CONTENTS

CHAPTER 3

RESULTS AND DISCUSSION

The human liver has the enzyme system to metabolize glycogen. The enzymes involved in glycogen metabolism are phosphorylase, glycogen synthetase (4), and Q enzyme (5) besides the enzymes involved in metabolism of nucleotide sugars (64). The enzymes involved in degradation of glycogen namely, α -amylase, glucoamylase, debranching or α α -1,6 glucosidase, maltase as a group are termed as amylolytic enzymes. Phosphorylase plays a degradative role, its regulatcry behaviour being fully established (4). The metabolic or physiological conditions reflecting nutritional status of organism decides the conditions favouring either synthesis or degradation of glycogen. The simultaneous synthetic and degradative operations in an organism are unlikely. The regulatory characteristics and synergic actions are the subjects of detailed investigation.

Hers et al in 1963 (65), supported the presence of glucoamylase activity in human liver. Their further studies showed the absence of glucoamylase activity in Type II glycogenoses. Human liver glucoamylase assists in the maintenance of blood sugar level. The effect of toxicants

on the activity of glucoamylase is of prime importance. The present investigation contributes to the study of the characteristics of glucoamylase in human liver and its response to the drugs.

The human liver tissue was obtained from local biopsy source. The washed liver tissue was homogenised using 0.25 M sucrose-2 mM EDTA buffer at pH 7.2 and centrifuged at 7000 rpm at 4°C for 30 minutes. The supernatent having glucoamylase activity was recovered by fractional precipitation with ammonium sulphate between 42 and 82 percent saturation resulted with an active precipitate. The precipitate was dissolved in minimum quantity of 5 mM Tri- 2 mM EDTA - 5 mM β-mercapto ethanol buffer at pH 7 and dialyzed against the same buffer. DEAE-cellulose column chromatography was carried out with dialyzed protein sample using Tri-HCl buffer pH 7. The enzyme was eluted with same buffer containing 0.25 M KCl and enzyme activity and protein concentration were estimated. Subsequently elution profile was obtained as shown in Fig.3.

High activity fractions were pooled, dialyzed against distilled water and lyophilized. The protein solution of lyophilized enzyme was sieved on Bio-gel P-100

DEAE cellulose-column Elution profile for Human liver glucoamylase.

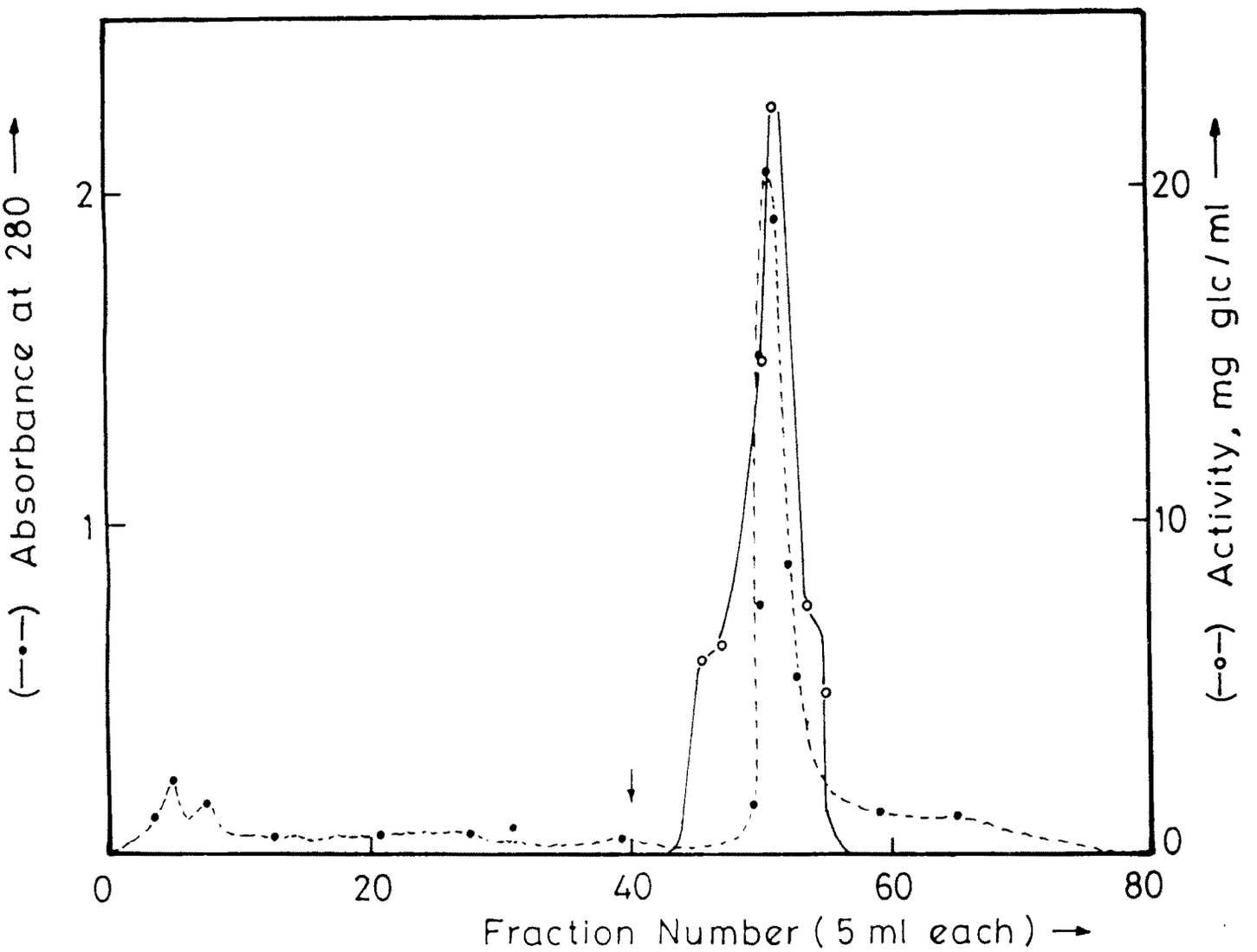


Fig. 3 Enzyme Sol.^{n} 5 ml column size 24x2 cm, equilibration & elution with tris HCl buffer pH 7·0, flow rate 40 ml / hour.

—o—Glucoamylase activity.

—•—Protein absorbed at 280 nm.

Molecular Sieving on Biogel-P-100
Human liver glucoamylase.

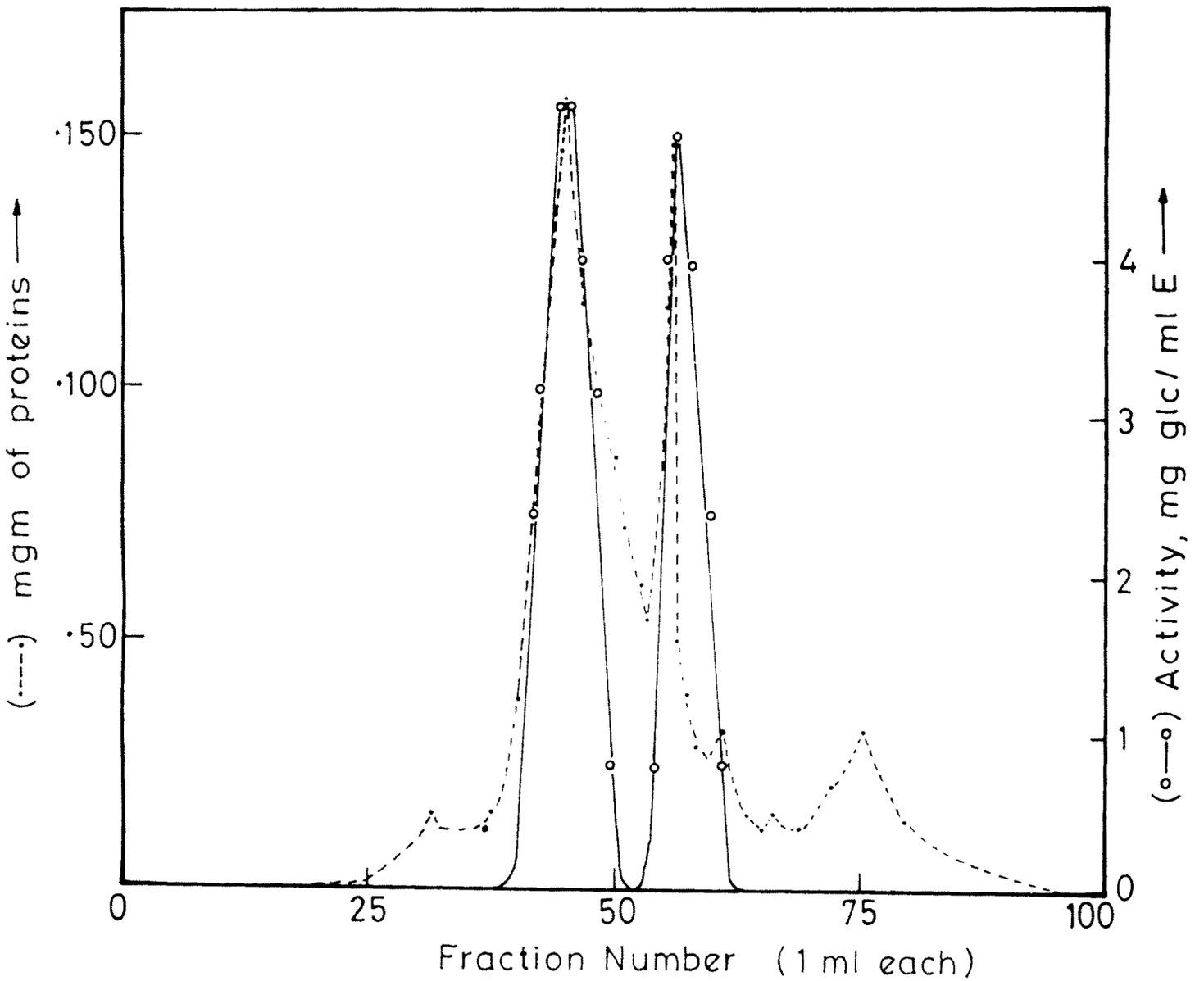


Fig. 4 Enzyme Sol^{n} 0·8 ml column size 70 x 16 cm equilibration & elution with 0·1 M pot.acetate buffer pH 4·2, flowrate 12 ml / hour.

Polyacryl amide gel electrophoresis.

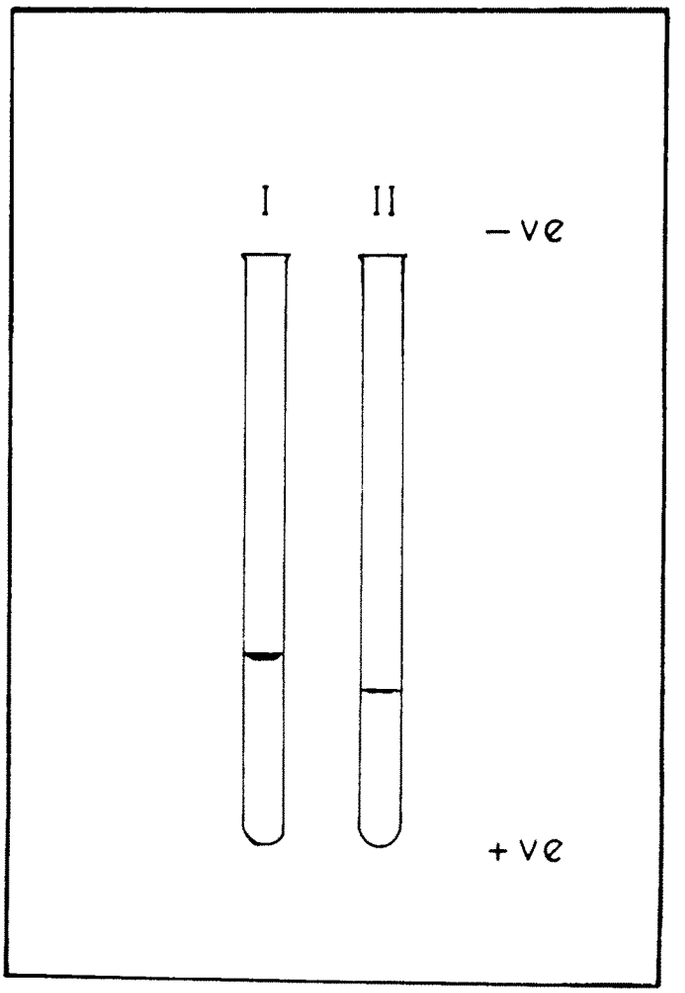


Fig. 5 Electrophoresis in poly acrylamide gel of human liver glucoamylase 100µl in glycine - NaOH buffer pH 8·5, Run carried out at pH 8·5, 3 m.A. per tube, 2·5 hour at room temp.

using potassium acetate buffer pH 4.2. The elution profile was obtained as shown in Fig. 4. Two separate peaks were observed by performing polyacrylamide gel electrophoresis as shown in Fig. 5.

The results were reproducible with fresh preparation. Molecular sieving on Bio-gel P-100 indicates that the two isoenzymes are almost of the same size.

The human liver glucoamylase was purified to more than a thousand fold as shown in the Table 1. The purification process was carried out in four steps :

(1) Fractional precipitation with ammonium sulphate
(2) DEAE-Cellulose column chromatography
(3) Gel filtration on Bio gel P-100
(4) Polyacrylamide gel electrophoresis

The protein concentration was determined using Folin phenol method, Glucoamylase activity was determined by using 3,5 dinitro salicylic acid reagent. The purification is calculated as the ratio of specific activity at each step relative to that of the homogenate fraction.

TABLE 1

PURIFICATION OF HUMAN LIVER GLUCOAMYLASE

FRACTION	PROTEIN CONC. MG|ML	TOTAL PROTEIN MG.	SPECIFIC ACTIVITY	PURIFICATION
Homogenate	15.00	15,000	0.029	-
Extract	14.00	11,200	0.10	3
Ammonium Sulphate Fractionation Dialysed	8.7	609	0.252	8.689
DEAE Cellulose Pool	0.733	47.64	10.91	376.2
Lyophilized & Redissolved	0.52	1.04	51.53	1777.0
Gel Filtration Bio-gel P-100				
Peak I	0.146	0.146	35.6	1227.5
Peak II	0.118	0.118	40.6	1400.0

Free human liver glucoamylase was immobilized on cyanogen bromide activated agaropectin. The free and immobilized human liver glucoamylase was studied for various parameters like pH (fig. 6), temperature (fig. 8) and substrate concentration (fig 12 and 13). The curves were plotted for dependance of activity on pH, temperature, and substrate concentration. Energy of activation was calculated from the Arrhenious plot (fig 10). The results are summarized in Table 2

TABLE 2

CHARACTERIZATION OF HUMAN LIVER GLUCOAMYLASE

PARAMETERS	FREE ENZYME	IMMOBILIZED ENZYME
1. Temperature in °C.	50	45
2. Activation energy in calories/mole	1176.4 & 615.34	1517.2 & 761.0
3. pH	4.4	5.6
4. Km (mg/ml)		
(i) Starch	26.4	15.4
(ii) Maltose	11.4	16.6

pH-activity curve for
Human liver Glucoamylase

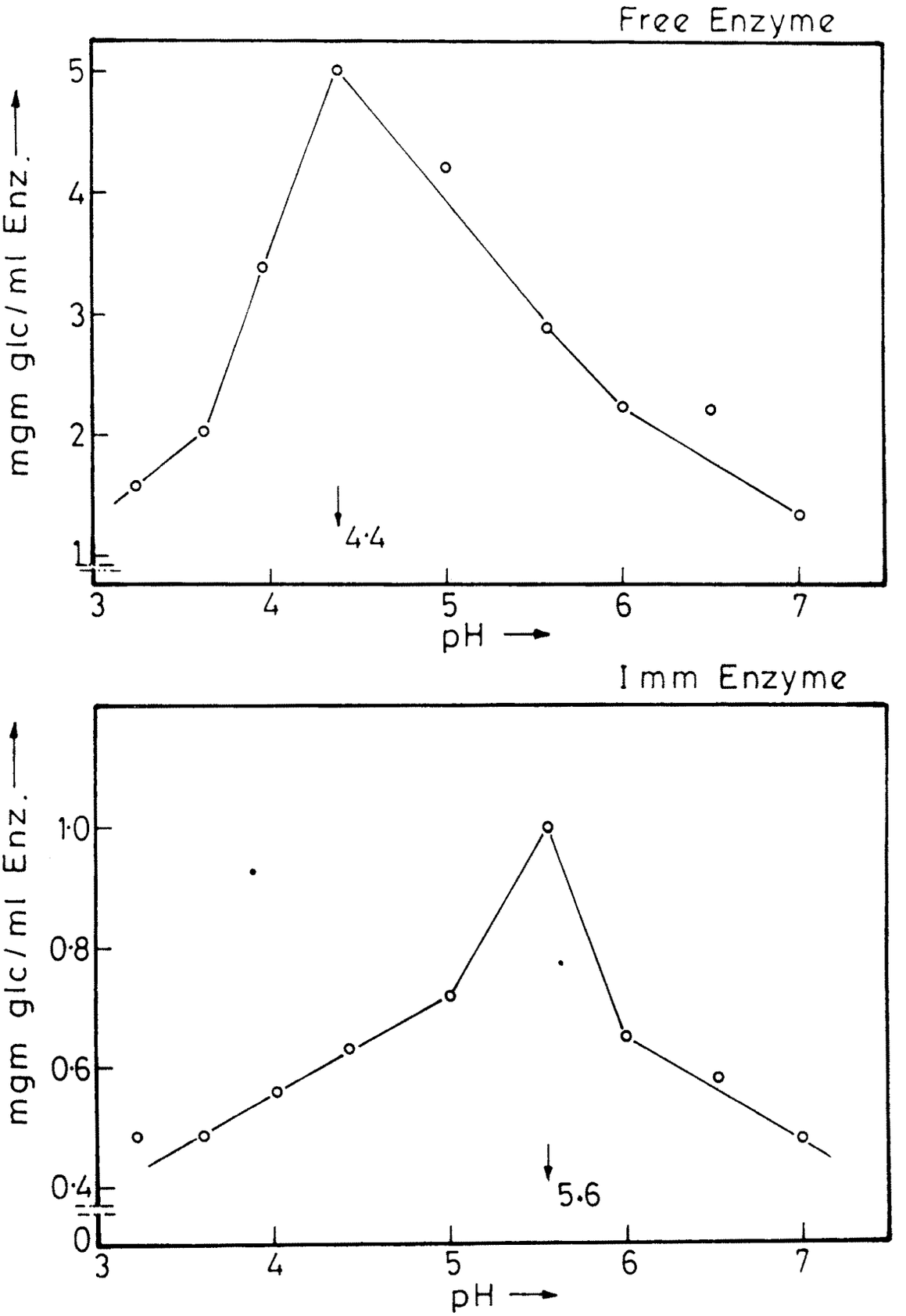


Fig. 6 Glucoamylase activity —o— mg glc/ml Enz. at 37°C/20', pot. acetate buffer pH 4·2.

Temperature-activity curve for
Human liver Glucoamylase

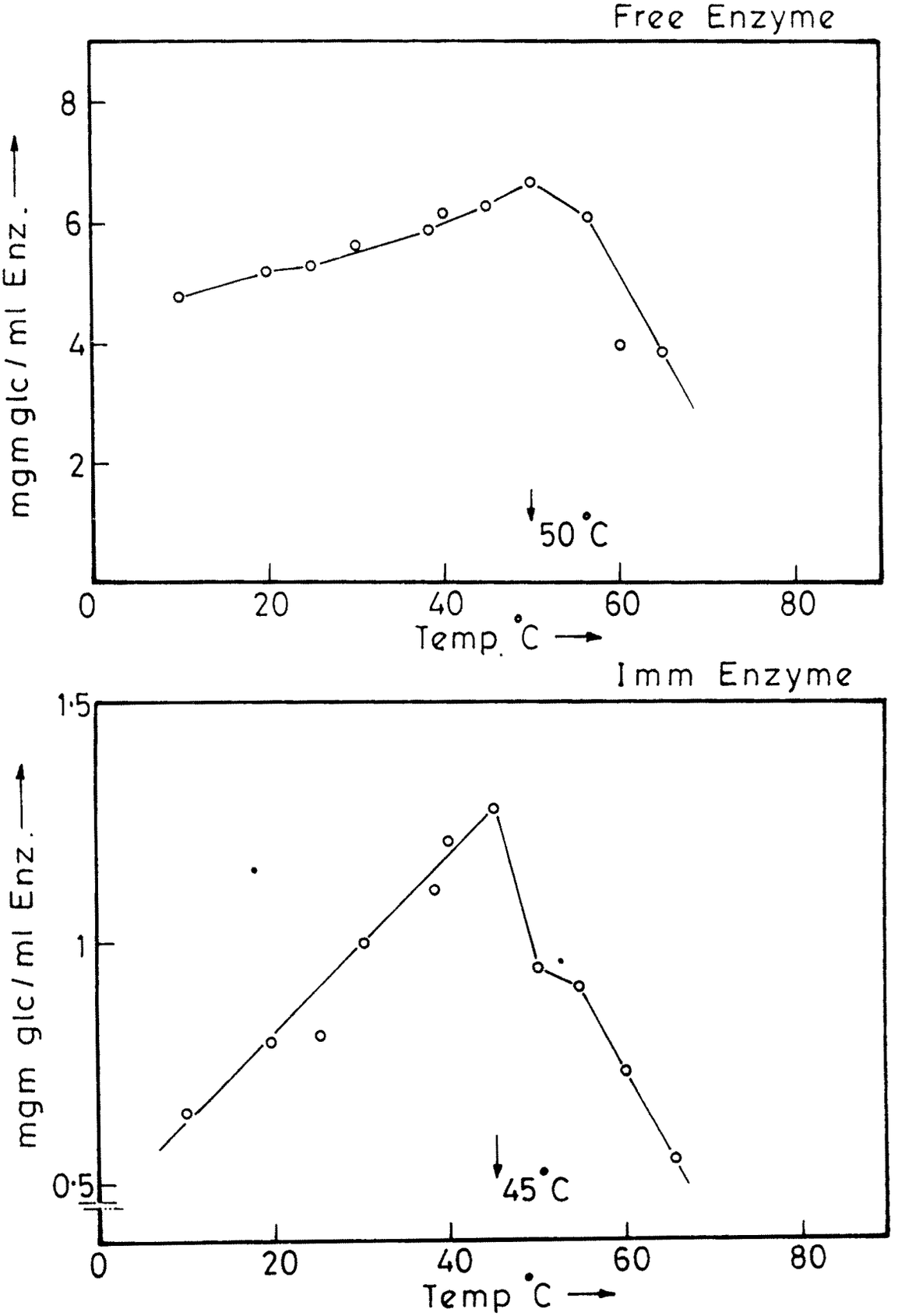
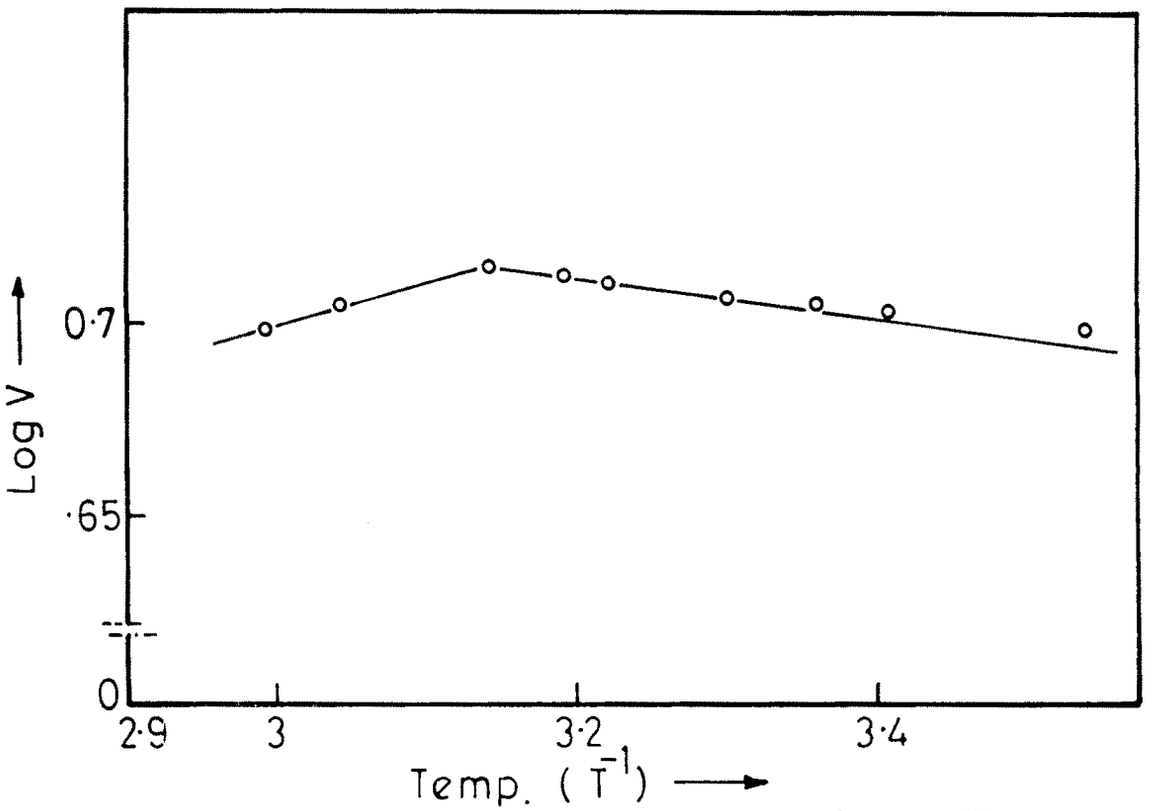


Fig. 8 Glucoamylase activity —o— mg glc/ml Enz. at 37°C/20', pot.acetate buffer pH 4·2 .

Arrhenious plot (Activation energy) for

Human liver glucoamylase Free Enzyme



1 mm Enzyme

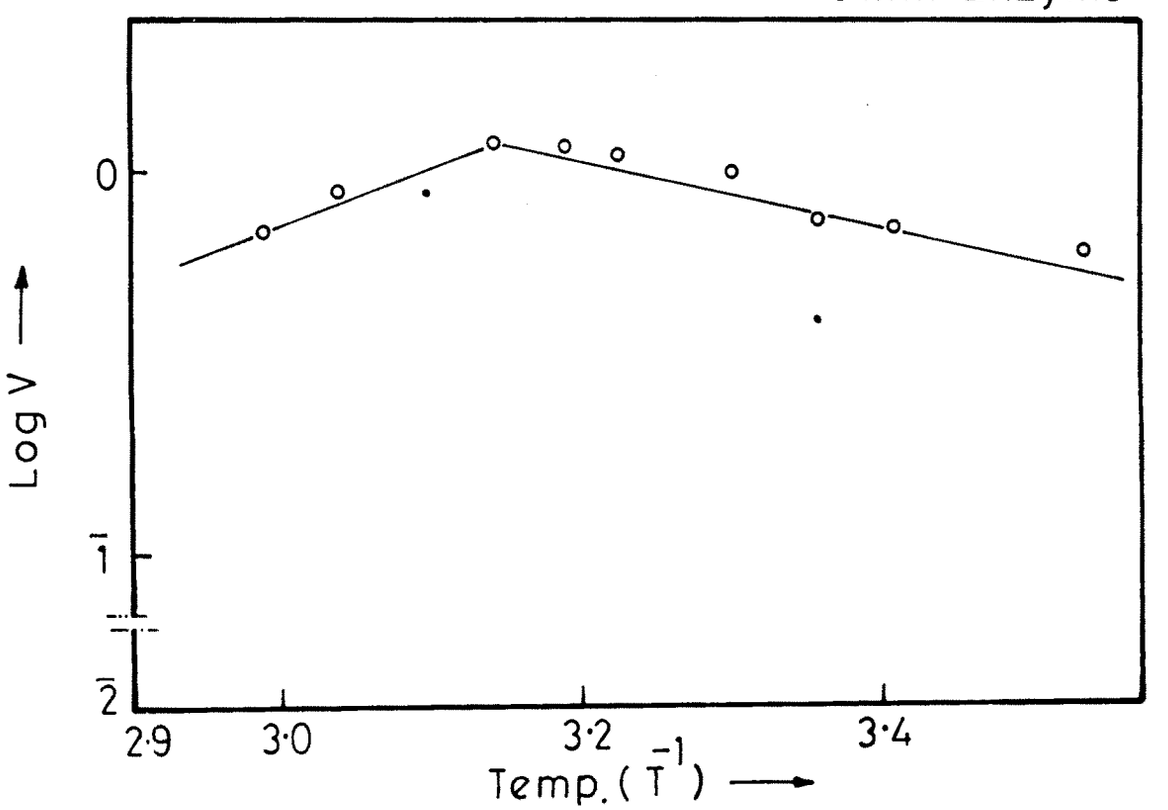


Fig. 10 Glucoamylase activity —o— mgm/ml Enz. at 37 °C / 20', pot. acetate buffer pH 4·2.

Line weaver-Burk plot for hydrolysis of starch by —
Human liver Glucoamylase

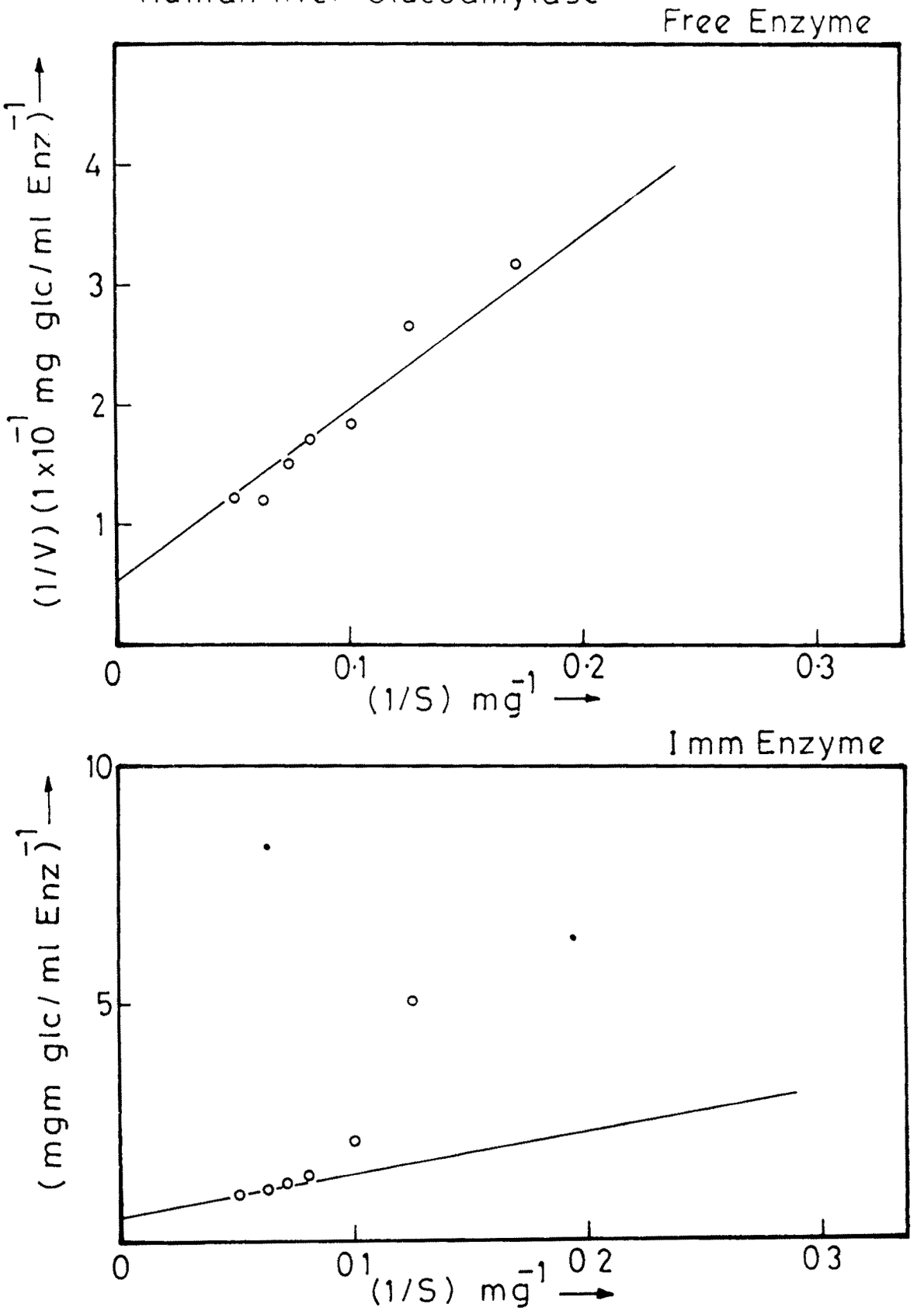


Fig.12 Glucoamylase activity —o— mg glc/ml Enz. at 37°C / 20', Pot. acetate buffer pH 4·2.

Edee Hofstee plot for hydrolysis of starch by Human liver Glucoamylase

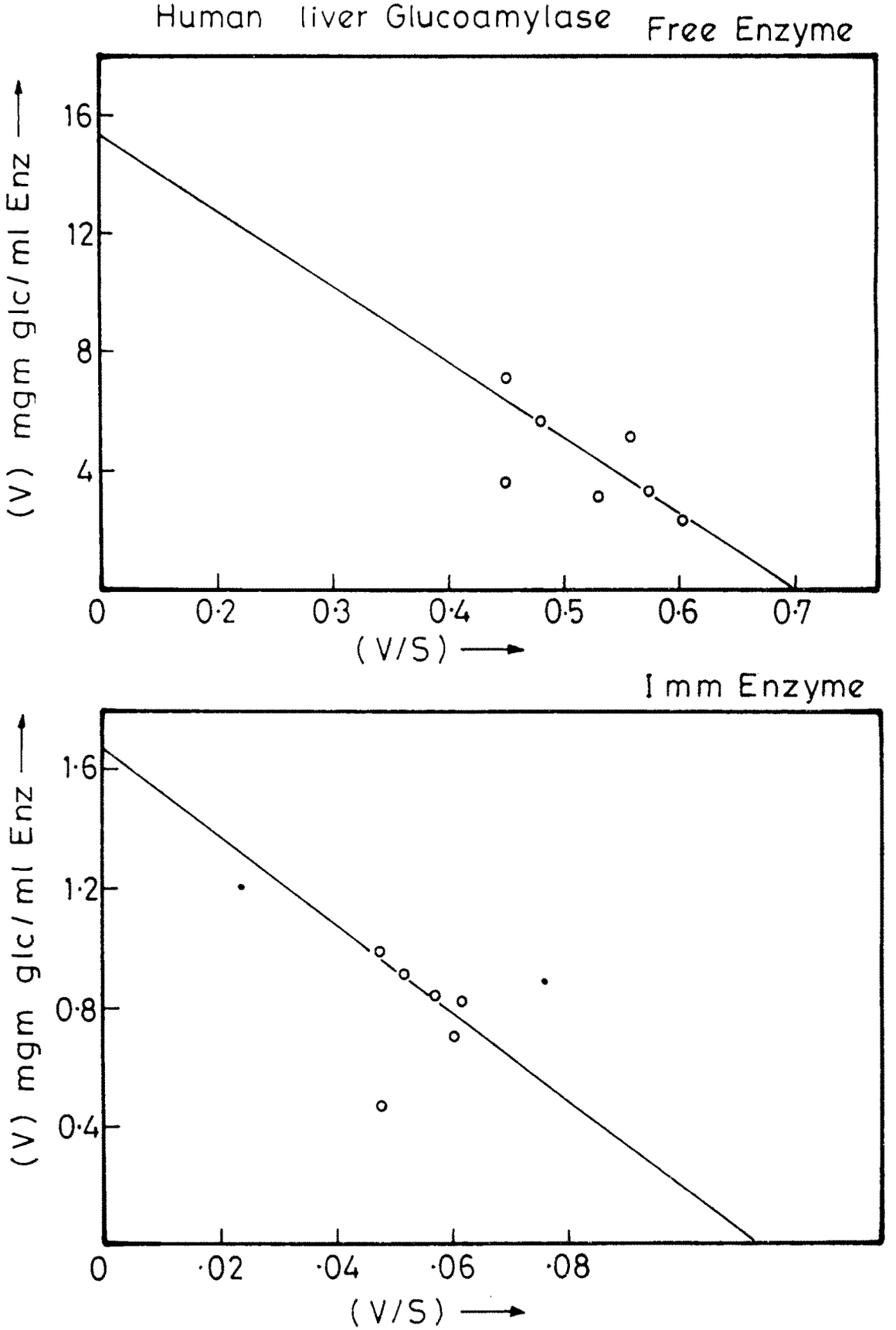


Fig. 14 Glucoamylase activity —o— mg glc/ml Enz. at 37°C / 20', pot. acetate buffer pH 4·2 .

Line weaver-Burk plot for hydrolysis of maltose by Human liver Glucoamylase

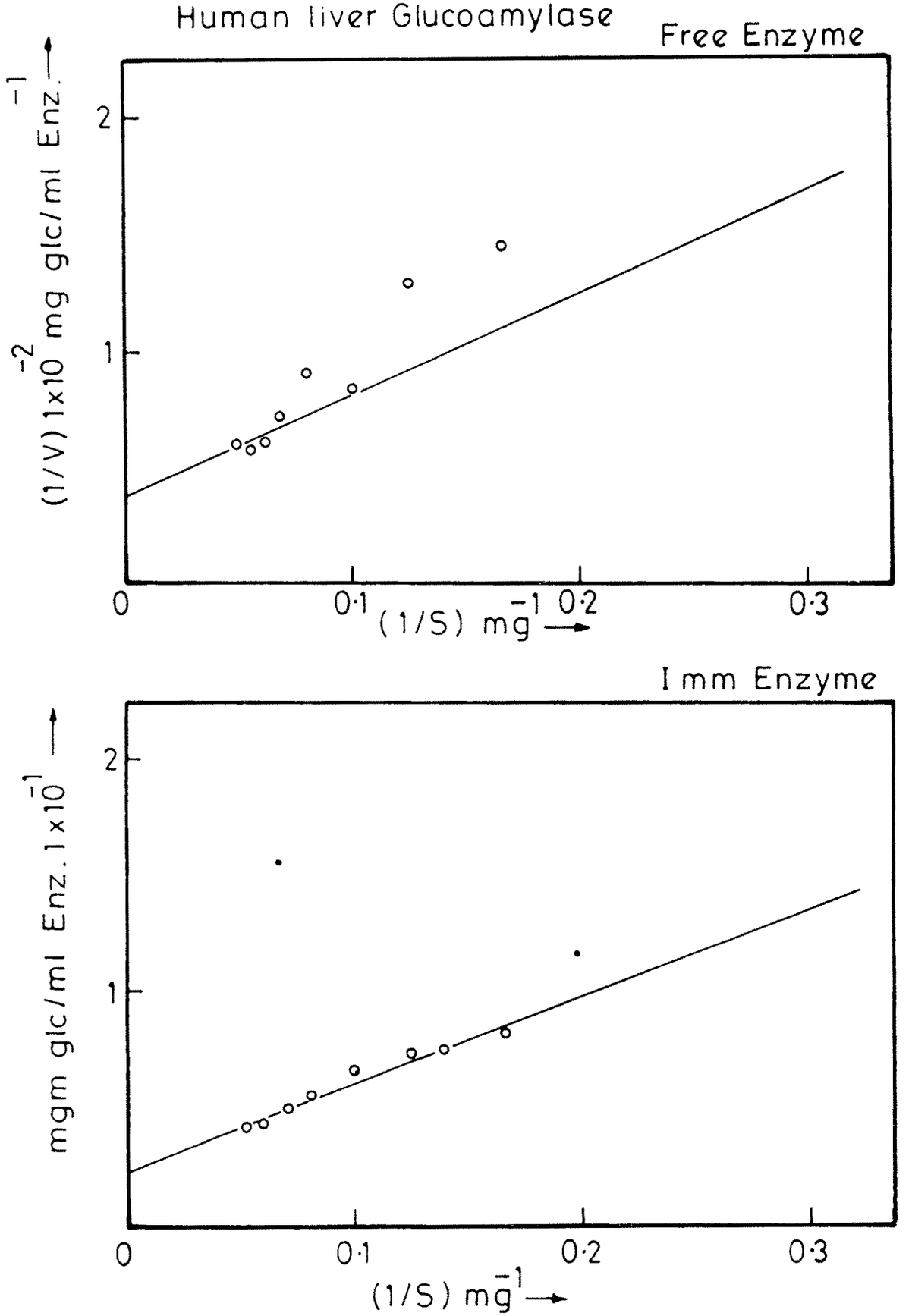


Fig.13 Glucoamylase activity —o— mg/ml Enz. at 37°C/20', pot. acetate buffer pH 4·2.

The commercially available Rhizopus glucoamylase was immobilized with cyanogen bromide activated agaropectin. The properties of the free and immobilized Rhizopus glucoamylase were studied and compared with those of human liver glucoamylase. Figures 7, 9, and 11 depict the effect of pH, temperature and substrate concentration. Figure 15 corresponds to Arrhenious plot.

The characteristics of Rhizopus glucoamylase are summarized in Table 3.

TABLE 3

CHARACTERIZATION OF RHIZOPUS GLUCOAMYLASE

NO.	PARAMETERS	FREE ENZYME	IMMOBILIZED ENZYME
1.	Temperature in °C.	45	50
2.	Activation energy in calories/mole	1250 & 594.5	2750 & 1090
3.	pH	4.0	6.0
4.	Km (mg/ml) : Starch	22.2	16.6

pH–activity curve for
Rhizopus glucoamylase

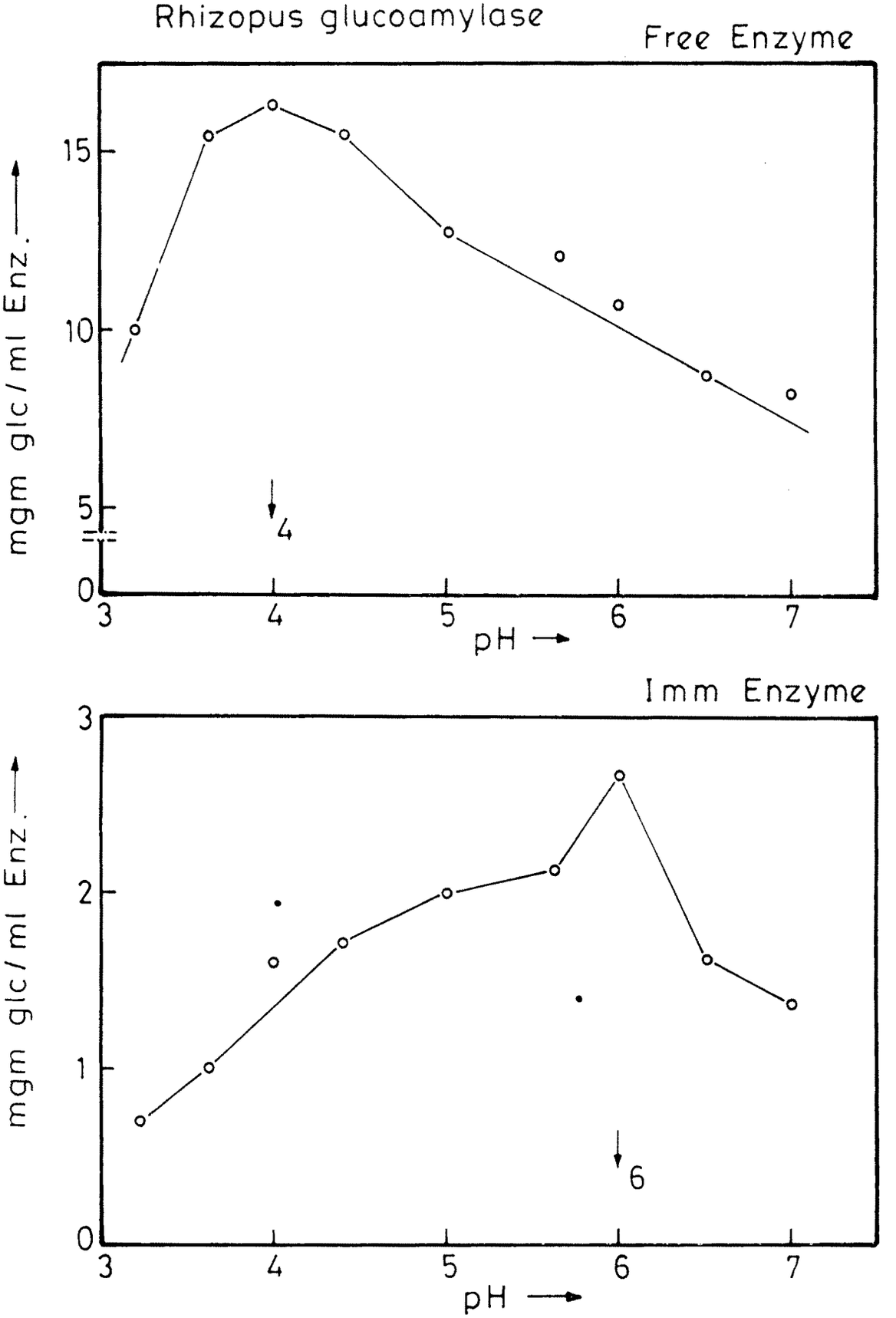


Fig. 7 Glucoamylase activity —o— mgm glc / ml Enz. at 37 °C / 20', pot. acetate buffer pH 4·4 .

Temperature-activity curve for
Rhizopus glucomylase

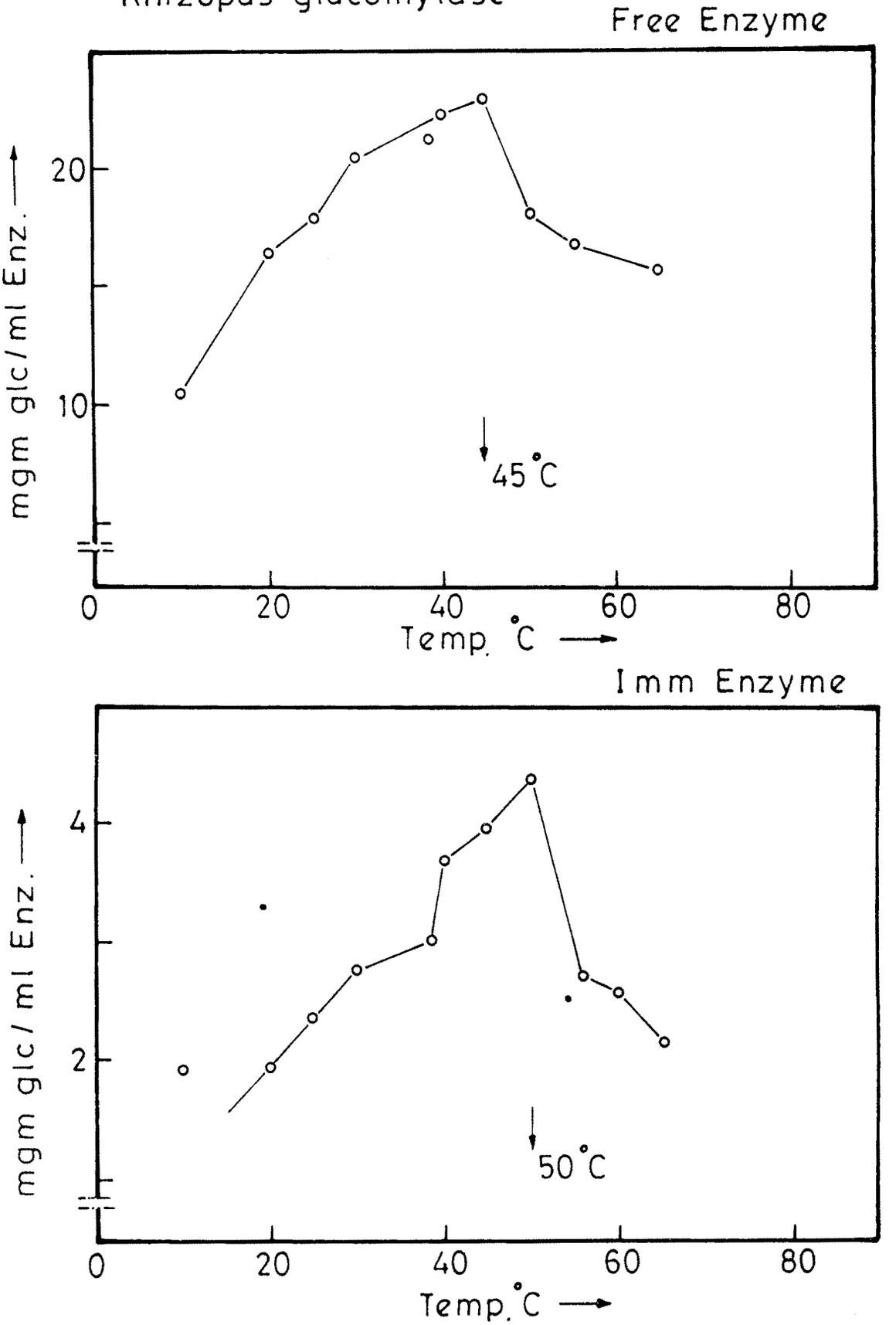


Fig.9 Glucoamylase activity —o— mgm/ml Enz. at 37°C/20', pot. acetate buffer pH 4·2.

Rhizopus Glucoamylase

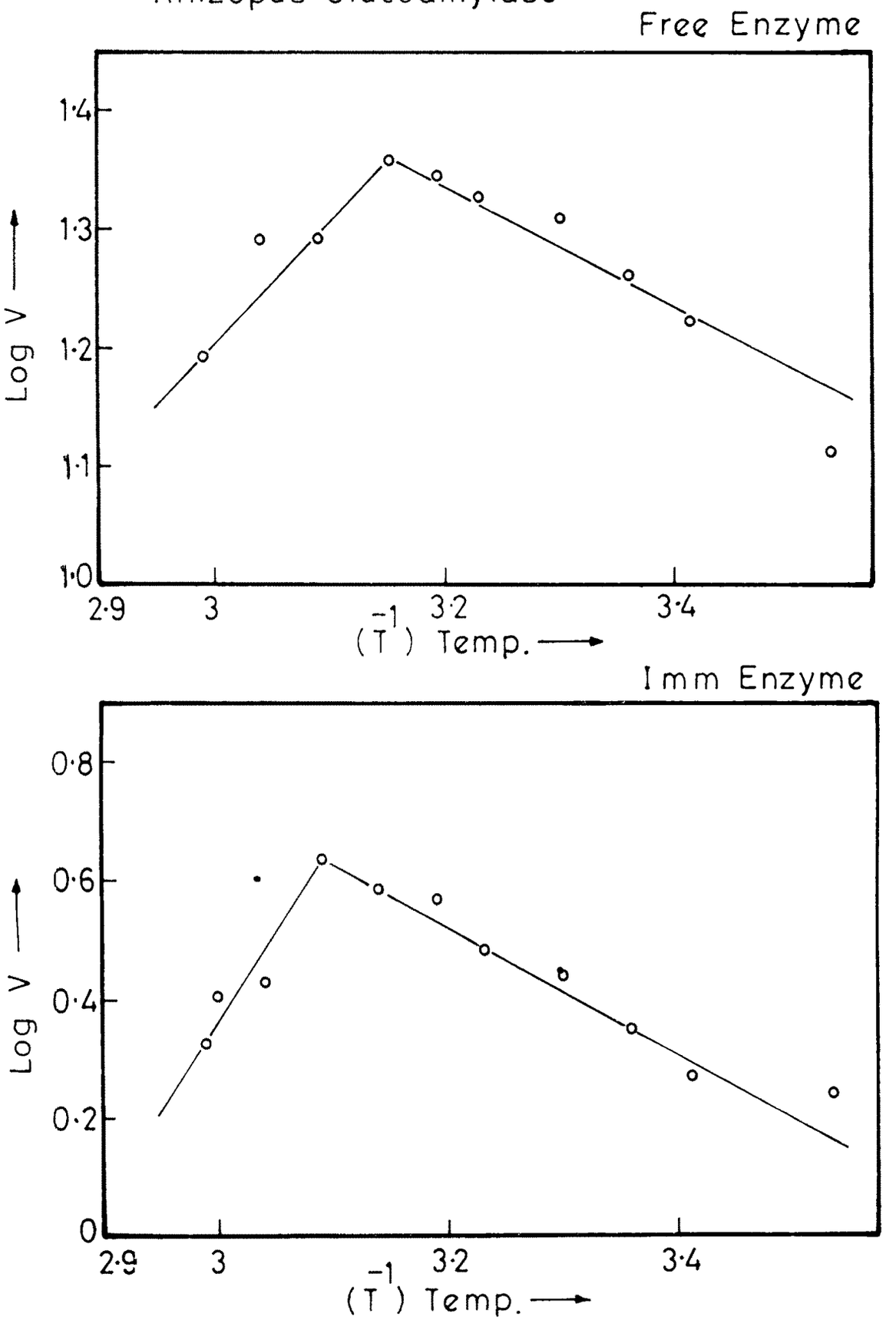


Fig.11 Glucoamylase activity—o—mgm glc/ml Enz. at 37°C/20', pot.acetate buffer pH 4·2.

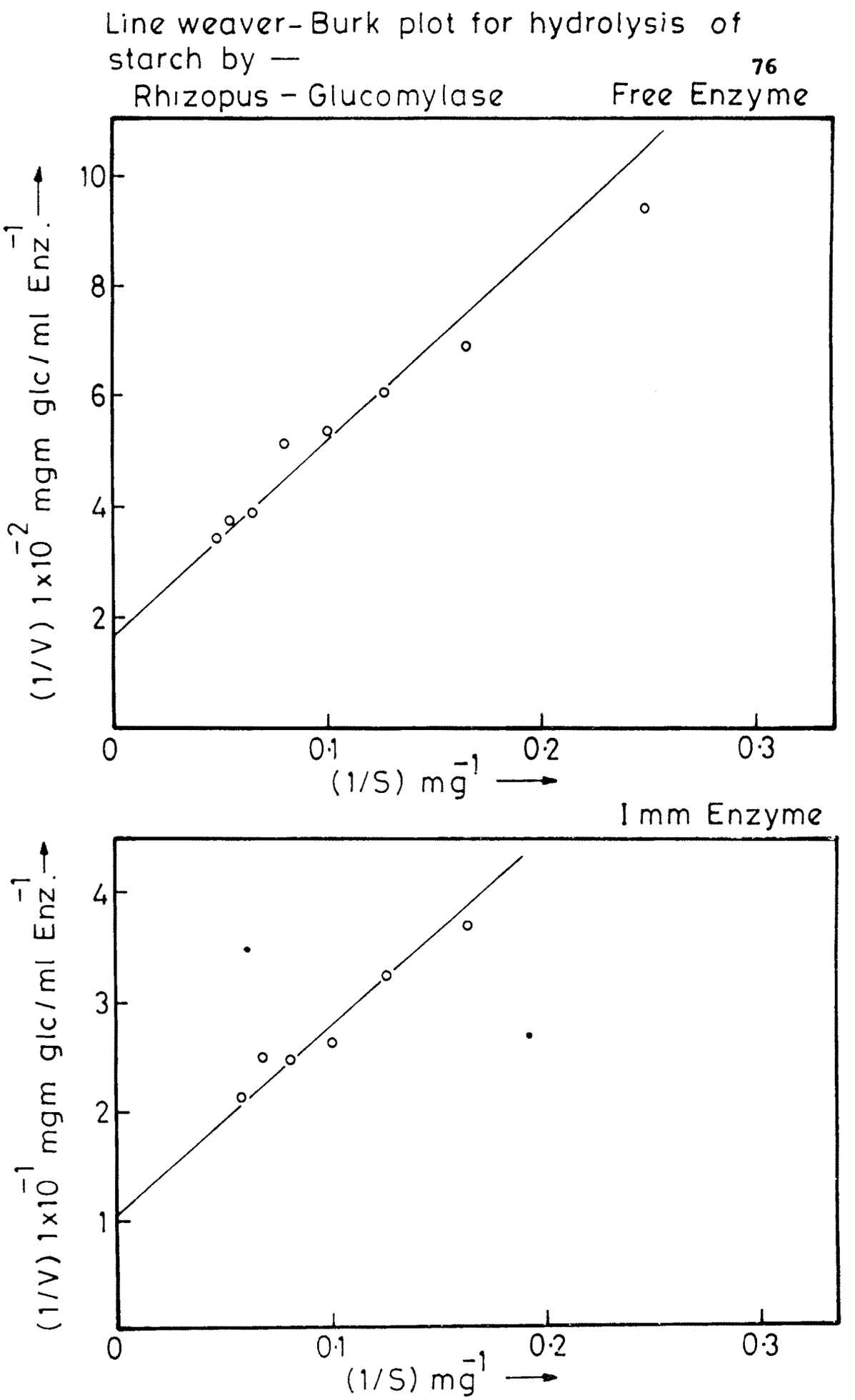


Fig.15 Glucoamylase activity —o— mgm glc/ml Enz. at 37°C/20', pot.acetate buffer pH 4·2.

DISCUSSION

The effect of environmental toxicants on organism could be better understood in molecular terms. In other words, the specific enzymic activity may be altered due to the presence of toxic molecules. Since heavy dosages of antibiotics are given to human subjects to control and inhibit the pathogenic infections, it is worth studying the effects of antibiotics on the catalytic activity of glycogen metabolizing enzymes.

The toxic environment has an effect on glucoamylase activity. The action of the drug penicillin is inhibitary to human liver glucoamylase. The inhibition of glucoamylase activity is of competitive type as shown in Figure 16. The streptomycin is found to accelerate the human glucoamylase activity as is evident in Figure 17. The histograph (Figurel8) depicts the action of penicillin and streptomycin.

Some of these enzymes may be membrane bound wherein, the freedom of the enzyme to undergo vast conformational changes is limited. Thus, the kinetics of free and membrane bound enzyme may differ. The glucoamylase in free state and immobilized state shows difference in kinetic parameters.

Line weaver-Burk plot for hydrolysis of starch by Human liver glucoamylase for inhibition by Penicillin.

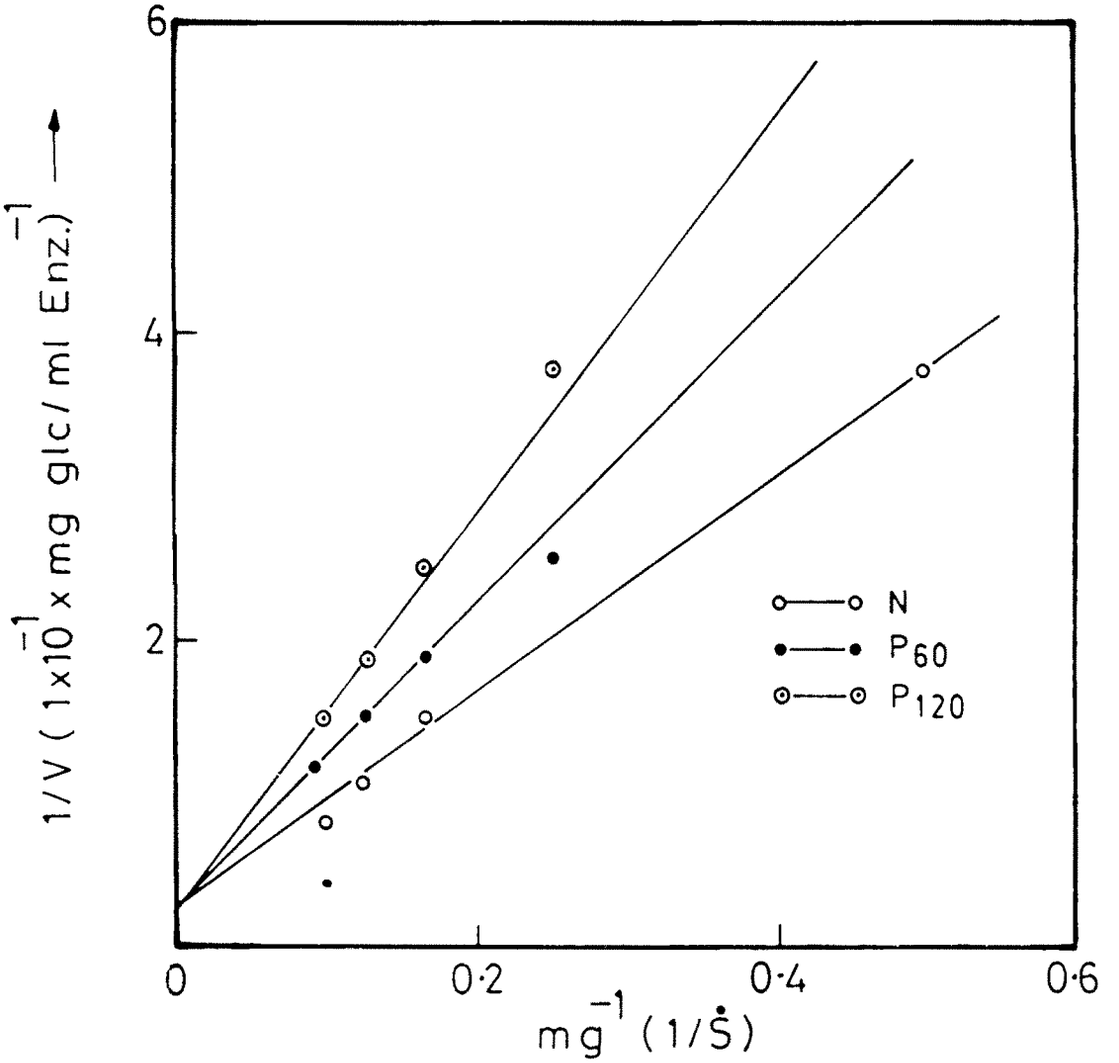


Fig. 16 Showing enzyme activity inhibition by penicillin.

—o— Normal activity plot,
—•— Penicillin (60 μgm),
—⊙— Penicillin (120 μgm) at 37°C / 20',

Line weaver-Burk plot for hydrolysis of starch by human liver glucoamylase activation by streptomycin.

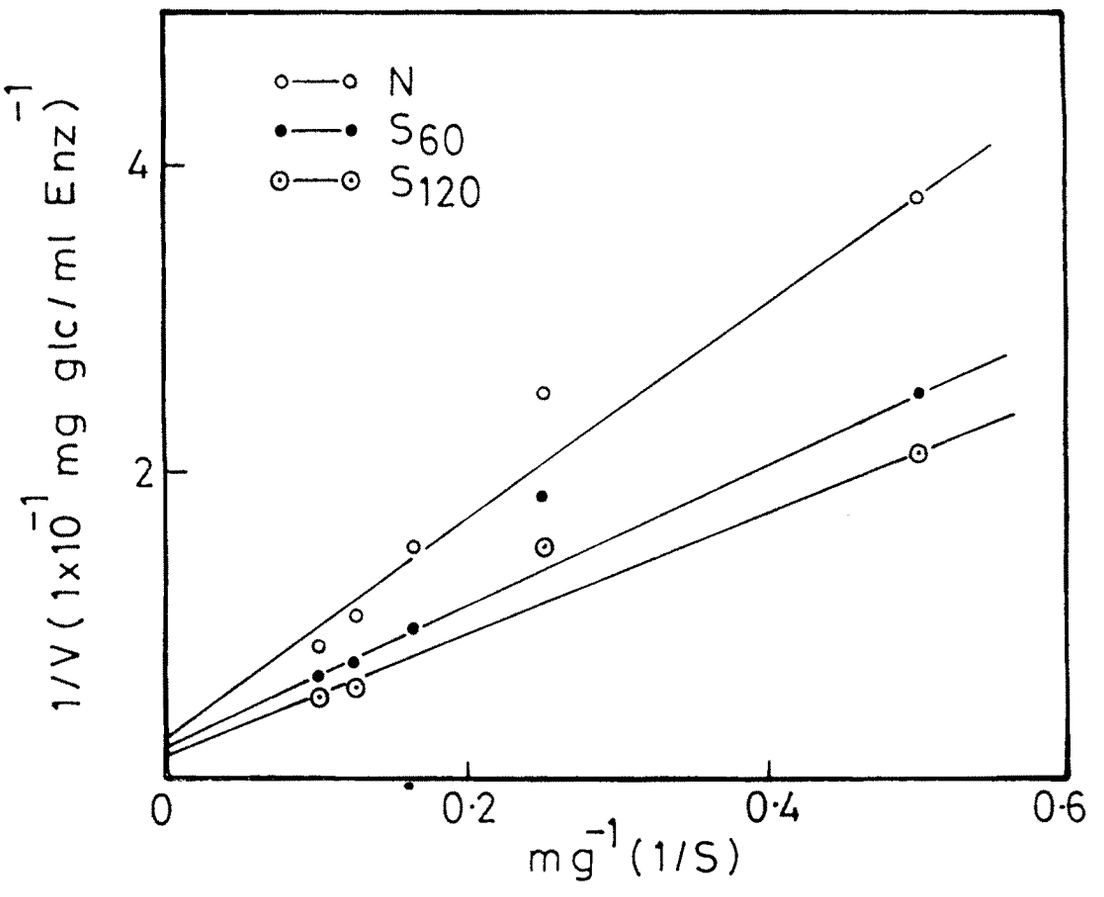


Fig.17 Showing enzyme activation by streptomycin.

—o— Normal activity plot.

—•— S_{60} streptomycin (60 ug) activity plot.

—⊙— S_{120} streptomycin (120 ug) activity plot.

Histograph showing effect of drugs on Human liver Glucoamylase activity.

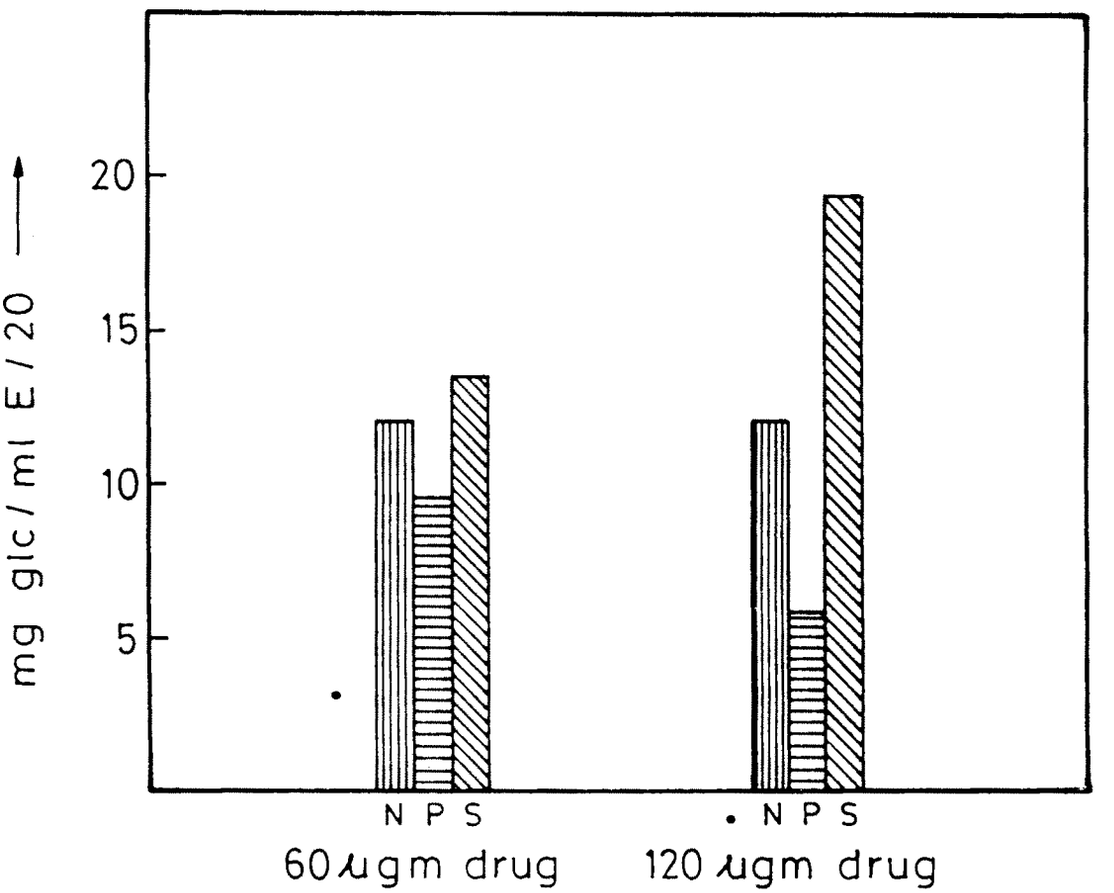


Fig. 18 Showing the effect of drugs on human liver glycoamylase.

N — Normal activity column.

P — Penicillin affected activity column.

S — Streptomycin affected activity column.

The results recorded in Table 2 and 3, and Figures 6, 7, 8, 9, 12 and 15 infer that pH optima of immobilized glucoamylase of human liver and Rhizopus glucoamylase showed an increase in pH from 4.4 to 5.6 and 4.0 to 6.0 respectively. Similarly, the K_m of the immobilized enzymes was lowered. The temperature behaviour of both differ in optimum value. In human liver glucoamylase the temperature optima is observed to be 5°C higher than that of the immobilized one. The Rhizopus glucoamylase on the other hand, exhibited reverse nature in that, the temperature optima of immobilized enzyme was higher by 5°C than that of the free enzyme.

1) Purification of Human Liver Glucoamylase

The human liver glucoamylase was purified to more than thousand folds by using simple techniques like ammonium sulphate fractionation, ion exchange chromatography with DEAE-cellusoe. Further purification was achieved with gel filtration onto Bio-gel P-100. Molecular sieving on Bio-gel P-100 resolved the enzyme into two activity peaks. The poly acrylamide gel electrophoresis supported the presence of isoenzymes. It is evident from the Table 1 that the two isoenzymes show a lower specific activity when separated from each other on gel filtration, while the combined

specific activity of the both the isoenzymes in the previous purification step is much higher. It infers that the combination of the isoenzymes of glucoamylase was more efficient than separated isoenzymes.

The two glucoamylase from P. oxalicum were observed by Yashiki Yamasaki and others (66). Similar results were observed with pig liver glucoamylase by Hirokozy (67).

2) Immobilization of Glucoamylase

There are various methods to immobilize the glucoamylase enzyme. Almost all types of immobilization methods are used by various workers.

Glucoamylase and glucose oxidase of Aspergilus niger were immobilized together by CNBr coupling to neutral support -sepharose by Stina Gesterlis et al (68). The pure glucoamylase was immobilized to polystyrene beads by W. M. Ledingham and others (69). Szajani B. and others covalently immobilized Aspergilus niger glucoamylase on polyacrylamide support containing carboxylic groups activated by water soluble Carbadimide (70).

In the present study the glucoamylase was immobilized by coupling to cyanogen bromide activated agaropectin. The method is advantageous as the process of immobilization is very simple. The agaropectin is non-biodegradable and stable sustance.

3) Dependance of activity on pH

The $[H^+]$ concentration has an effect on the activity of the enzyme. Maximum activity of the enzyme was observed in the acidic region. Bacterial glucoamylase have an optimum pH between 4.2 to 5.6 (12, 16, 17, 71, 72 & 73). yeast and Rhizopus glucoamylase have an optimum pH of 4.2 (66, 74, 75). Aspergilus glucoamylase was observed to be more stable in the acidic region than the neutral region (18). Jefrey and Brown (22) have shown that the enzyme has maximum activity towards maltose at pH 3.7 and towards glycogen at pH 4.4. The cattle liver glucoamylase (63) has an optimum pH of 4.5. As SH- groups are present in the glucoamylase, the enzyme is progressively inactivated by incubation at pH above 7.

Glucoamylase immobilized on polyacrylamide gel support has exhibited maximal stability at pH 4.0 to 4.5 (70) Glucoamylase immobilized on chitinous support showed optimum pH of 4.0 (76).

In the present study, it has been observed that pH optima of free and immobilized enzyme were 4.4 and 5.6 respectively as shown in figure 6. After immobilization pH optima shifted to a higher value. The shift is belived to be due to the aggregation of the anions at the centre of the immobilized molecule. The nature of the curve indicates that the enzyme was sensitive to pH.

4) Dependance of activity on Temperature

Temperature effects several factors that alter the velocity of the enzymic reaction. The factors are stability of enzyme, actual velocity of breakdown complex, substrate enzyme affinity and pH function of any or all of the components.

The thermal stability of glucoamylase was reported by Hayun (17) showing maximum activity at 75°C. Yeast glucoamylase has a temperature optima of 57°C (16). Cattle liver glucoamylase has optimal temperature range from 40-55°C (77). Szasani B. (70) reported optimum temperature of immobilized glucoamylase on polyacrylamide gel support as 60°C, which was 5°C less than the free enzyme. While V. S. Nithianandan et al (83) reported the increase in the optimum

temperature of immobilized enzyme by 5°C to that of the free enzyme at 55°C. Glucoamylase immobilized on chitin and its derivatives, and further cross linked with glutaraldehyde had optimum temperature of 50°C (76).

In the present investigation the optimum activity of the human liver glucoamylase was observed at 50°C and of the immobilized enzyme at 45°C as shown in figure 8. The curves indicate that the activity progressively increases after maximal activity and then it falls rapidly.

The plot of log V versus 1/T is not always a straight line. In number of cases the graph has a discontinuity of slope and approximated to two straight lines meeting at an angle (78). It indicates that there is a change from one value of activation energy to another at transition temperature. According to Mussey (79), it might be associated with an effect on temperature on orientation of water molecules involved in the reaction.

The values of activation energy of free and immobilized human liver glucoamylase observed were 1176.4, 615.34, and 1517.2, 761 respectively as per figure 10.

V. Baseveswara Rao et al (73) calculated the activation energy of reaction from Arrhenious plot as 61.5 KJ/mole. The enzyme was found to be thermostable.

5) Dependance of activity on Substrate

The vast majority of the enzyme catalysed reactions involved more than one substrate. It is true even for hydrolases which are frequently treated as being single molecule reaction. Since second substrate water is present in large excess, that is usually assumed that enzyme is saturated with this substrate all times.

The K_m values of T. Lonuginus glucoamylase (73) towards glucogen are 22 mg/ml, and towards maltose 8.3 mg/ml, and towards starch 44 mg/ml. Rhizopus glucoamylase has K_m towards starch 19.2 mg/ml, 14.0 mg/ml for free and immobilized form respectively (80).

Sugar beet glucoamylase has K_m value 1.9 mg/ml towards starch whereas, towards maltose it was 68 mg/ml (81). Pig liver glucoamylase (72) has K_m towards glycogen 9.1×10^{-3} M, and towards maltose 1 x 10-4 M. Cattle liver glucoamylase displayed K_m as 1×10^{-2} M (76).

Glucoamylase immobilized on polyacrylamide gel has K_m 16.0 mg/ml with soluble starch (70). Glucoamylase immobilized on chitinous support has K_m 1.3 x 10^{-1} gm/lt (76).

The K_m characteristic to free and immobilized human liver glucoamylase from reciprocal plots (Figures 12 and 13) observed were towards starch 26.6 mg/ml and 15.4 mg/ml; towards maltose 11.4 mg/ml and 16.6 mg/ml respectively. The lowering of K_m value on immobilization of enzyme indicates the increase in affinity to substrate.

6) Parallel Experiments

The parallel experiments were run to characterize Rhizopus glucoamylase of commercial source and the results are shown in Table 3.

On comparison, it was observed that pH optima shifted towards neutral region for both the human liver glucoamylase and Rhizopus glucoamylase on immobilization. The pH optima of human liver glucoamylase (free) was higher than that of free Rhizopus glucoamylase by 0.4 unit. While, it was less by 0.4 unit after immobilization. The K_m characteristics were observed slight higher to human liver

glucoamylase. The temperature optima was seen higher by 5°C to free human liver glucoamylase than that of free Rhizopus glucoamylase, the reverse was observed on immobilization.

7) The effect of penicillin on human liver glucoamylase activity is shown in Figures 16 and 18. The drug showed the increasing apparant K_m of reaction therefore, it would be a competitive inhibition. It is of interest to note that though penicillin does not have a proper structural similarity to the substrate, the kinetics indicate a competitive inhibition which may be due to the non-covalent interaction of penicillin with one of the amino acids involved in the active centre of the enzyme.

The effect of streptomycin on human liver glucoamylase is shown in Figure 17 and 18. It was observed that the velocity of glucoamylase reaction increased with decrease of K_m. The streptomycin would be a accelerator of enzyme activity.

The action of these two drugs on human liver glucoamylase is of great importance since both the drugs are widely used in the treatment of various diseases.

It has been observed that in patients suffering from Mc Ardle's disease, the muscle phosphorylase is defective, but even then there is no storage of glycogen, hence, there is a possibility that other α - glucosidases may be playing a significant role.

In such diseases if glucoamylases are proved to play the main role in the glycogen degradation, then the effect of toxicants on the enzyme activity gain a wider dimension. Though only a speculation, owing to the limitations of this investigation, which could not pursue studies in vivo; the inhibitory or activating effect of these antibiotics on glucoamylase, especially in pateints suffering from any of the diseases of glycogen metabolism could lead to serious clinical manifestations.