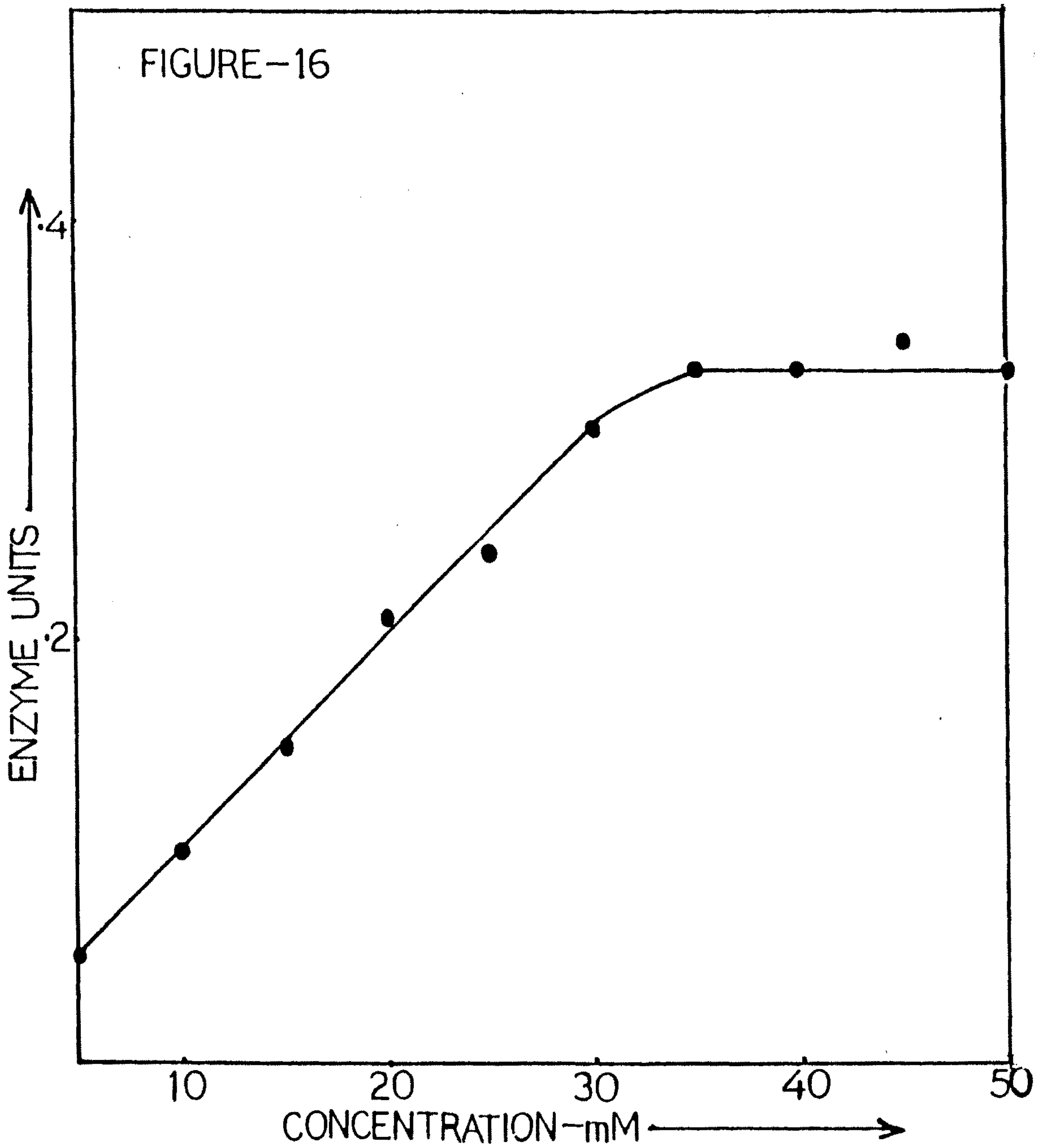
FIGURE-16
ENZYME UNITS
·4
·2
10
20
30
40
50
CONCENTRATION-mM

2) Effect of Manganese Sulfate :

Effect of manganese sulfate is studied on Enzyme II activity at various concentrations (5mM, 10mM, 15mM, 20mM, 25mM, 30mM, 400mM, 45mM, 50mM and 55mM).

The results obtained were in units per mg protein of the sample.

The values of the experimental results are given in Table 18 and its alterations in graphic forms are illustrated in Figure 17.

The alterations indicated that manganese sulfate linearly activated the enzyme upto 44mM of concentration and further the activities were practically constant.

3) Effect of $CuSO_4$:

The enzyme activity of Enzyme II was also studied using various concentrations of $cuSO_4$ (upto 0.1mM). But at any of the concentrations studied, the enzyme was inhibited.

4) Effect of $CaCl_2$:

The varied concentrations of calcium chloride (upto 0.1mM) were studied for their influence on Enzyme II activity. The

TABLE - 18

(Effect of $MnSO_4$)

Sr.No.	Concentration (mM)	AcPase activity (Units/mg protein)
1.	5	0.132 ± 0.00528
2.	10	0.266 ± 0.01064
3.	15	0.400 ± 0.01600
4.	20	0.500 ± 0.02000
5.	25	0.670 ± 0.02680
6.	30	0.800 ± 0.03200
7.	35	0.900 ± 0.03600
8.	40	1.060 ± 0.04240
9.	45	1.170 ± 0.04680
10.	50	1.170 ± 0.04675
11.	55	1.170 ± 0.04682

Values are mean ± SE of five experiments.

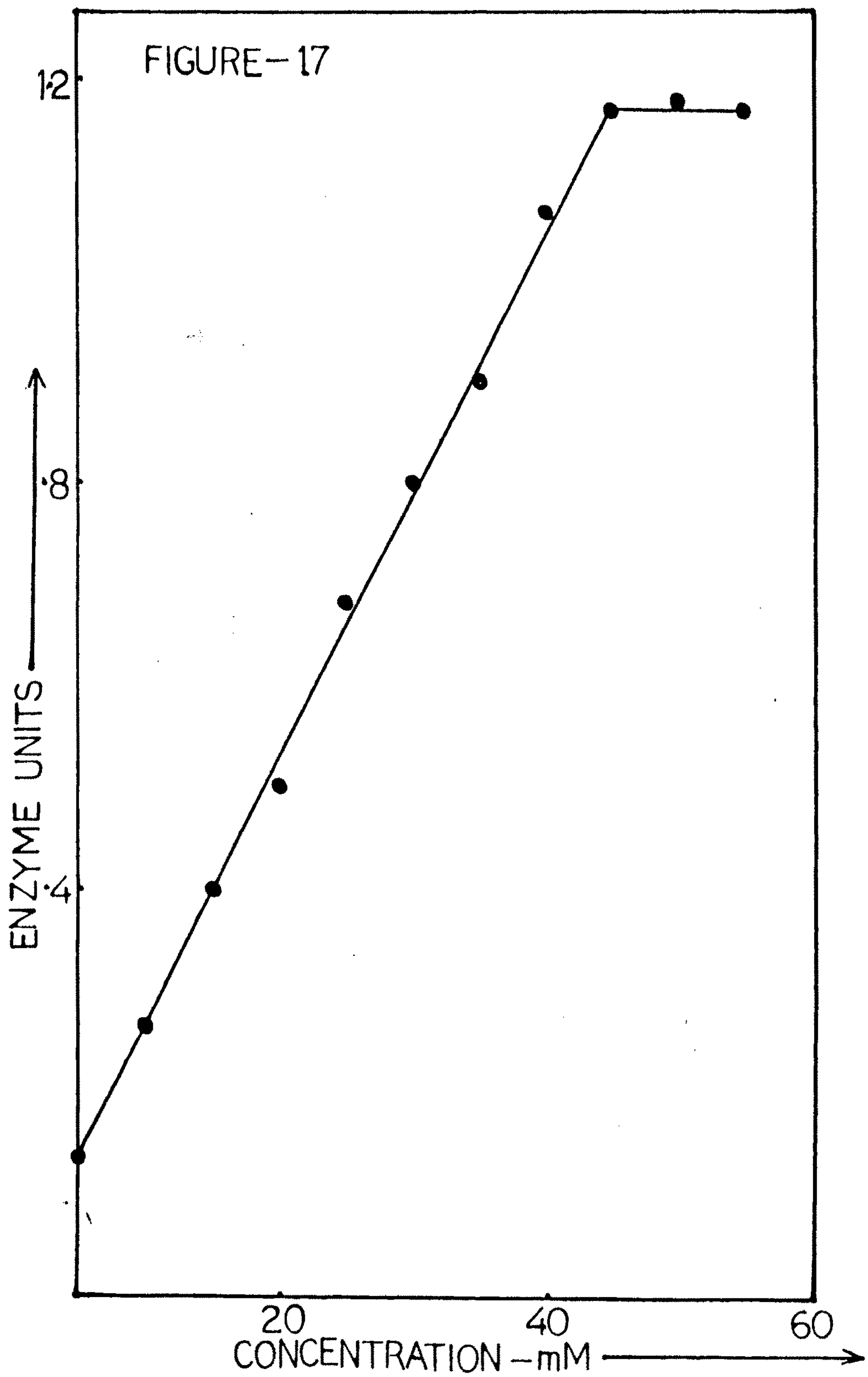
FIGURE-17
1·2
·8
·4
ENZYME UNITS
20
40
60
CONCENTRATION-mM

results indicated that at any concentration of the calcium chloride the activity of Enzyme II was inhibited.

Effect of EDTA and EGTA :

The enzyme activities were inhibited totally at 20mM., The activities were also inhibited in presence of magnesium sulfate by EDTA.

In presence of EDTA (20mM) the activity was not altered which indicated its failure to inhibit enzyme activities.

Similarly even in presence of magnesium sulfate the activities were not altered by EGTA.

Effects of other chemicals :

The other modifiers of enzyme activities were also studied to see the alterations in Enzyme II activities in presence of these modifiers. Following were the modifiers that were utilized for the studies :

1. Formalin
2. Triton X-100
3. Methanol
4. Ethanol
5. Acetone
6. Tartarate

7. Citrate
8. Glycerol
9. NaF.

The observations are given in Table 19.

Effect of preincubation of Enzyme II on enzyme activity :

In the initial work, Enzyme II was preincubated at the 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 activity. The results indicated that the preincubation upto 60^{o}C retained enzyme activities in preincubated Enzyme II. At further temperatures enzyme activities were not retained on preincubation.

Therefore, further studies were done using time variations at 60^{o}C preincubation of Enzyme II.

Enzyme II was preincubated at 60^{o}C for various time intervals. This preincubated enzyme at 60^{o}C for 3', 5', 7', 10', 15', 20', 25', 30', 35', 40'. These samples were further used for enzymatic studies.

The enzyme activities were estimated as units per mg protein. The alterations occurred in the enzyme activities from the above experiments are given in Table 10 and the graphical illustrations are depicted in Figure 17.

TABLE - 19

(Effect of Other Chemicals)

Sr.No.	Name of the chemical	Inhibition of Enzyme-II	No Influence
1.	1 % Formalin	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 M Citrate	Total	-
8.	1 % Glycerol	-	No influence
9.	$<$ 20mM NaF	Total	-

TABLE - 20

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

Sr.No.	Time (Minutes)	AcPase activity (Units/mg protein)
1.	3	0.12 ± 0.0048
2.	5	0.13 ± 0.0052
3.	7	0.16 ± 0.0064
4.	10	0.18 ± 0.0072
5.	15	0.91 ± 0.0364
6.	20	0.98 ± 0.0392
7.	25	1.14 ± 0.0456
8.	30	1.15 ± 0.0460
9.	35	1.14 ± 0.0450
10	40	1.14 ± 0.0451

Values are means ± SE of five experiments.

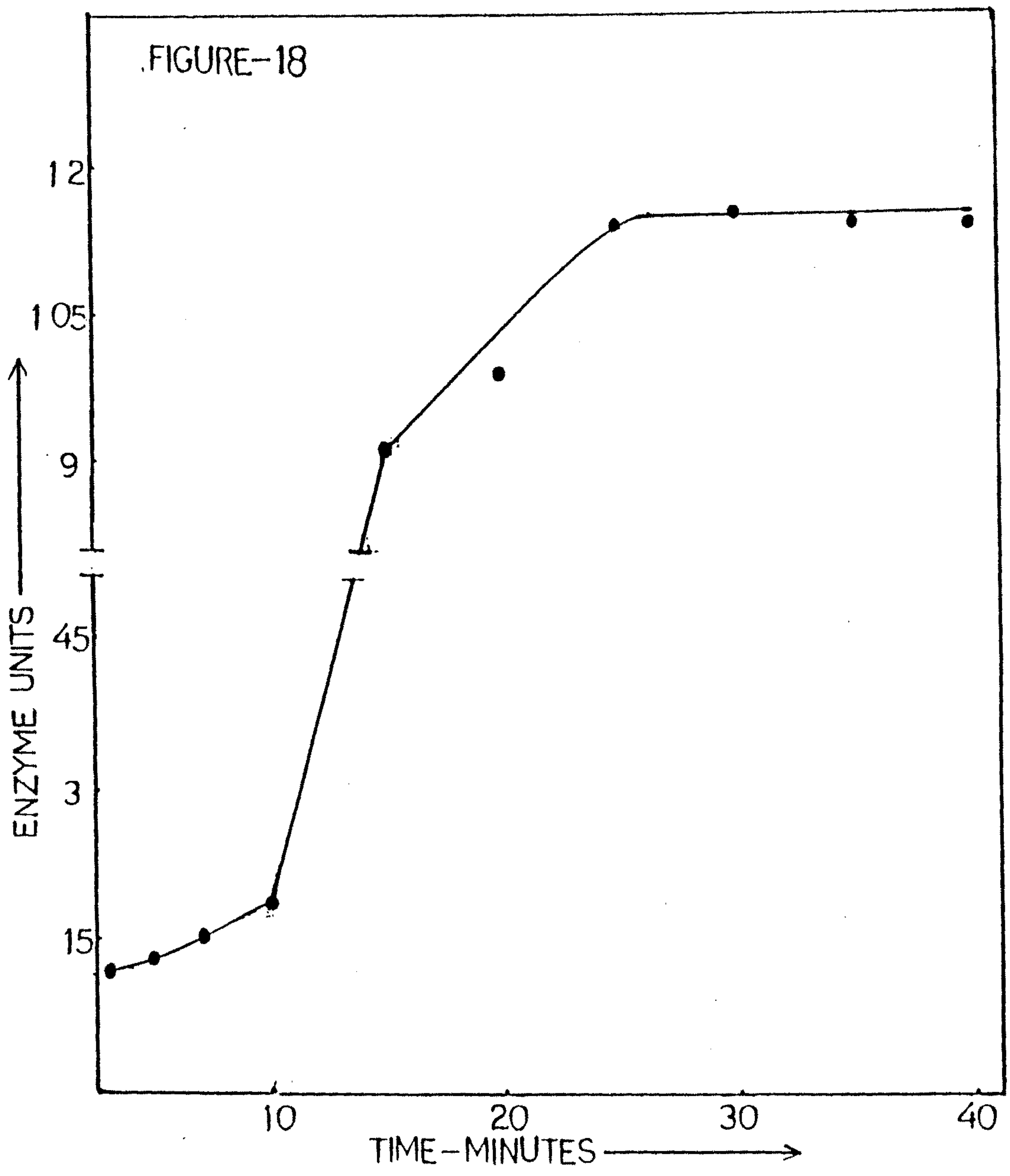
FIGURE-18
12
1 05
9
45
3
15
ENZYME UNITS
10
20
30
40
TIME-MINUTES

The results of above work indicated that the preincubation of enzyme at $60^{o}C$ for 3', 5', 7' and 10' showed increase in the expression of enzyme activities as compared to normal purified Enzyme II.

At 15' of preincubation of the enzyme II resulted in the burst of enzyme activity. This burst was practically maintained by Enzyme II samples preincubated at 20', 25', 30', 35', 40' without any further alteration.

Thus the results indicated from the kinetic studies that were conducted using Enzyme II isolated from Rana cyanophlyctis ovary during prebreeding conditions showed the following characters:

1. pH Optimum - 4.4
2. Temperature Optimum - $30^{o}C$
3. Km for p-nitrophenyl-phosphate - 34 mM.
4. Activated linearly by
   $MgSO_4$ (upto 35 mM)
   $MnSO_4$ (upto 44mM)
5. Inhibited by
   $CuSO_4$ (totally inhibited)
   $CaCl_2$ (totally inhibited)
   EDTA (totally inhibited)
6. Not influenced by EGTA

7. Totally inhibited by

1 % Formalin

1 % Triton X-100

1 % Methanol

1 % Acetone

1 % Ethanol

0.05 M Citrate

(1mM and above) NaF.

8 Not influenced by

20mM tartarate

1 % glycerol.

9. The effect of preincubation of enzyme sample on enzyme activity indicated that the enzyme activity resulted into burst on 15' of preincubation which was retained upto 40' of preincubation.

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