C H A P T E R - III

E N Z Y M E - I

C H A P T E R – III

ENZYME I

Tiplon and Dixon (1979) have discussed the effects of pH on enzymes in which they have discussed the phenomenon of "The presence of more than one enzyme - substrate intermediates". This mechanism can be extended to activity for more than two protonic states of the enzyme-substrate complex. Provided the pK values are separated enough so that plateaus exist; their values can be determined as described by the peaks of the activities.

According to the above mechanism the pH optima studies were carried out for the isolated enzyme sample using the buffer system and substrate concentration used for the acid phosphatase studies for the frog tissues, (*Kubicz* , et. al.,1981; Mest er, et. al., 1985; Jan ska et. al.,1989), so that multiple forms of enzymes if any based on the pH variations could be determined.

pH optima

The isolated and purified enzyme samples were used in the Acetate buffer of 0.2 M at various pH (3.25, 3.5, 3.7, 3.8, 4.0, 4.2, 4.4, 4.6, 4.8, 5, 5.2, 5.4, 5.6 as per Dawson et at ,1978). The substrates used were p-nitrophenyl phosphate

and Na-β-glycerophosphate, glucose-6-phosphate , and ATP. P-nitrophenyl phosphate and Na-β-glycerophosphate, both expressed the equal units of enzymes and therefore results of p-nitrophenyl phosphate only are expressed in present project. Glucose-6-phosphate and ATP were not susceptible for hydrolysis by enzymes in the above pH series. The isolated enzyme samples were prepared dissolving the precipitate in acetate buffers of respective pH with known protein content.

Table 1 shows the enzyme activities expressed as units per mg protein at different pH. Figure 1 shows, the graphic representation of the Table 1.

As the maximum activities were considered; there were three types of acid phosphatases acting at three different pHs- 3.7, 4.4 and 5.

These three forms of enzymes acting at different pH had been used for the kinetic studies of the enzymes.

In this chapter acid phosphatase I (Enzyme I) was further confirmed for its optimum pH using enzyme samples prepared in acetate buffer of 0.2 M and pH 3.7 and using the acetate buffer of 0.2 M of varied pH for assay (3.25, 3.5, 3.7, 3.8, 4, 4.2, 4.4,4.6, 4.8, 5, 5.4, 5.6 as per Dawson et.al.,1978).

TABLE - 1

(Effect of pH)

Sr.No.	pH	AcPase activity (Units/mg protein)
1	3.25	0.020 ± 0.0008
2.	3.5	0.032 ± 0.0013
3.	3.7	0.068 ± 0.0027
4.	3.8	0.030 ± 0.0012
5.	4.0	0.02 ± 0.0003
6.	4.2	0.020 ± 0.0007
7.	4.4	0.050 ± 0.0020
8.	4.6	0.020 ± 0.0005
9.	4.8	0.020 ± 0.0002
10.	5.0	0.040 ± 0.0016
11.	5.2	0.020 ± 0.0004
12.	5.4	0.020 ± 0.0006
13.	5.6	0.020 ± 0.0009

Values are mean ± SE of five experiments

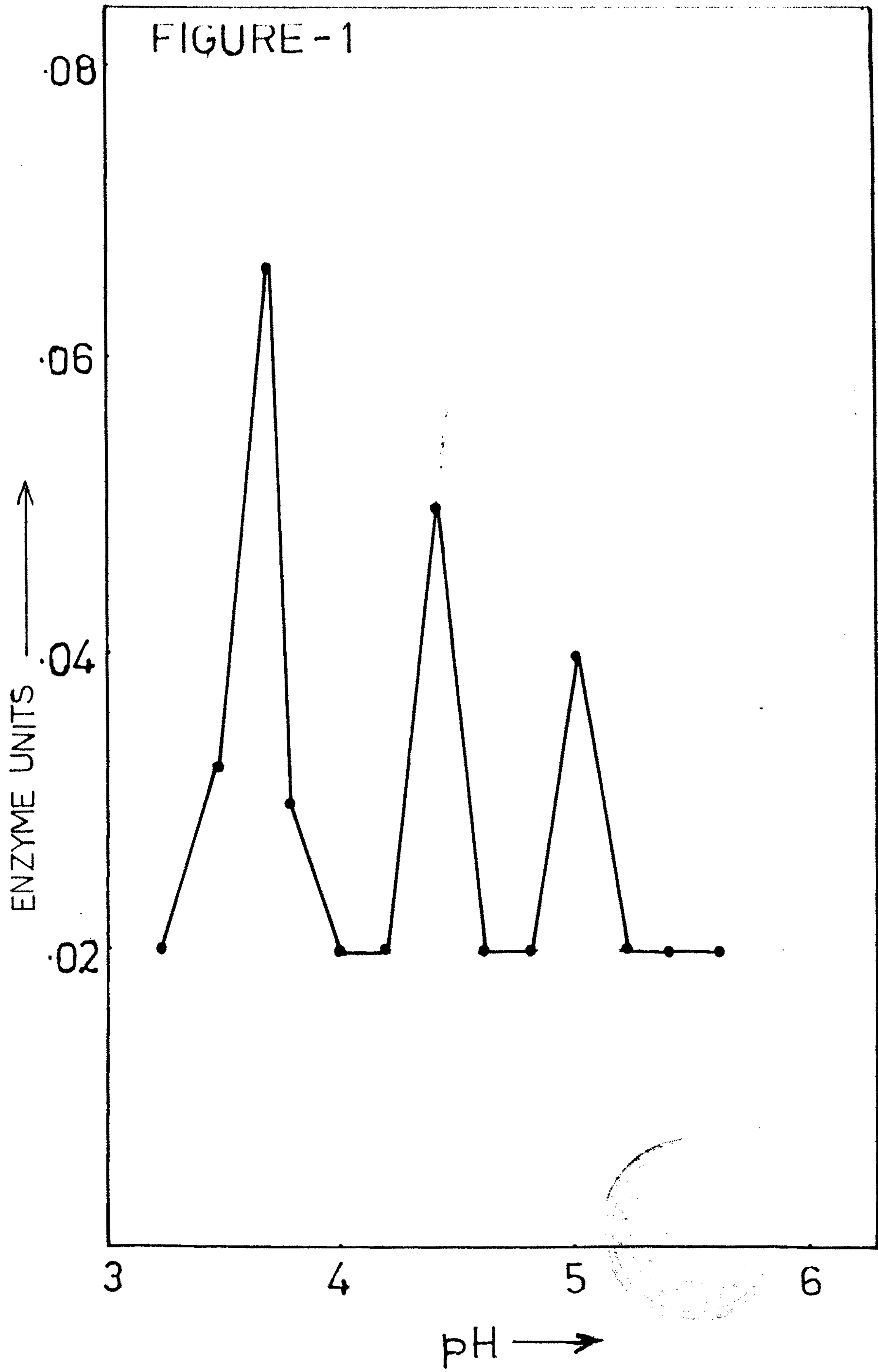
FIGURE-1
·08
·06
·04
·02
ENZYME UNITS
3
4
5
6
pH

Table 2 shows the enzyme activities and Figure 2 shows the graphic illustration.

Optimum temperature

To study the optimum temperature of Enzyme I, the enzyme activities were carried out at different temperatures (15°C, 20°C, 25°C, 30°C, 33°C, 37°C, 39°C, 40°C) and the optimum temperature for Enzyme I was noted.

Table 3 gives the enzyme activities per mg protein andFigure 3 gives the gra phic illustration of theTable 3.

The observations indicated from the graph . Were that Enzyme I showed the optimum temperature of 30°C for its activity.

Therefore further kinetics was done using 30°C as the optimum temperature for enzyme activity.

To study the optimum incubation time for assay the assay system was studied with varying time intervals of incubation period. Table 4 shows the enzyme activities/mg protein with variations in time of incubations and Table 4 is represented graphically in Figure 4.

The Figure 4 indicated linearly increasing enzyme activity upto 20 minutes of incubation and further upto 30 minutes maintained plateau at its maximum.

TABLE - 2

(Effect of pH)

Sr.No.	pH	AcPase activity (Units/mg protein)
1.	3.25	0.020 ± 0.0006
2.	3.5	0.022 ± 0.0010
3.	3.7	0.080 ± 0.0032
4.	3.8	0.020 ± 0.0008
5.	4.0	0.020 ± 0.0005
6.	4.2	0.019 ± 0.0007
7.	4.4	0.021 ± 0.0009
8.	4.6	0.020 ± 0.0004
9.	4.8	0.022 ± 0.0011
10.	5.0	0.020 ± 0.0012
11.	5.2	0.020 ± 0.0003
12.	5.4	0.019 ± 0.0003
13.	5.6	0.020 ± 0.0002

Values are mean ± SE of five experiments.

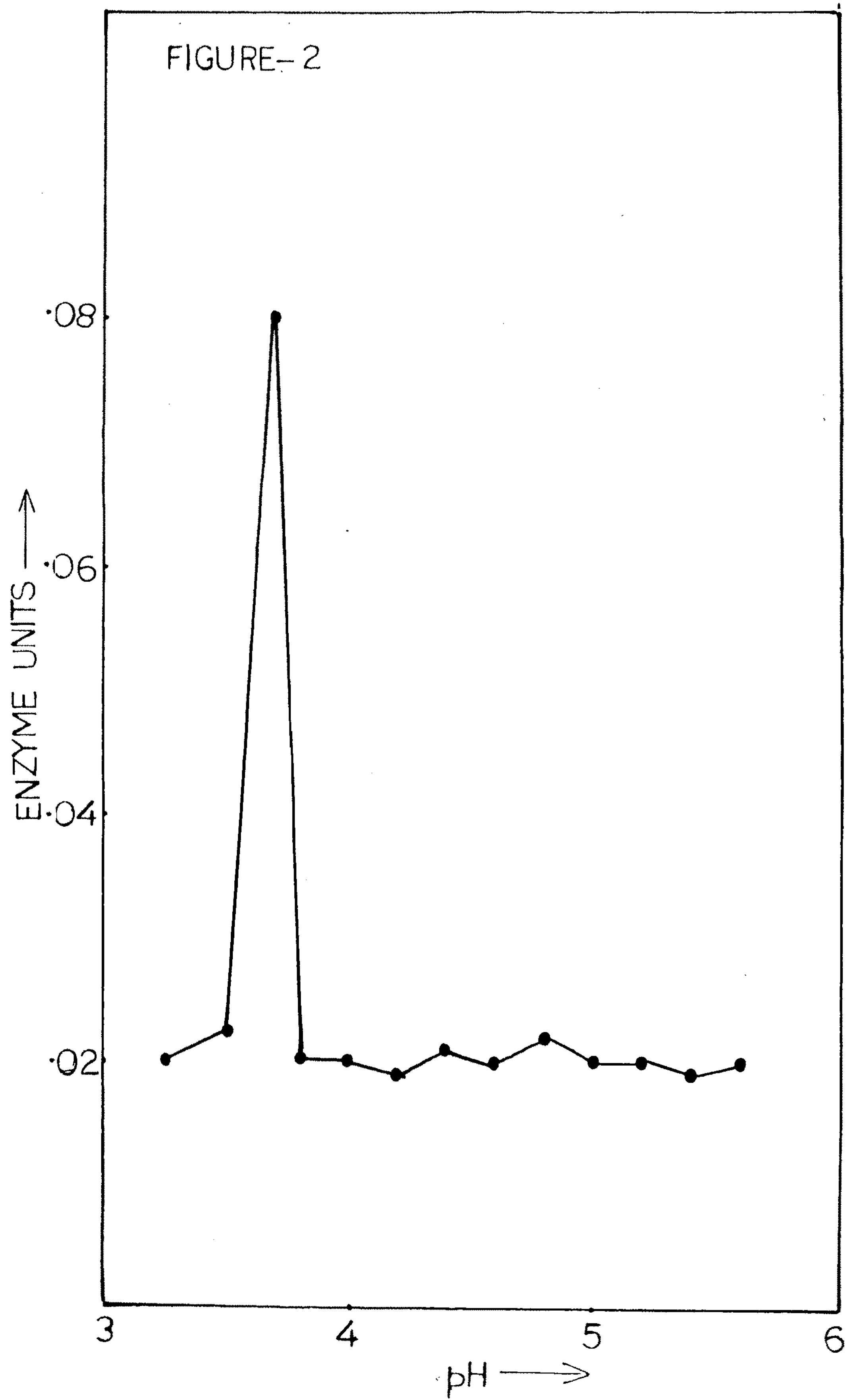
FIGURE- 2
ENZYME UNITS
·08
·06
·04
·02
3
4
5
6
pH

TABLE - 3

(Effect of Temperature)

Sr.No.	Temperature (^{o}C)	AcPase activity (Units/mg protein)
1.	$15^{o}C$	0.0075 ± 0.00030
2.	$20^{o}C$	0.0150 ± 0.00060
3.	$25^{o}C$	0.0500 ± 0.00200
4.	$30^{o}C$	0.0600 ± 0.00240
5.	$33^{o}C$	0.0600 ± 0.00210
6.	$37^{o}C$	0.0590 ± 0.00230
7.	$39^{o}C$	0.0610 ± 0.00260
8.	$40^{o}C$	0.0600 ± 0.00220

Values are mean ± SE of five experiments.

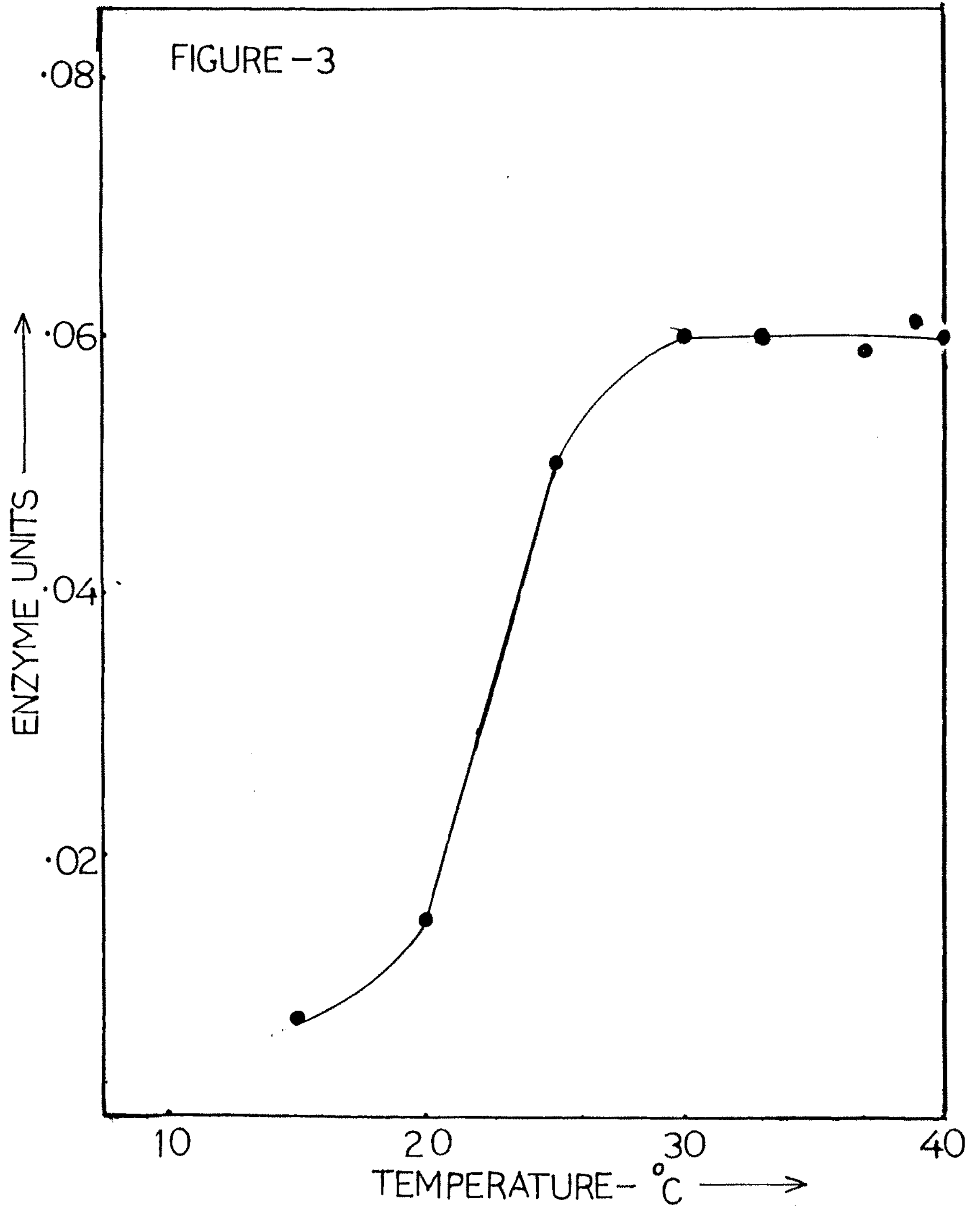

FIGURE - 3
·08
·06
·04
·02
ENZYME UNITS
10
20
30
40
TEMPERATURE - °C

TABLE - 4

(Effect of Incubation Time)

Sr.No.	Time (Minutes)	AcPase activity (Units/mg protein)
1.	5	0.14 ± 0.0056
2.	10	0.28 ± 0.0112
3	15	0.44 ± 0.0176
4.	20	0.58 ± 0.0232
5.	25	0.58 ± 0.0228
6.	30	0.58 ± 0.0230
7.	35	0.56 ± 0.0224
8.	40	0.58 ± 0.0229

Values are mean ± SE of five experiments.

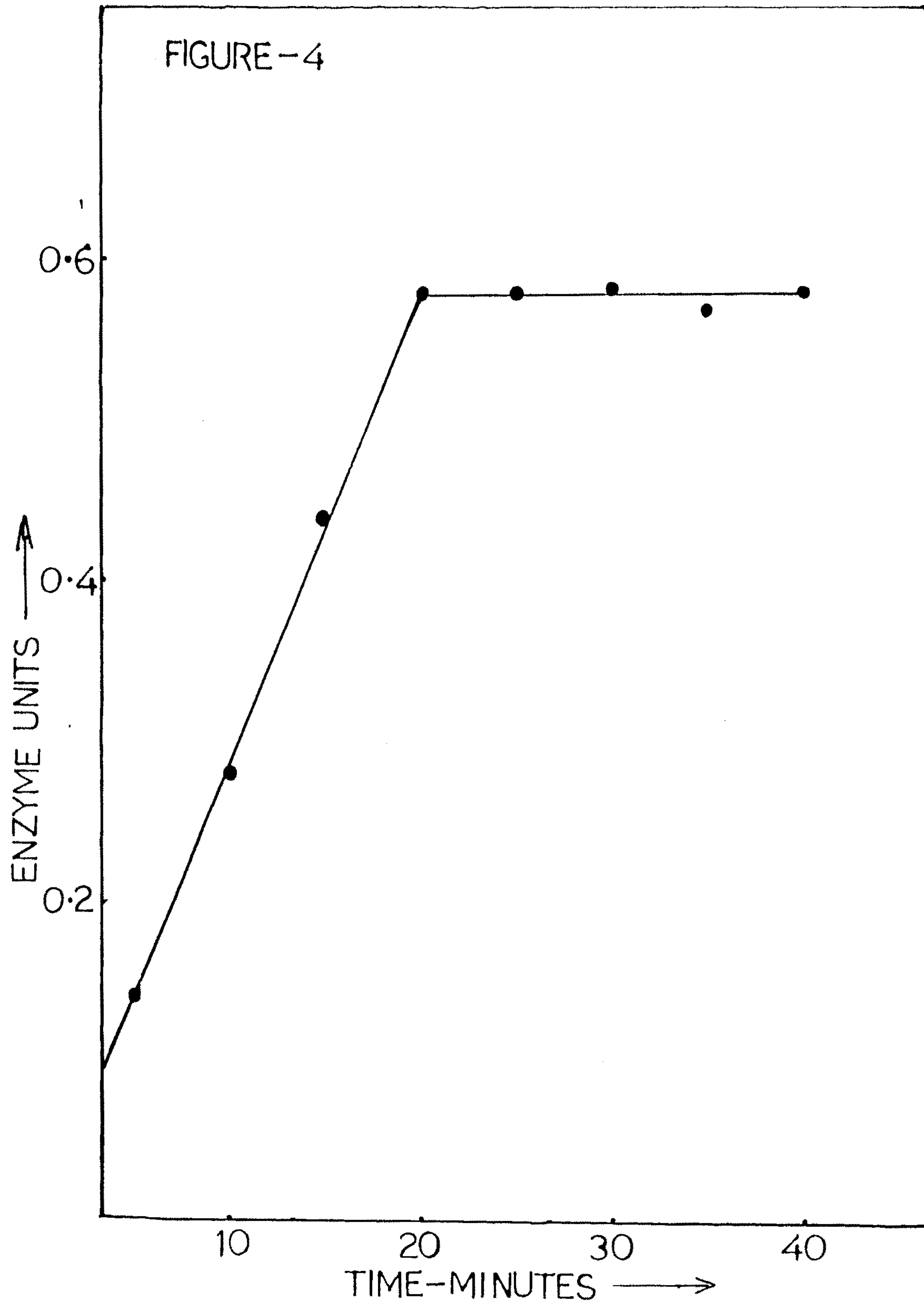
FIGURE-4
0·6
0·4
0·2
ENZYME UNITS
10
20
30
40
TIME-MINUTES

For the further Kinetic studies ten minutes of incubation period was chosen.

Km determination

Effect of substrate concentration on the enzyme activities of Enzyme I were studied.

p-nitrophenyl phosphate was used in varying concentration (5 mM, 10mM, 15 mM, 20mM, 25 mM, 30mM, 35 mM, 40mM, 45 mM) in 0.2 M acetate buffer at pH 3.7. The activities were estimated as per mg protein and given in Table 5 and its graphical illustration is given in Figure 5.

The data from the above experiment indicated that upto 15 mM concentration the rate of increase in enzyme activity was slow and further it smoothly increased upto 30mM of substrate concentration to achieve its maximum which was retained for further concentrations of p-nitrophenyl phosphate.

All the kinetic studies were done by using the substrate concentration of 20.8mM of p-nitrophenyl phosphate.

Effect of divalent ions on the enzyme activity :

To study the effects of divalent ions on enzyme activity following salts were used for the studies -

TABLE - 5

(Effect of Substrate concentration)

Sr.No.	Concentration (mM)	AcPase activity (Units/mg protein)
1.	5 mM	0.00 ± 0.0000
2	10 mM	0.05 ± 0.0020
3.	15 mM	0.10 ± 0.0040
4	20 mM	0.23 ± 0.0092
5.	25 mM	0.33 ± 0.0132
6.	30 mM	0.44 ± 0.0176
7.	35 mM	0.45 ± 0.0180
8.	40 mM	0.45 ± 0.0178
9.	45 mM	0.45 ± 0.0175

Values are mean ± SE of five experiments.

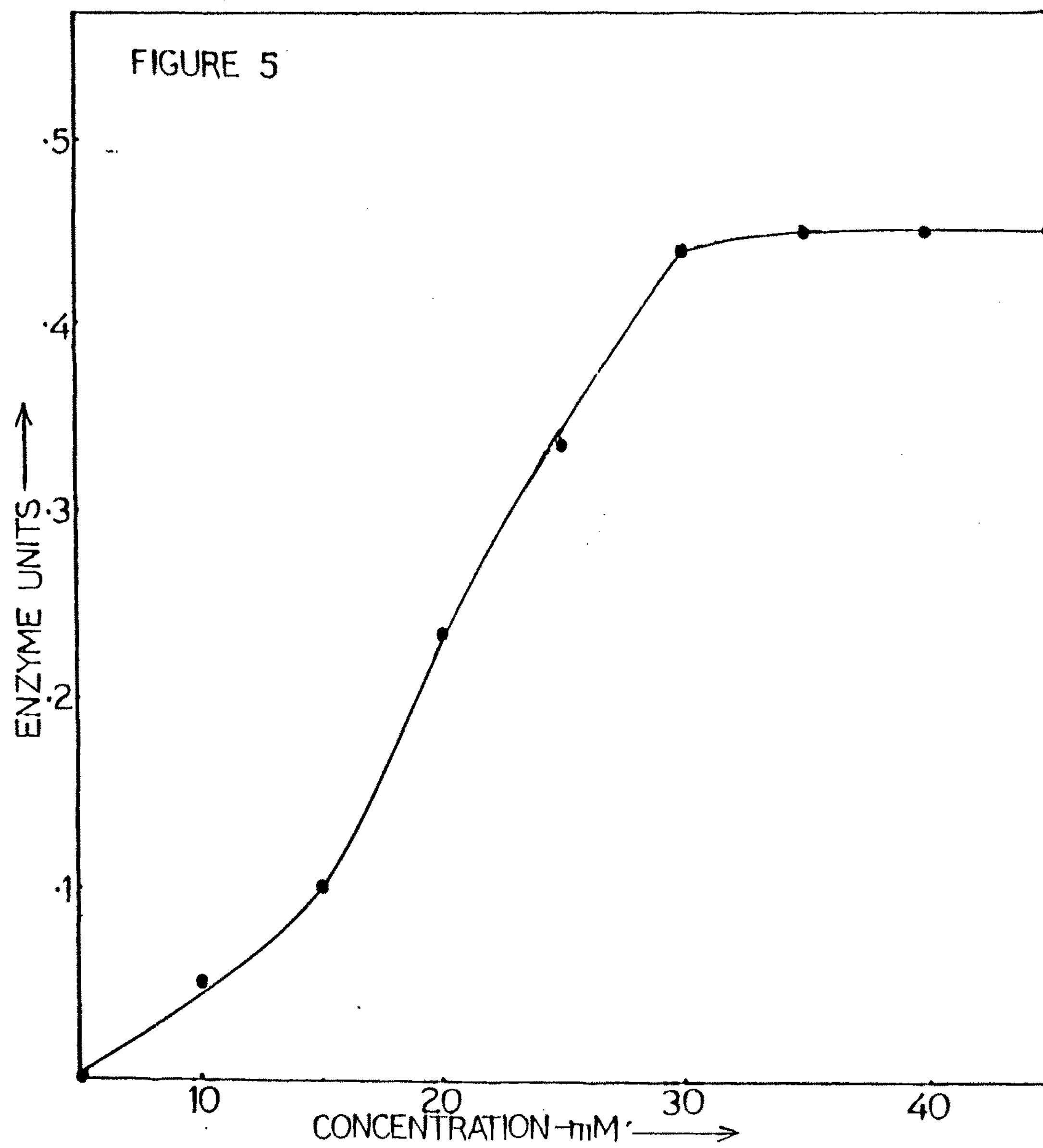

FIGURE 5
·5
·4
·3
·2
·1
ENZYME UNITS
10
20
30
40
CONCENTRATION-mM

1) $MgSO_4$ (Magnesium Sulfate)

2) $MnSO_4$ (Manganese Sulfate)

3) $CuSO_4$ (Copper Sulfate)

4) $CaCl_2$ (Calcium Chloride)

1) Effect of Magnesium Sulfate:

The effect of Magnesium sulfate with varied concentrations (5mM, 10mM, 15mM, 20mM, 25mM, 30mM, 35mM, 40mM, 45mM, 50mM,) were studied on the enzyme activities of the Enzyme I.

The results were obtained as enzyme activities per mg protein and are given in Table 6.

The graphical representation of the Table 6 is given by Figure 6.

The results indicated that Mg^{++} activated the Enzyme I. The activation was directly proportional to the concentration of Mg^{++} upto 40mM.

2) Effect of Manganese Sulfate :

The effect of Manganese Sulfate on the enzyme activities were studied with various concentrations of Manganese sulfate (10mM, 20mM, 30mM, 40mM, 50mM, 60mM, 70mM, 80mM, 90mM, 100mM, 110mM, 120mM, 130mM).

TABLE - 6

(Effect of $MgSO_4$)

Sr.No.	Concentration (mM)	AcPase activity (Units/mg protein)
1.	5	0.020 ± 0.0008
2.	10	0.040 ± 0.0016
3.	15	0.067 ± 0.0027
4.	20	0.080 ± 0.0036
5.	25	0.100 ± 0.0040
6.	30	0.110 ± 0.0044
7.	35	0.140 ± 0.0056
8.	40	0.160 ± 0.0064
9.	45	0.160 ± 0.0060
10.	50	0.160 ± 0.0062

Values are mean ± SE of five experiments.

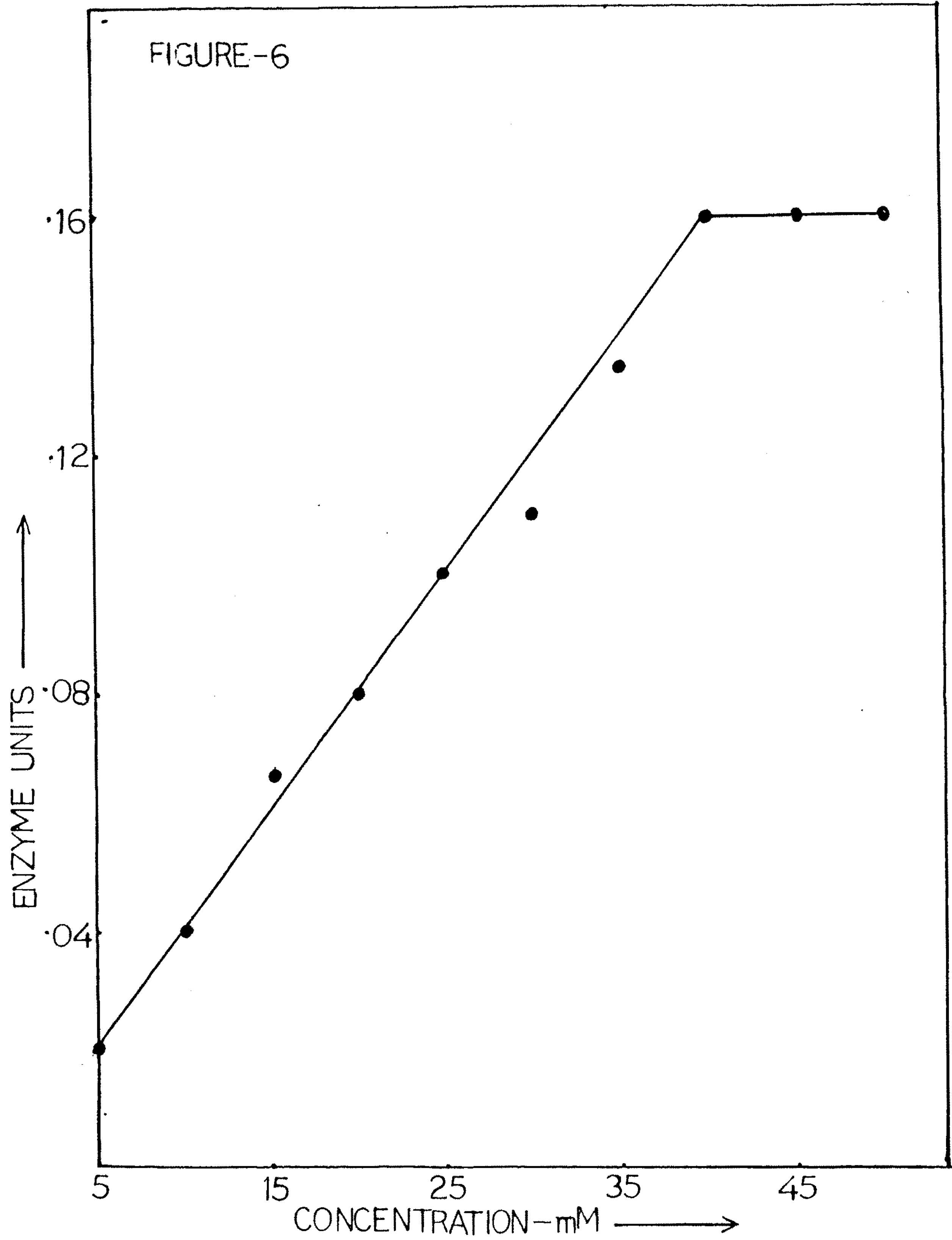
FIGURE-6
·16
·12
·08
·04
ENZYME UNITS
5
15
25
35
45
CONCENTRATION-mM

The enzyme activities were obtained as per mg protein.

The alterations there in are expressed in Table 7 and the respective graphical illustration is given in Figure 7.

The results indicated that the Mn^{++} activated the Enzyme I with the linear proportion upto 100mM concentrations. Further activities were retained at its maximum.

3) <u>Effect of Copper Sulfate</u> :

With the varying concentrations of Copper Sulfate (10mM, 15mM, 20mM, 25mM, 30mM, 35mM, 40mM, 45mM, 50mM,). The Enzyme I activity was studied.

The activity in terms of per mg protein is exhibited in Table 8 and graphic illustration is given in Figure 8.

The alterations indicated that the increase in activity is proportional to the increase in Cu^{++}. The increase continued upto the 35mM concentrations of Copper ions and in further concentrations the activity remained steady.

4) <u>Effect of Calcium Chloride</u> :

The activity of Enzyme I was estimated using various concentrations of Calcium chloride (upto0.1 mM) but at any value of Calcium chloride Enzyme I activity observed was 0.00.

TABLE - 7

(Effect of $MnSO_4$)

Sr.No.	Concentration (mM)	AcPase activity (Units/mg protein)
1.	10	0.10 ± 0.0040
2.	20	0.20 ± 0.0080
3.	30	0.30 ± 0.0120
4.	40	0.43 ± 0.0172
5.	50	0.52 ± 0.0208
6.	60	0.62 ± 0.0248
7.	70	0.76 ± 0.0304
8.	80	0.81 ± 0.0324
9.	90	0.87 ± 0.0348
10.	100	0.91 ± 0.0364
11.	110	0.91 ± 0.0360
12.	120	0.91 ± 0.0362
13.	130	0.91 ± 0.0368

Values are mean ± SE of five experiments.

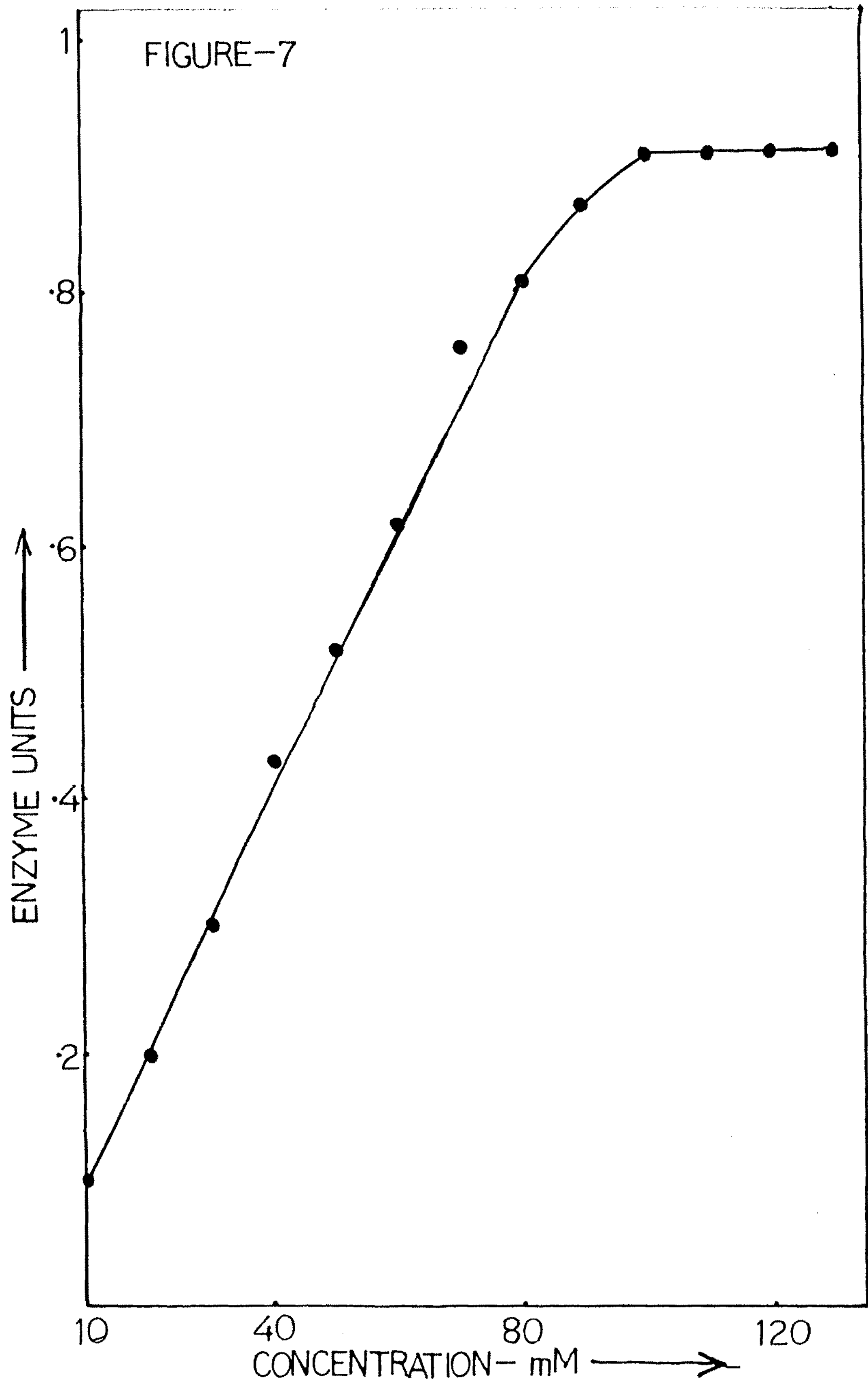
FIGURE-7
ENZYME UNITS
1
·8
·6
·4
·2
10
40
80
120
CONCENTRATION— mM

TABLE - 8

(Effect of $CuSO_4$)

Sr.No.	Concentration (mM)	AcPase activity (Units/mg protein)
1.	5	0.13 ± 0.0052
2.	10	0.30 ± 0.0120
3.	15	0.46 ± 0.0184
4.	20	0.63 ± 0.0252
5.	25	0.82 ± 0.0328
6.	30	0.92 ± 0.0368
7.	35	1.10 ± 0.0440
8.	40	1.10 ± 0.0410
9.	45	1.10 ± 0.0430
10	50	1.10 ± 0.0425

Values are mean ± SE of five experiments.

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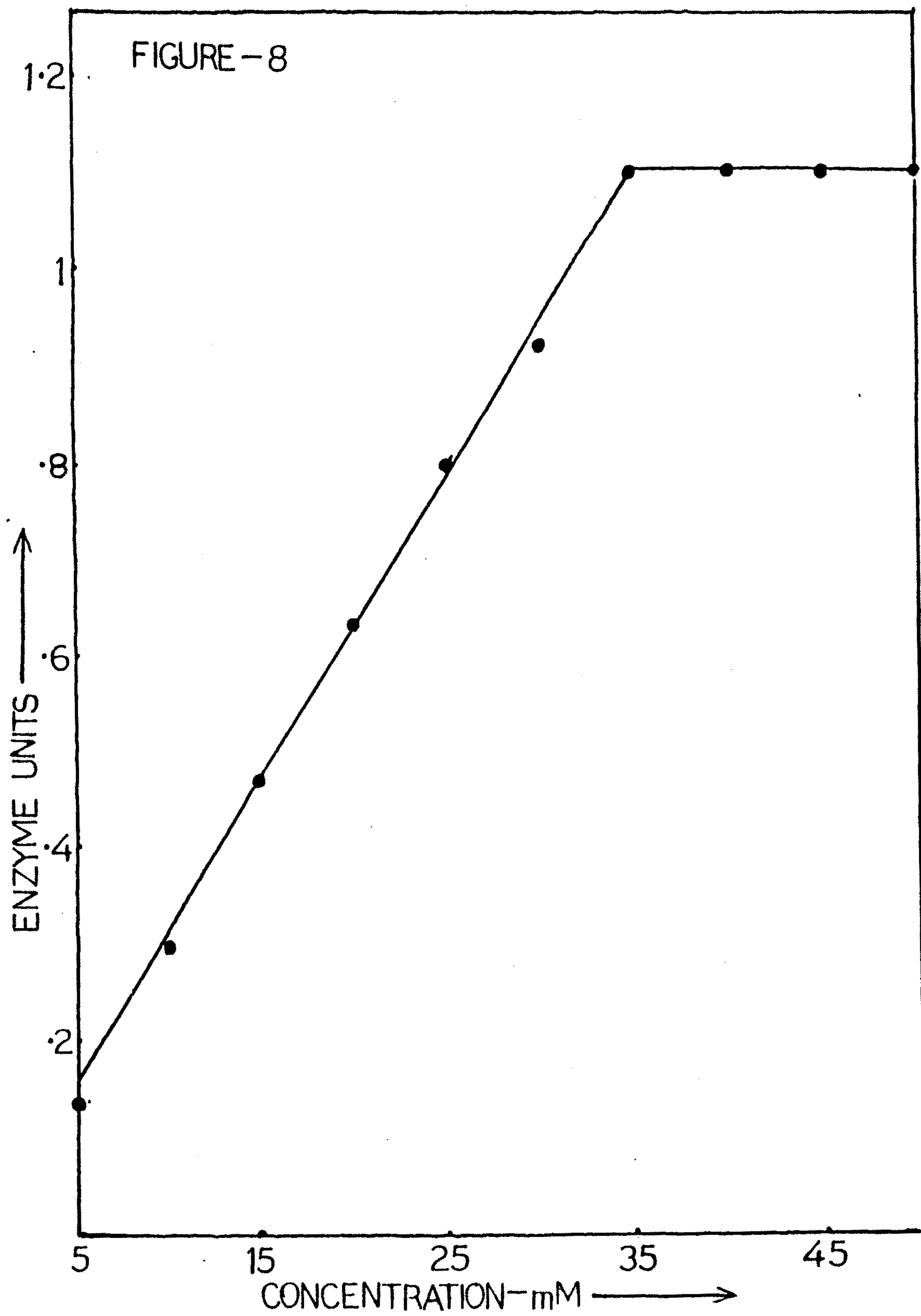
FIGURE-8
1·2
1
·8
·6
·4
·2
ENZYME UNITS
5
15
25
35
45
CONCENTRATION-mM

These results indicated the total inhibition of enzyme activity by Calcium chloride.

Effect of Citrate :

The activity of the Enzyme I was estimated in presence of increasing concentrations of citrate (0.01M, 0.02M, 0.03M, 0.04M, 0.05M, 0.06M, 0.07M, 0.08M, 0.09M , 0.1M)

The enzyme activities were calculated as units per mg protein and are given in Table 9 with its graphic representattion in Figure 9.

The results indicated that the Enzyme I is activated upto 0.05M of citrate in linear proportion. In presence of higher concentrations of citrate the activities remained steady.

Effect of Glycerol :

Enzyme I activity is also influenced by glycerol. The varying concentrations of glycerol (0.1 %, 0.2 %, 0.3 %, 0.4 %, 0.5% 0.6 %, 0.7 %, 0.8 %, 0.9 %, 1.0 %, 1.2 %, 1.4 %, 1.6 %).

The results are given in Table 10 and figure 10 represents graphical illustration.

The activities showed enhancement directly proportional to the increase in concentration of glycerol upto 1% concentration

TABLE - 9

(Effect of Citrate)

Sr.No.	Concentration (M)	AcPase activity (Units/mg protein)
1.	0.01 M	0.020 ± 0.0008
2.	0.02 M	0.028 ± 0.0011
3.	0.03 M	0.048 ± 0.0019
4.	0.04 M	0.060 ± 0.0024
5.	0.05 M	0.080 ± 0.0032
6.	0.06 M	0.078 ± 0.0031
7.	0.07 M	0.081 ± 0.0034
8.	0.08 M	0.080 ± 0.0030
9.	0.09M	0.079 ± 0.0029
10.	0.1 M	0.080 ± 0.0035

Values are mean ± SE of five experiments.

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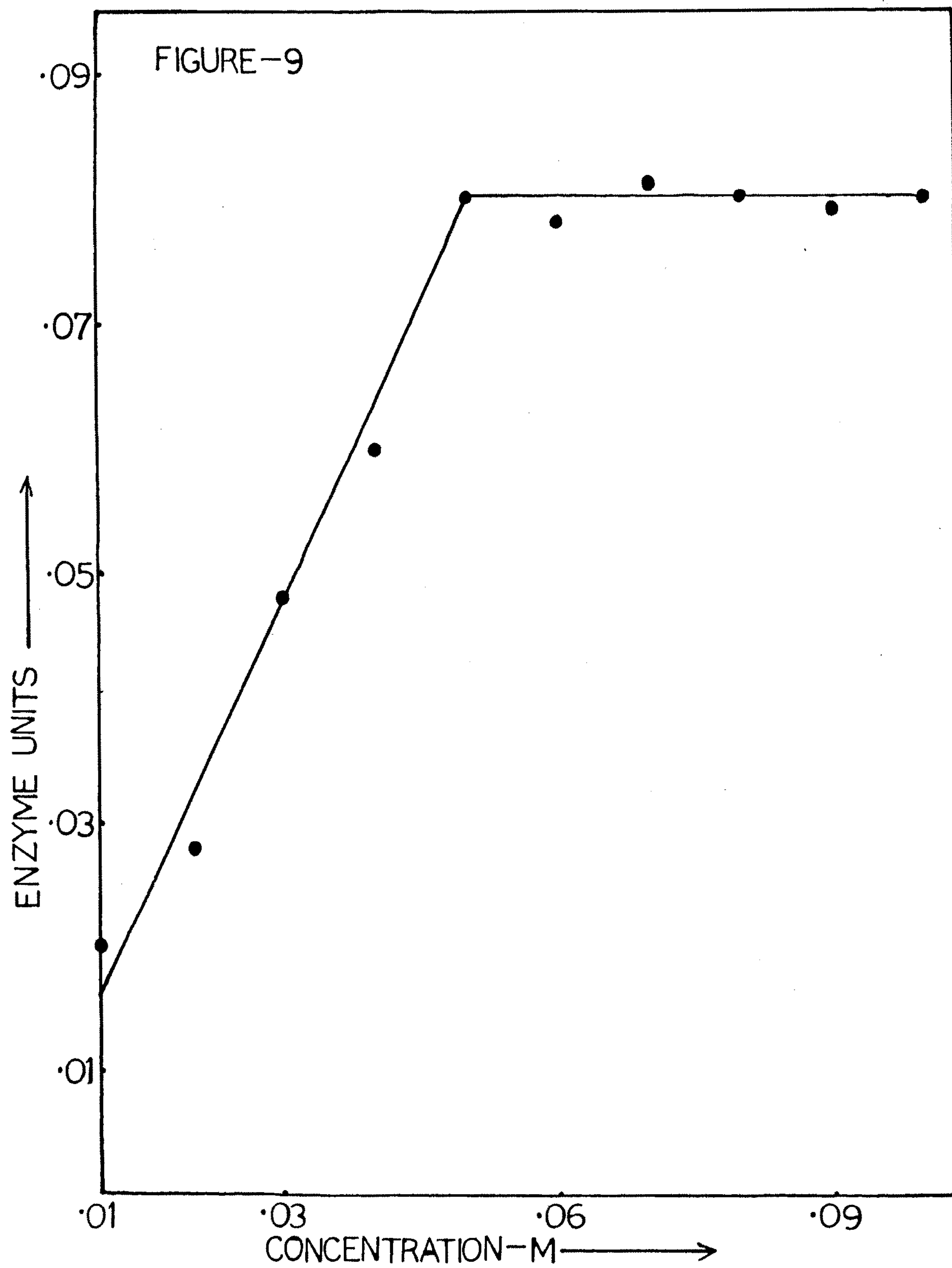
FIGURE-9
·09
·07
·05
·03
·01
ENZYME UNITS
·01
·03
·06
·09
CONCENTRATION-M

TABLE - 10

(Effect of Glycerol)

Sr.No.	Concentration (%)	AcPase activity (Units/mg protein)
1.	0.1	0.010 ± 0.0004
2.	0.2	0.020 ± 0.0008
3.	0.3	0.035 ± 0.0014
4.	0.4	0.050 ± 0.0020
5.	0.5	0.060 ± 0.0024
6.	0.6	0.075 ± 0.0030
7.	0.7	0.084 ± 0.0034
8.	0.8	0.100 ± 0.0040
9.	0.9	0.105 ± 0.0042
10.	1.0	0.120 ± 0.0048
11.	1.2	0.120 ± 0.0044
12.	1.4	0.122 ± 0.0049
13.	1.6	0.120 ± 0.0046

Values are mean ± SE of five experiments.

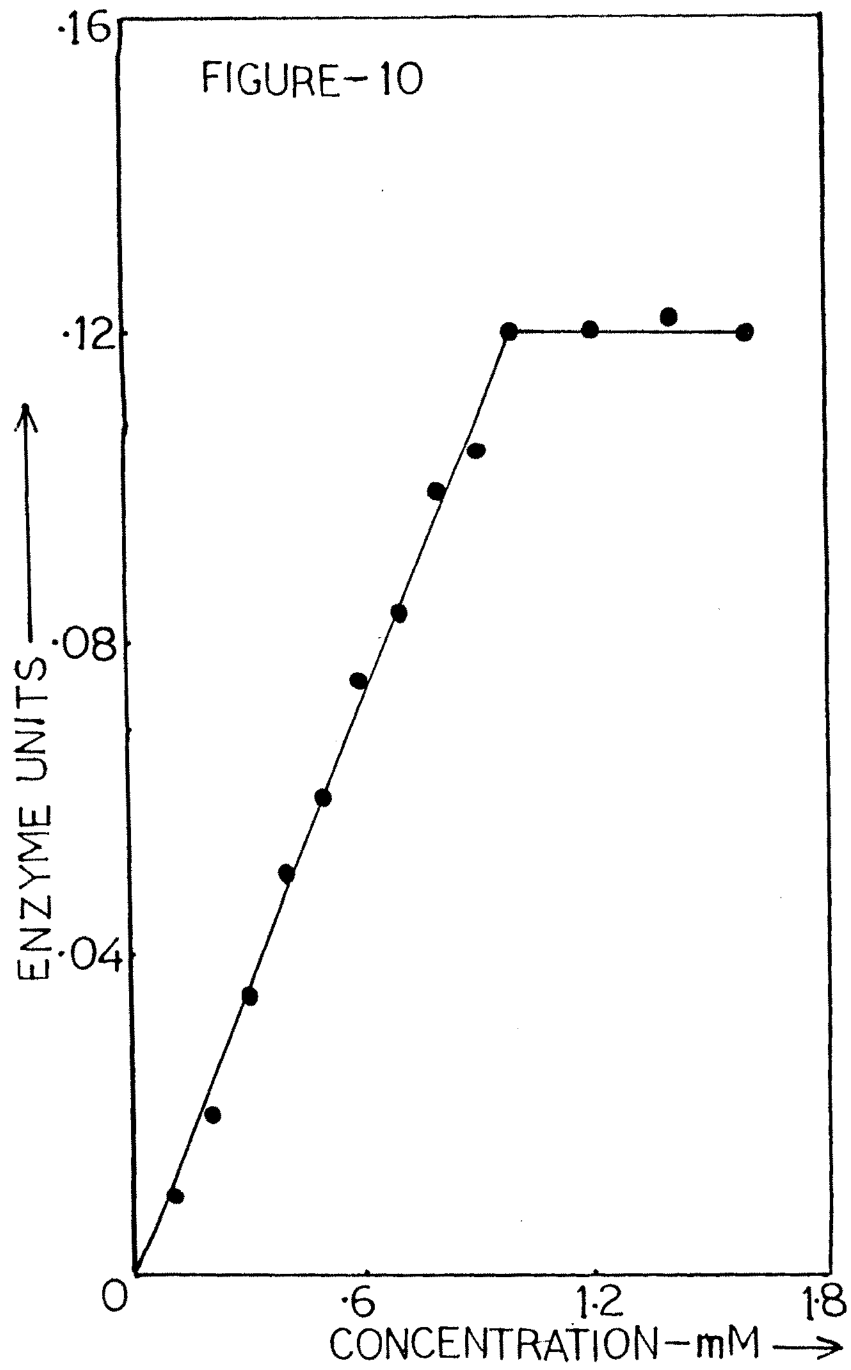

FIGURE-10
·16
·12
·08
·04
0
ENZYME UNITS
·6
1·2
1·8
CONCENTRATION–mM

of glycerol. The further concentrations of glycerol showed maintenance of the highest activities observed in 1 % glycerol.

Effect of EDTA & EGTA

In presence of EDTA of various concentrations the enzyme activities reported were 0.00.

The results indicated that Enzyme I is totallyinhibited by EDTA.

In presence of EGTA (20mM) the enzyme activities were studied with varied concentrations of Magnesium sulfate which were used earlier. The activities observed were similar to the activities of Enzyme I that were reported with only magnesium sulfate. These observations indicated that EGTA was unable to influence enzyme activities independently or with Magnesium Sulfate.

Effects of other chemicals :

Influence of the following chemicals was also studied on the enzyme activities of Enzyme I.

1) Formalin,
2) Triton X-100,
3) Methanol,
4) Ethanol,

TABLE - 11

(Effect of other chemicals)

Sr.No.	Name of the chemical	Inhibition of Enzyme I	No Influence
1.	1 % Formalin	Total	-
2.	1 % Triton X-100	Total	-
3.	1 % Methanol	Total	-
4.	1 % Ethanol	Total	-
5.	< 20 mM Tartarate	-	No influence
6.	1 % Acetone	-	-
7.	< 20 mM NaF	Total	-

5) Tartarate,

6) Acetone,

7) NaF (Sodium Fluoride)

The results are given in Table 11.

The results indicated that 1% formalin, 1% Triton X-100 1% Methanol, Ethanol, Acetone, and NaF inhibited the enzyme activity in total.

Tartarate (20 mM) was not influencing the enzyme activity.

Effect of preincubation of Enzyme I on enzyme activity:

In the preliminary work, Enzyme I was preincubated at various temperatures (40°C, 45°C, 50°C, 55°C, 60°C, 65°C, 70°C, 75°C, 80°C) and was used for the determination of the enzyme activity. Upto 60°C preincubation of the enzyme, the enzyme activity was retained but for the preincubation of further temperatures no activity was expressed by the preincubated Enzyme I sample.

Therefore to study Enzyme I further; it was preincubated at 60°C for varied time intervals and the stability of the enzyme to express its function was determined.

Enzyme I was preincubated at 60°C for various time intervals. This preincubated enzyme at 60°C (for 3 min., 5 min., 7 min., 10 min., 15 min., 20 min., 25 min., 30 min., 35 min.,

40 min.) was further used for enzyme activity studies.

The results are given in Table 12 and they are graphically represented in Figure 11.

The results indicated that 3 min., 5 min., 7 min., and 10 min. cf incubations showed small increase in the enzyme activities as compared to the non-incubated Enzyme I. But after 15 min. of preincubation at 60°C resulted in the burst of enzyme activity which continued to increase upto 25 minutes of incubation retained at steady expression of the enzyme activity.

The enzyme activities were calculated as units per mg protein.

Thus the results indicated that Enzyme I isolated from Rana cyanophlyctis ovary during prebreeding conditions showed the following characters.

1. pH-Optimum - 3.7 .
2. Temperature optimum - 30°C.
3. Km for p-nitrophenyl phosphate - 20.8 mM.
4. Activated linearly by

   $MgSO_4$ (upto 40 mM)

   $MnSO_4$ (upto 100 mM)

   $CuSO_4$ (upto 35 mM)

   Citrate (upto 0.05 M)

   Glycerol (upto 1 %).

TABLE - 12

(Effect of Preincubation at 60^{0}C)

Sr.No.	Time (minutes)	AcPase activity (Units/mg protein)
1.	3	0.060 ± 0.0024
2.	5	0.075 ± 0.0030
3.	7	0.094 ± 0.0038
4.	10	0.130 ± 0.0052
5.	15	1.090 ± 0.0436
6.	20	1.185 ± 0.0474
7.	25	1.310 ± 0.0524
8.	30	1.310 ± 0.0520
9.	35	1.330 ± 0.0532
10.	40	1.340 ± 0.0536

Values are mean ± SE of five experiments.

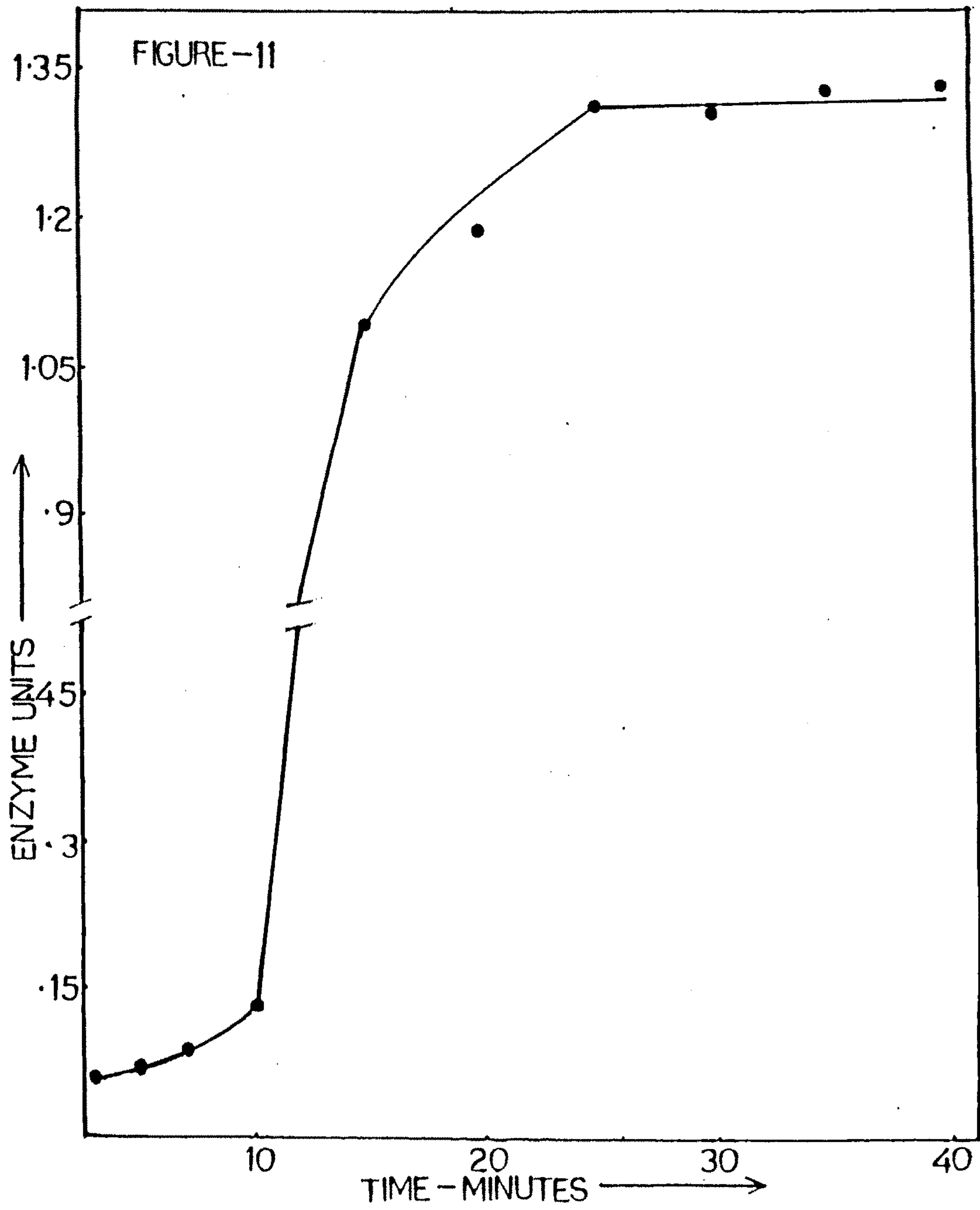
FIGURE-11
1·35
1·2
1·05
·9
·45
·3
·15
ENZYME UNITS
10
20
30
40
TIME - MINUTES

5. Totally inhibited by Calcium chloride.

6. Totally inhibited by EDTA.

7. Not influenced by EGTA.

8. Totally inhibited by

   1% Formalin

   1% Methanol

   1% Ethanol

   1% Acetone

   1% Triton X-100

   Sodium fluoride (< 0.1 mM)

9. Not influenced by tartarate.

10. The effect of preincubation of enzyme sample on enzyme activity indicated that the enzyme activity was burst on 15 min. of preincubation which was retained upto 40 min. of preincubation.

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