RESULTS

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Comparative study of the enzymes involved in ornithine metabolism in normal and tumour tissue of Rumex acetosa.

A comparative study made on the enzymes of ornithine metabolism showed (Table 4) that arginase and ornithine aminotransferase are high in tumour than in normal tissue whereas ornithine carbamoyl transferase is low in tumour tissue.

Previous studies in this laboratory (Naik, 1970) have shown that the level of arginine is low in tumour tissue and no ornithine or citrulline could be detected. These studies would thus indicate that ornithine formed by a high arginase activity in tumour tissue might be channelled more towards glutamate or proline formation via ornithine aminotransferase reaction rather than going for citrulline formation. The presence of an increased ornithine to glutamate pathway has also been reported in this tumour tissue (Srivastava and Naik, 1971).

As pointed out in the introduction, ornithine carbamoyl transferase is a key enzyme involved in the recycling of ornithine to arginine. This enzyme has a competing role with aspartate transcarbamylase for a common substrate, carbamyl

Table 4 : Comparision of enzymes of ornithine metabolism in normal and tumour tissues of Rumex acetosa cultivated in vitro.

	Specific activity	
	Normal	Tumour
Arginase	6.19	10.54
Ornithine transcarbamylase	5.78	2.97
Ornithine aminotransferase	1.05	3.02

phosphate. Since a larger pool of pyrimidines is required for an enhanced synthesis of nucleic acids expected in tumour tissues, it is reasonable to assume that there might be some sort of control operating on the enzyme ornithine carbamoyl transferase for a balanced partitioning of carbamyl phosphate towards the biosynthesis of arginine or pyrimidine nucleotides. Studies reported below have been carried out with a view to find whether this enzyme has such characteristics.

Purification of ornithine carbamoyl transferase from tumour tissue

The data reported in Table 5 show that the enzyme can be enriched to about 140-150 fold with 28% recovery by alumina C_γ and calcium gel treatment and fractionation on DEAE-cellulose column.

The purified enzyme was found to be essentially free of arginase and ornithine aminotransferase activity.

Stability of enzyme on storage

The purified enzyme preparation was quite unstable and loses its complete activity in 24 hours when stored frozen or at 0° and could not be stabilized by any of the various methods tried. Due to unstability and very low protein content of the purified fraction further attempts at purification have not been successful. The purification was

Table 5 : Purification of ornithine carbamoyl transferase from Rumex tumour tissue.

Procedure	Total volume (ml)*	Total activity units**	Total protein (mg)	Specific activity units/mg protein	Purification (fold)	Recovery %	
						Enzyme units	Protein
1. Homogenate	140	238	327	0.73	1.0	100	100
2. Triton X-100 supernatant	130	222	133	1.67	2.3	93	41
3. Alumina C_γ gel eluate	120	118	28	4.21	5.8	50	9
4. Calcium gel supernatant	120	118	15	7.87	10.8	50	5
5. DEAE - fractions							
1	10	16	0.13	123.08	169.2	7	0.04
2	10	23	0.22	104.55	143.8	9	0.07
3	10	14	0.14	100.00	137.5	6	0.04
4	10	13	0.15	86.67	119.2	6	0.04
Combined DEAE fraction	40	66	0.64	103.57	142.4	28	0.19

* Starting from 42 grams of tissue (20 days old).

** Unit is defined as the amount of enzyme which will form 1 µmole of citrulline in 30 min. under the assay conditions.

carried out in batches and any batch differing more than ± 10 percent in specific activity was not used for further studies.

Reversibility of reaction

In the present case it has only been possible to study the reaction in the direction of citrulline synthesis and all attempts made to show the reversibility have been unsuccessful.

Substrate specificity

The enzyme was found to be specific for L-ornithine. The results reported in Table 6 show that the enzyme activity at equimolar concentration of DL-ornithine is half that of L-ornithine. Also the presence of D-ornithine does not seem to influence the velocity of reaction. Apart from carbamyl phosphate other carbamyl compounds also did not serve as carbamyl donor.

Effect of pH and buffer ion concentration

The pH studies for the forward reaction were carried out in the range of 7.0 to 10.5 by using two buffers (Table 7 and Figure - 1A). The pH optimum lies between 9.0 - 9.5. It can also been seen that tris buffer gives a higher activity compared to bicarbonate buffer.

The data reported in Table 8 show that tris buffer upto a concentration of 50 µmoles has no inhibitory effect but

Table 6 : Effect of L-ornithine and DL-ornithine on ornithine carbamoyl transferase activity.*

Substrate**	Concentration (μ moles)	Enzyme activity (citrulline formed, μmoles)
DL-ornithine	0.50	0.20
	1.00	0.36
	2.00	0.46
L-ornithine	0.25	0.15
	0.50	0.31
	1.00	0.43

* 6 μg of enzyme protein was used in this experiment.

** Carbamyl phosphate was 2 μmoles.

Table 7 : Effect of pH on ornithine carbamoyl transferase activity.

pH	Enzyme activity (citrulline formed, µmoles)	
	Tris-HCl buffer*	Carbonate - bicarbonate buffer*
7.0	0.10	-
7.5	0.29	-
8.0	0.38	-
8.5	0.42	-
9.0	0.47	0.42
9.5	0.48	0.27
10.0	-	0.11
10.5	-	0.00

* 50 µmoles of buffer was used.

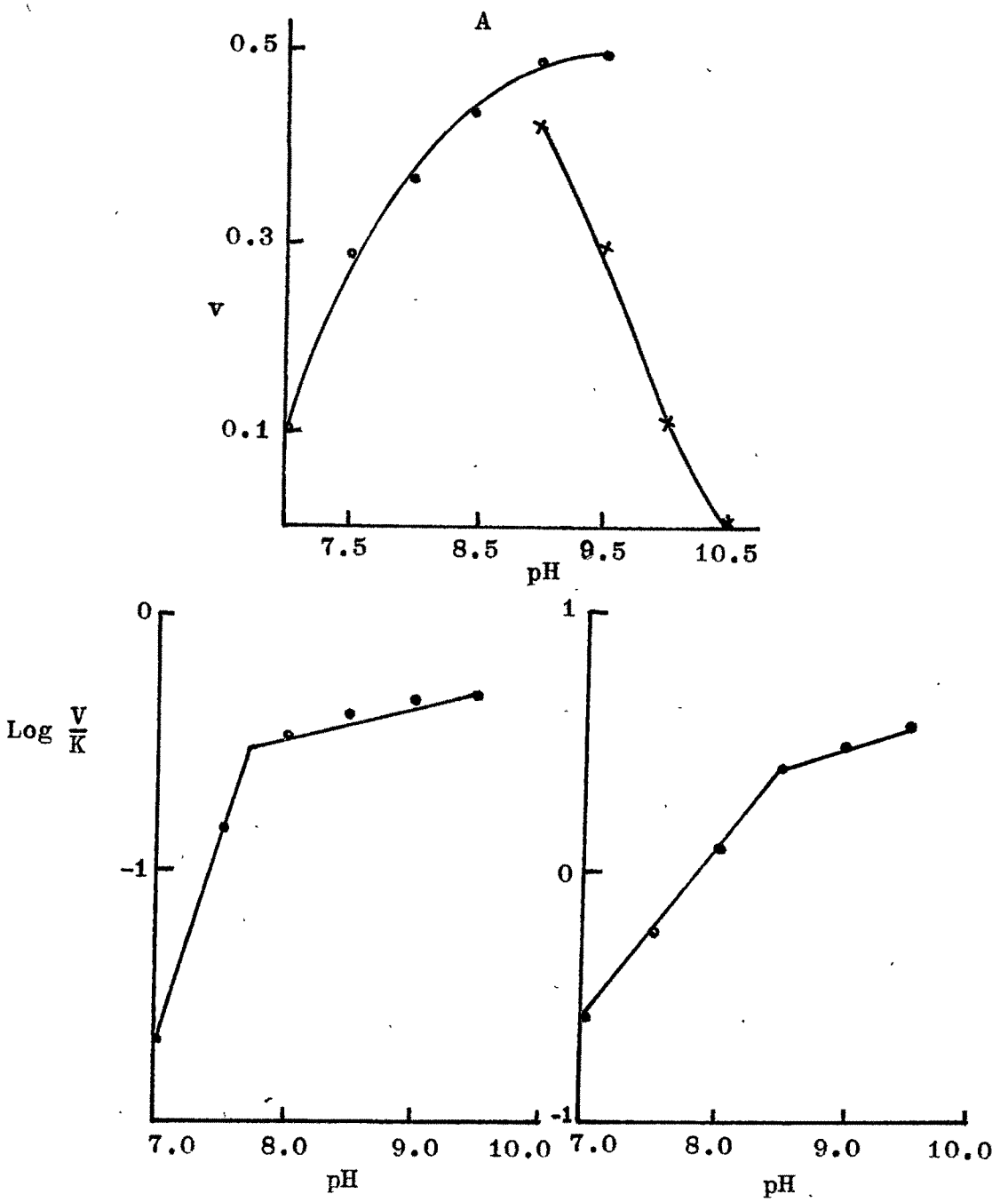


Fig.1: Effect of pH on reaction velocity (A) and effect of pH on first order rate constant with (B) ornithine and (C) carbamyl phosphate as variable substrates.

Table 8 : Effect of buffer ion concentration on ornithine carbamoyl transferase activity.

Buffer concentration (µmoles)*	Enzyme activity (citrulline formed, µmoles)
10	0.64
20	0.61
30	0.62
40	0.62
50	0.62
100	0.50
150	0.50
200	0.50

* Tris-HCl buffer of pH 9.0 was used.

20 percent inhibition was observed by raising the concentration to 100 μmoles.

Variation of kinetic parameter with pH

The two entities which show independent variation with pH are V (the maximum velocity when all substrates are saturating) and V/K (the apparent first order rate constant for reaction of enzyme with substrate at very low substrate concentration but with all other substrate saturating).

The pH variation of the Michaelis constant (Km), in particular, is not a simple function but rather displays the effect of dividing the expression for V as a function of pH by the expression for V/K. Data are usually plotted in the form of log V or log V/K Vs. pH. However, there are certain reservations concerning the interpretation of log V Vs. pH (Cleland, 1970). Thus while the theory of variation of V is fairly straightforward, interpretation is usually not.

The pH variation of V/K is as informative as that of V, since V/K reflects the ability of enzyme (specially the form that combines with the substrate) and substrate to form a complex. Thus any ionizations occuring on either the substrate or the enzyme form which combines with the substrate will show up in the log V/K Vs.pH plot if they effect the ability of these two to combine but they will not show up if they do not. The advantage of this analysis is that the

observed pKs will be true values since they reflect only one step the combination of enzyme and substrate.

When such a study was made on the purified enzyme and log V/K values were plotted for ornithine or carbamyl phosphate as the varied substrate at saturating levels of nonvaried substrate, it was found that ornithine as varied substrate gave a pK of 7.7 whereas carbamyl phosphate as varied substrate gave a pK of 8.5 (Figure - 1B,C).

Effect of enzyme concentration

The enzyme activity increased almost linearly upto an enzyme concentration of 3.2 µg protein after which the increases were not linear (Table 9 and Figure - 2).

Effect of period of incubation

The enzyme activity increased almost linearly upto 30 minutes of incubation (Table 10 and Figure - 3) and there was no significant increase thereafter.

Effect of incubation temperature

The enzyme activity increased with rise in temperature upto 40°C and then decreased as the temperature was raised further (Table 11 and Figure - 4). The temperature coefficient value (Q10) between 5 - 37° was found to range between 2.1 - 2.2.

Table 9 : Effect of enzyme concentration on ornithine carbamoyl transferase activity.

Enzyme protein (µg)	Enzyme activity (citrulline formed, µmoles)
0.4	0.04
0.8	0.11
1.2	0.19
1.6	0.26
2.4	0.38
3.2	0.48
4.0	0.54
4.8	0.59
5.6	0.62
6.4	0.64

Table 10 : Effect of period of incubation on ornithine carbamoyl transferase activity.

Incubation time (minutes)	Enzyme activity (citrulline formed, μmoles)
5	0.08
10	0.15
15	0.18
20	0.25
25	0.29
30	0.33
35	0.36
40	0.37
45	0.39
50	0.41
55	0.43
60	0.43

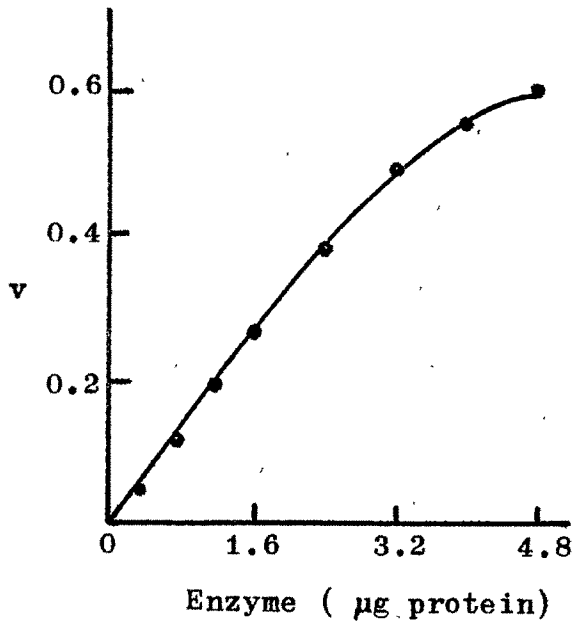


Fig.2: Effect of enzyme concentration on reaction velocity

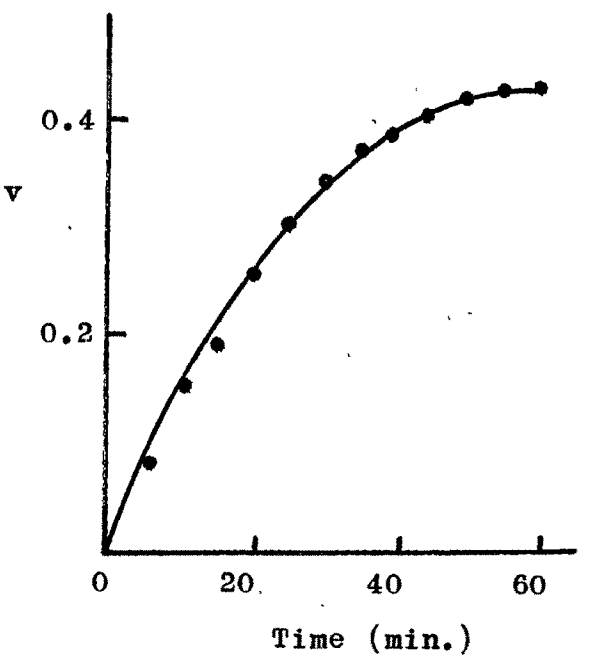


Fig.3: Effect of period of incubation on reaction velocity.

Table 11 : Effect of temperature of incubation on ornithine carbamoyl transferase activity.

Temperature ^{o}C	Enzyme activity (citrulline formed, µmoles)
5	0.03
10	0.05
15	0.07
20	0.11
25	0.15
30	0.24
37	0.34
40	0.36
45	0.26
50	0.19
60	0.02
70	0.02

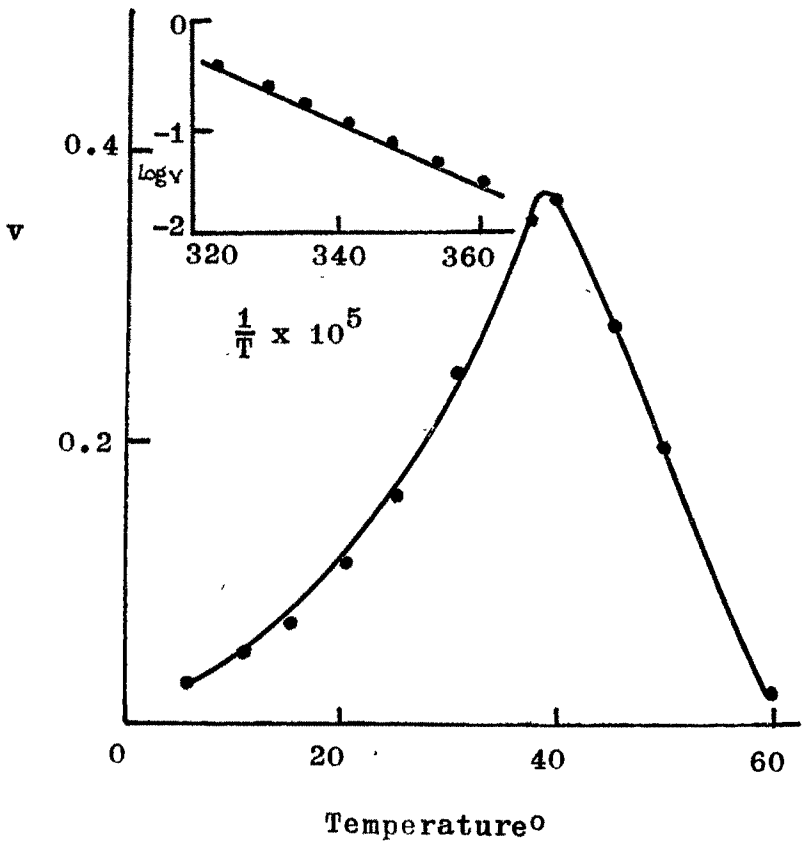


Fig.4: Effect of incubation temperature on reaction velocity.

The inset shows the Arrhenius plot of the same data upto 37°.

The energy of activation was calculated according to Arrhenius equation

$$E = \Delta H + RT$$

$$\text{and } \log V = \frac{\Delta H}{2.303} + \frac{RT}{R} \cdot \frac{1}{T}$$

When log V was plotted against 1/T a straight line was obtained (Figure - 4 inset) giving a slope of $\frac{H}{2.303} + \frac{RT}{R}$. From this energy of activation was calculated. It was found to be 13200 calories.

Thermal properties of enzyme

The heat stability of the purified enzyme is reported in Table 12. The enzyme was fully active at 40^{o} and retained more than 80 percent activity upto 50^{o}. Above this temperature the enzyme was extremely heat-labile and was completely inactivated at 65^{o} within 10 minutes.

Kinetics of substrate saturation

For most enzyme - catalysed reactions, plots of initial reaction velocity against substrate concentrations yield hyperbolic curves as is predicted by the Michaelis-Menten equation

$$v = \frac{VA}{Ka + A}$$

Table 12 : Thermal inactivation of ornithine carbamoyl transferase.

Inactivation temperature* °C	Enzyme activity (citrulline formed, µmoles)
0	0.28
40	0.27
45	0.25
50	0.23
55	0.21
60	0.15
65	0.00

* Partially purified enzyme was incubated for 10 minutes at the specified temperature, chilled for 10 minutes in an ice bath. An aliquot was then used for assay at 37°C.

However regulatory enzymes often exhibit a sigmoidal rather than hyperbolic substrate saturation function, i.e. a plot of reaction velocity against substrate concentration is concave upward in the region of low velocity. Such complex kinetics is explained with the existence of two or more interacting substrate binding sites on the enzyme such that the binding of one substrate molecule facilitates the binding of the next (Koshland, 1970). Monod, Wyman and Changeux (1965) advanced a hypothesis to explain these abnormal kinetics. In the meantime, several other theories have also been advaneed to explain the sigmoidal kinetics (Adair, 1925; Kirtley and Koshland, 1967; Koshland, Nemethy and Filmer, 1966; Hill, 1913; Atkinson, Hathaway and Smith, 1965; Frieden, 1959; Weber, 1965; Weber and Anderson, 1965). Whatever be the explanation, the sigmoidal response is probably of fundamental importance in cellular regulation because there is a threshhold concentration below which the enzyme activity is relatively insensitive to variations in concentration of substrate and effectors and above which relatively slight change in concentration evoke marked effects in enzyme activity (Changeux, 1963). It was also suggested that the legands could effect the neighbouring active sites and bring about conformational changes in the subunits of such enzymes, which will alter the kinetic patterns. If the energy of the interaction between subunits is changed by this conformational change e.g. if there is a net stabilizing energy then

the second molecule of substrate will bind more readily than the first as more and more substrate is bound. This is the basis of cooperative effect observed so frequently in allosteric proteins.

If subunit interactions occur, there will also be deviations in the double reciprocal plots. In cases of positive cooperativity a sigmoid appearance of v Vs. s plot is observed and the double reciprocal plot is concave upward. In negative cooperativity the double reciprocal plot is concave downwards.

Table 13 and Figure -5A,B show substrate saturation data for ornithine or carbamyl phosphate as variable substrate at a fixed concentration of nonvaried substrate. In both cases the plots did not follow a normal hyperbolic pattern but showed a definite sigmoidal response.

Tables 14 and 15 show the effect of substrate saturation data at several fixed levels of nonvaried substrate. In case of ornithine as varied substrate the activity reached a maximum at 2 µmoles and remained constant upto 4 µmoles but decreased at high concentrations (Table 16 and Figure 5C) with carbamyl phosphate as variable substrate the activity reached a maximum at 1 µmole and further increase upto 20 µmoles did not show any significant effect on activity (Results after 0.5 µmoles are not shown in Figure). This

Table 13 : Effect of substrate variation on ornithine carbamoyl transferase activity.

Varied substrate concentration* (µmoles)	Enzyme activity (citrulline formed, µmoles) with variable substrate	
	ornithine	carbamyl phosphate
0.050	0	0.02
0.100	0	0.04
0.125	0	0.07
0.150	0	0.09
0.175	0.01	0.11
0.200	0.03	0.14
0.225	0.03	0.16
0.250	0.05	0.18
0.275	0.06	0.21
0.300	0.07	0.22
0.350	0.11	0.24
0.400	0.14	0.26
0.500	0.18	0.28
0.600	0.23	0.30
0.700	0.28	0.31
0.800	0.31	0.33
0.900	0.33	0.34
1.000	0.35	0.35
1.250	0.37	0.36
1.500	0.39	0.36
1.750	0.40	0.38
2.000	0.41	0.38

* The concentration of nonvaried substrate was 2 µmoles.

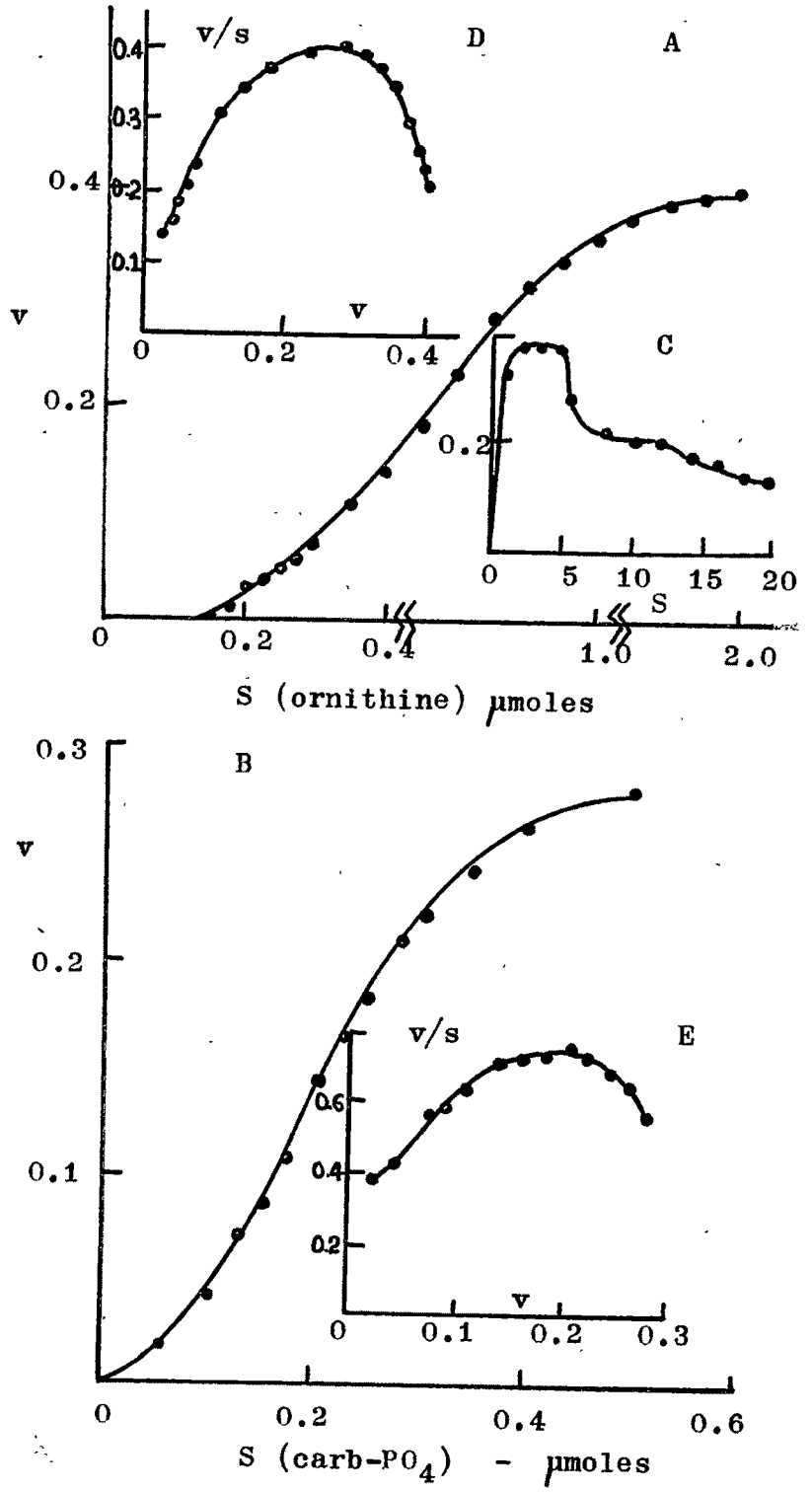


Fig.5 : Effect of substrate concentration on reaction velocity, (A) with ornithine and (B) with carb-PO_4 as variable substrates. (C) Shows the inhibition by high ornithine concentration. (D) and (E) show the Woolf plots of ornithine and carb-PO_4 as variable substrates.

Table 14 : Effect of ornithine variation on ornithine carbamoyl transferase activity at different nonvaried concentrations of carbamyl phosphate.

Ornithine concentration µmoles	Enzyme activity (citrulline formed, µmoles) at carbamyl phosphate concentration (µmoles)			
	0.50	0.75	1.0	1.50
0.50	0.16	0.19	0.21	0.23
0.75	0.20	0.24	0.26	0.30
1.00	0.23	0.29	0.32	0.36
1.50	0.28	0.34	0.41	0.43
2.00	0.30	0.36	0.43	0.47
2.50	0.33	0.38	0.45	0.48
3.00	0.36	0.40	0.45	0.48
4.00	0.38	0.41	0.47	0.50

Table 15 : Effect of carbamyl phosphate variation on ornithine carbamoyl transferase activity at different nonvaried concentrations of ornithine.

Carbamyl phosphate concentration (µmoles)	Enzyme activity (citrulline formed, µmoles) at ornithine concentration (µmoles)			
	0.50	0.75	1.00	1.50
0.50	0.18	0.23	0.27	0.34
0.75	0.19	0.25	0.30	0.39
1.00	0.19	0.26	0.32	0.41
1.50	0.20	0.27	0.33	0.43
2.00	0.21	0.27	0.35	0.45
2.50	0.21	0.28	0.37	0.45
3.00	0.21	0.28	0.37	0.45
4.00	0.21	0.28	0.38	0.46

Table 16 : Inhibition of ornithine carbamoyl transferase activity at high ornithine concentrations as variable substrate*.

Ornithine concentration (µmoles)	Enzyme activity (citrulline formed, µmoles)
1	0.28
2	0.39
3	0.38
4	0.38
5	0.31
8	0.22
10	0.21
12	0.20
14	0.19
16	0.17
18	0.15
20	0.14

* Carbamyl phosphate concentration was 2 µmoles.

sigmoidal substrate saturation response would thus indicate a cooperative substrate binding which is further substantiated by concave upward curvature in double reciprocal plots (Figure 6A,B).

Several other diagnostic tools have been used to study the cooperative interactions (Koshland, 1970). One of them is by plotting the data according to Hill equation :

$$\log \frac{V}{Vmax-V} = n \log (S) - \log K$$

Where v = velocity

Vmax = Maximum velocity

(S) = Substrate concentration

K = Constant

n = Hill coefficient

If $\log \frac{V}{Vmax-V}$ is plotted against log (S) this results in a straight line, the slope of which is represented by n. The slope n of lines obtained in Hill plots has been interpreted by a number of workers as a function of the interaction of substrate or effector binding sites and of the strength of this interaction (Taketa and Pogell, 1965; Atkinson, 1966; Corwin and Fanning, 1968; and Changeux, 1963).

In order to calculate Vmax in the present case it was necessary to know the intercept value (intercept = 1/Vmax). Since the data of the double reciprocal plots (Figure - 6)

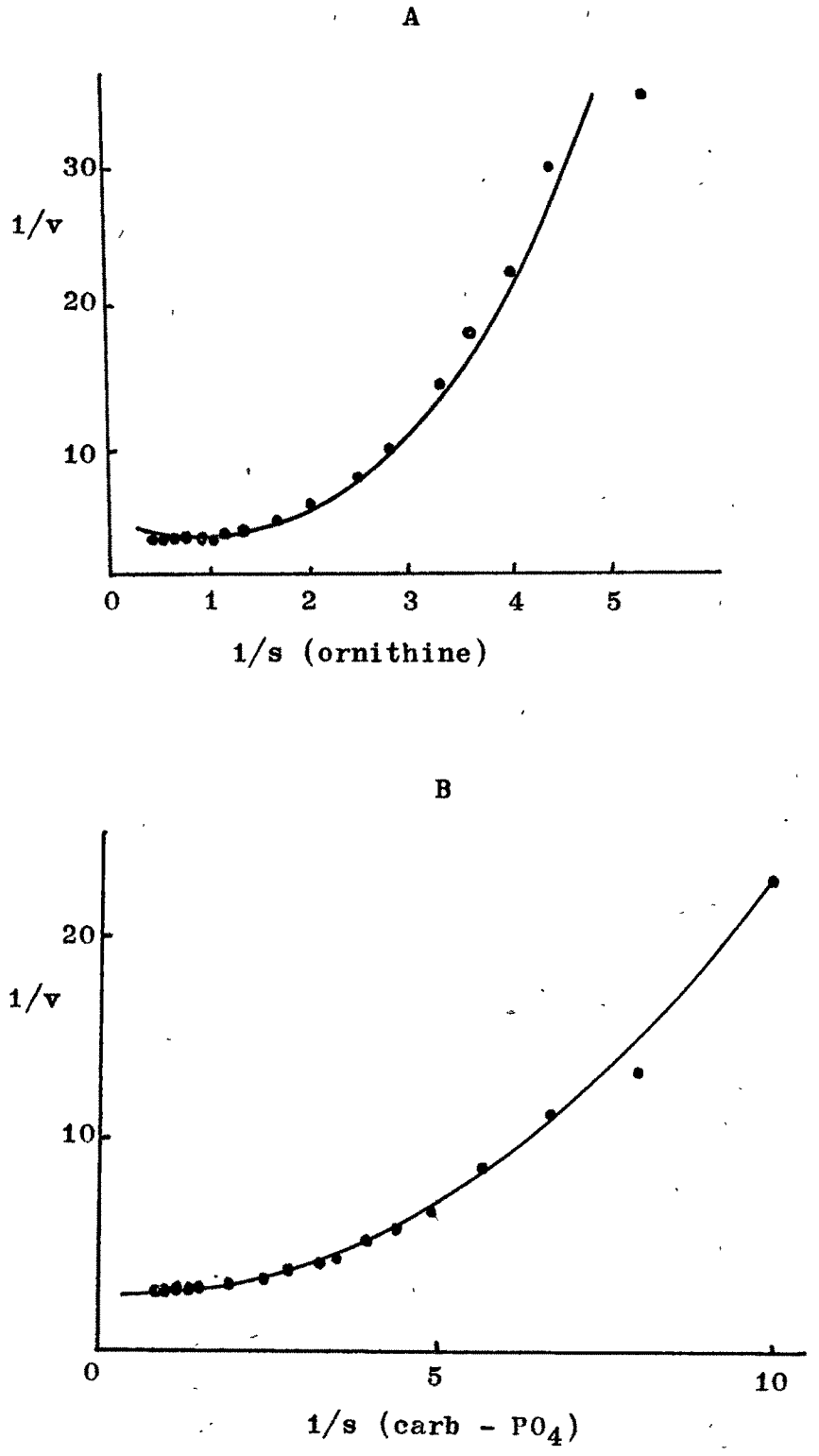


Fig. 6 : Double reciprocal plots of substrate concentration on reaction velocity with (A) ornithine and (B) carbamyl phosphate as the variable substrate.

did not fit to a straight line equation, it was fitted to the equation of a parabola.

$$y = a + b + c^2$$

Statistical analysis showed that experimental values fit very well with the theoritical values. However, in case of ornithine as variable substrate it can be seen that the vertex of the parabola (Figure - 6A) lies in the first quardant itself and therefore in this case the vertex was taken for calculating Vmax.

Taking these calculated values of Vmax a plot was made according to Hill equation. The points gave a straight line and the slope of line was calculated (Figures - 7A,B) and found to be 3.1 in case of ornithine and 1.5 in case of carbamyl phosphate. If the substrate saturation curve would have been following the Michaelis-Menten equation the n value should have been 1.0. However, in the present case the slope deviates significantly from 1.0 and on this basis one might assume an interaction of at least 3 binding sites for ornithine and 2 for carbamyl phosphate. This again indicates a cooperative interaction of substrate binding sites.

A further proof of cooperative substrate binding was indicated when substrate saturation data (Figure-5A,B) was plotted according to the method of Woolf (quoted by Haldane

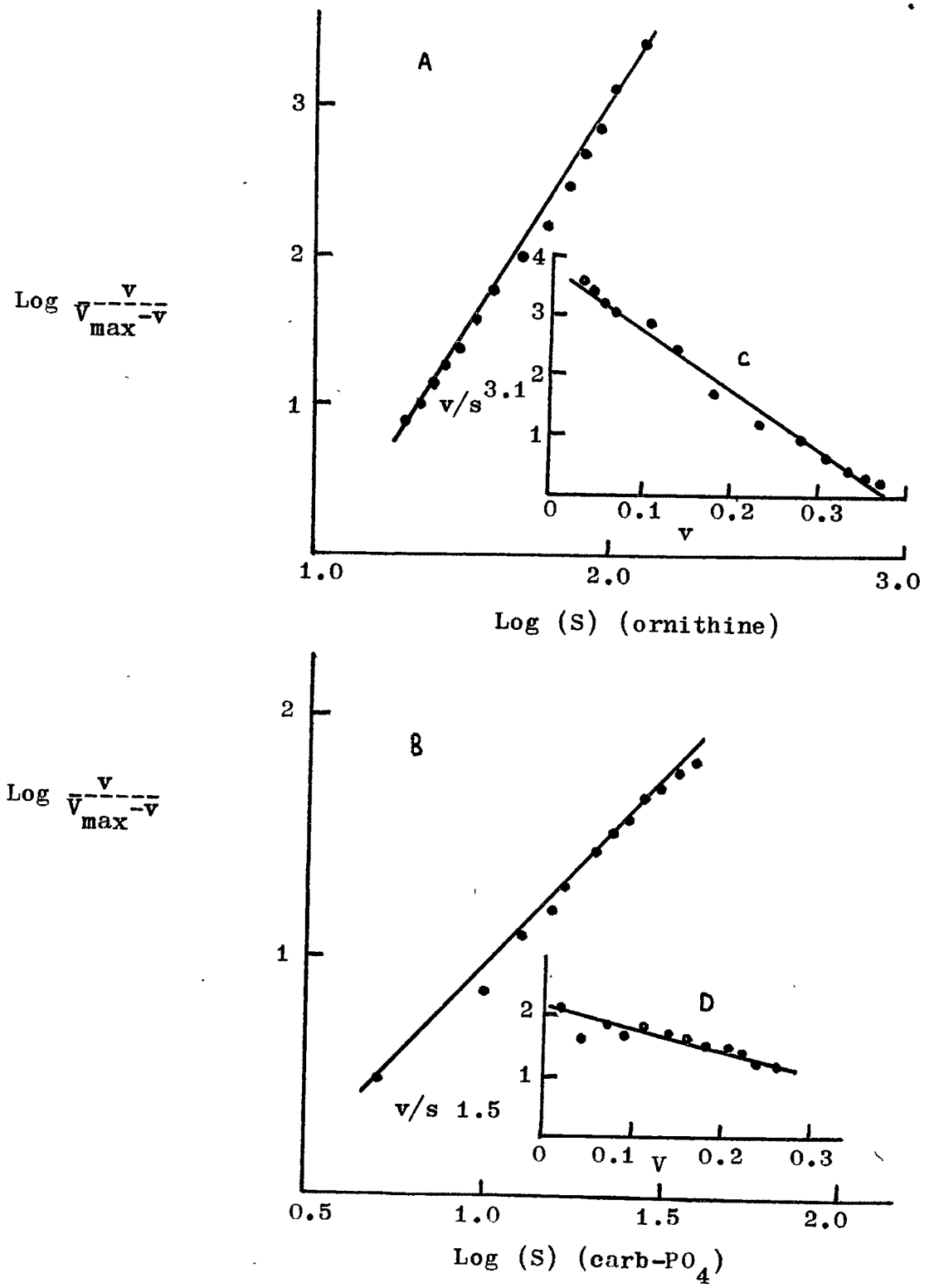


Fig.7: Hill plots of the reaction velocity.
The insets show the Woolf plots when plotted as v/s^n vs v.

and Stern, 1932) i.e. plotting v/s against v. If the data follows normal Michaelis-Menten kinetics, a straight line would be obtained. However, in case of a cooperative binding a curved line as shown in Figure-5D,E will be obtained. If the same data is plotted as v/s^n against v (n being the Hill coefficient value, obtained from Hill plot) the curved plot now changes almost fitting to a straight line (Figure - 7C,D).

Bireactant initial velocity kinetics

Kinetic mechanisms usually fall into two major groups. Those in which all the reactants must combine with the enzyme before reaction can take place and any product be released are called ordered or sequential. Mechanisms in which one or more products are released before all substrates have added are called ping pong; in such mechanisms the enzyme oscillates between two or more stable enzyme forms, while in the sequential mechanism there is only one stable enzyme form- the free enzyme (Cleland, 1970).

A determination of initial velocity patterns usually involves variation of the concentration of one substrate at different fixed levels of the other one, and in the absence of products.

Bireactant mechanisms give one of the three patterns. Most sequential mechanisms will show a rate equation of the form

$$v = \frac{VAB}{Kia\ Kb + KaB + KbA + AB}$$

where A and B are substrate concentrations, Ka and Kb are Michaelis constants of A and B; and Kia is the dissociation constant of A.

A plot of 1/v Vs. 1/A or 1/B under these conditions is a straight line and if such reciprocal plots are made Vs.1/A. at various B levels or vice versa it gives a series of lines intersecting to the left of vertical axis. The crossover point has a horizontal coordinate of -1/Kia when A is the substrate being varied.

Ping pong bireactant mechanisms give an initial velocity rate equation, similar to the above except that there is no constant term in the denominator. The result is that the pattern of reciprocal plots becomes parallel.

Initial velocity kinetic studies carried out on the enzyme purified in the present case are reported in Table 14 for ornithine as the varied substrate and carbamyl phosphate as the changing fixed substrate. This data when plotted in double reciprocal form gave an intersecting pattern (Figure - 8A) with lines crossing above the horizontal axis. It has been pointed out that this happens when Kia is greater than Ka value (Cleland, 1970). The slope and intercept replots given in Figure - 8 inset gave Kia, Ka and Kb values as 1.10, 0.87 and 0.41 μmoles respectively which again shows that Kia is greater than Ka value. Thus it can be concluded

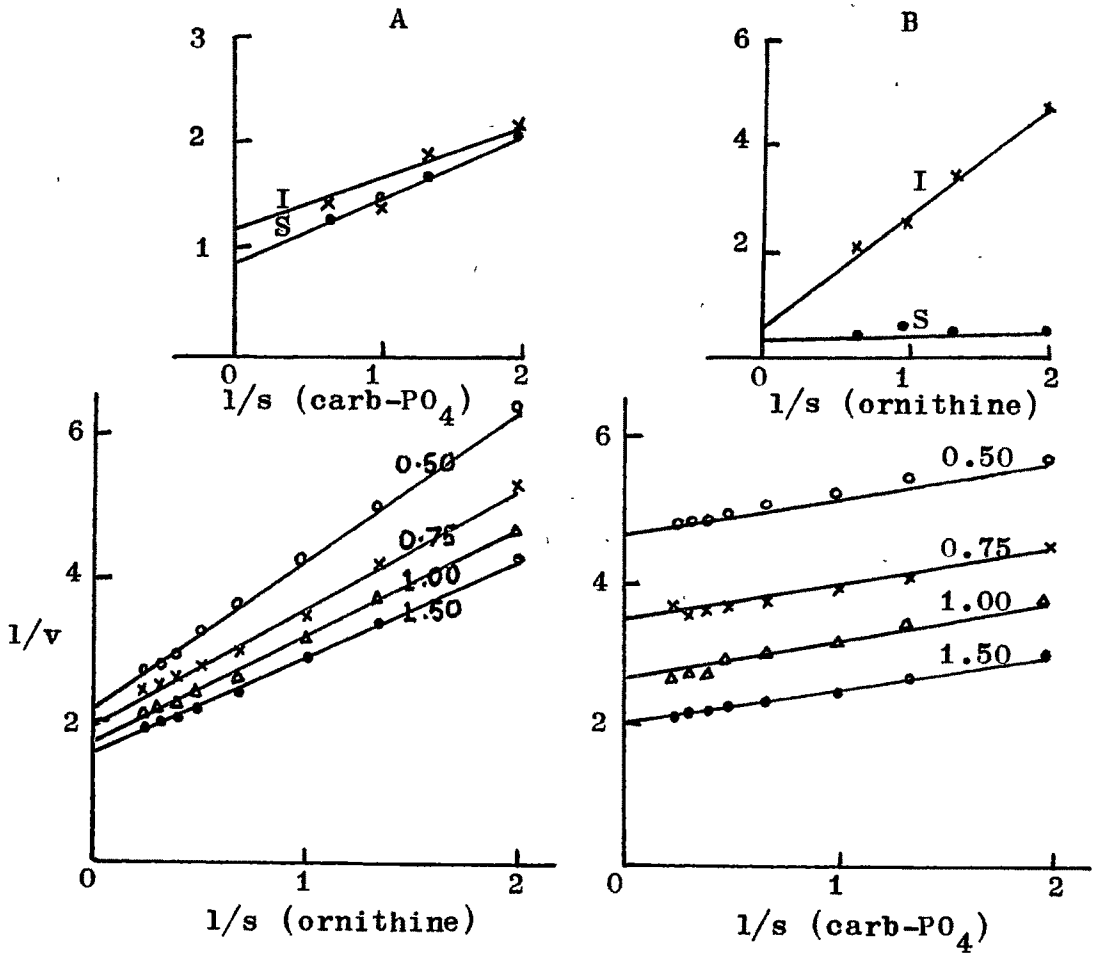


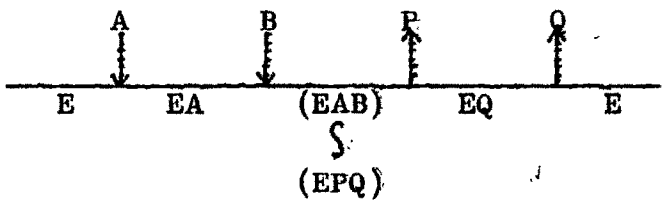
Fig.8: Initial velocity patterns with (A) ornithine and (B) carb-PO_4 as the varied substrates at several fixed levels of the nonvaried substrate.

Concentrations are shown on the lines.

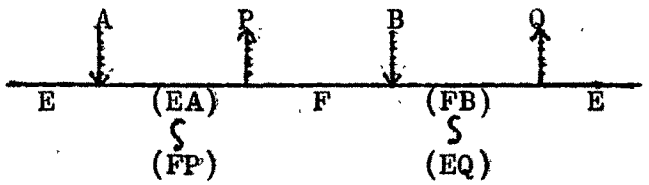
from this data that the binding of ornithine is independent of carbamyl phosphate concentration.

However, when carbamyl phosphate was taken as the varied substrate at changing fixed levels of ornithine the pattern (Table 15 and Figure - 8B) was found to be a family of almost parallel lines with no statistically significant difference in the slopes (Figure - 8 inset). This type of initial velocity pattern is a characteristic of ping pong mechanism. This means that there is no reversible connection between point of addition in the reaction sequence of the variable and the changing fixed substrate.

The classic sequential or intersecting pattern will confirm to a mechanism (Cleland, 1963) as follows :



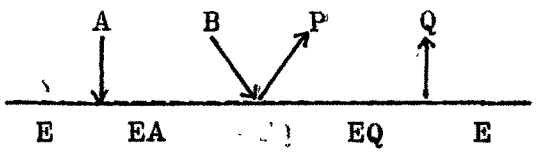
whereas, that of ping pong will be as follows :



(A and B represent the reactants and P and Q are the products in the order in which they bind or leave the enzyme molecule).

For a sequential mechanism the initial velocity patterns for both substrates are expected to be intersecting whereas for ping pong mechanism both the patterns should show parallel pattern.

In the present case however, one pattern is intersecting while the other is a parallel one. This would argument point that the mechanism for ornithine carbamoyl transferase may be similar to that proposed by Theorell and Chance (1951) for peroxidase.



This mechanism is really a special case of an ordered mechanism where substrate A combines with the enzyme to form complex EA but the steady state level of the central complex (EAB ⇌ EPQ) is very low. As the B combines a product comes out leaving complex EQ and then the next product is released. A further proof to this mechanism is obtained from the product inhibition studies reported below.

Inhibition studies

Inhibition is another major tool available for studying the kinetic mechanisms of enzyme catalysed reactions. Inhibitors may be products, substrates or other molecules. There are three basic types of inhibitions depending upon the effect the

inhibitor has on the slope and intercept of reciprocal plots. If the slope of the reciprocal plot is affected, but the intercept is not, the result is competitive inhibition fitting to an overall rate equation.

$$v = VS/K\ (1 + I/Ki) + S$$

If the intercepts are affected and the slopes are not, the inhibition is uncompetitive according to rate equation.

$$v = VS/K + S\ (1 + I/Ki)$$

If both slope and intercepts are increased, the inhibition is non-competitive

$$v = VS/K\ (1 + I/Kis) + S(1 + I/Kii)$$

The sequential Bi-Bi mechanism predicts three non-competitive product inhibitions and one competitive product inhibition, with one pattern becoming uncompetitive at high levels of the other substrate (Cleland, 1970). However, if the rate limiting step is solely the breakdown of EQ and the steady state level of the (EAB⇝EPQ) central complex is very low, the mechanism reduces to the Theorell-Chance type described earlier. In this case B-P pattern changes to competitive and there will be two competitive and two non-competitive patterns. All inhibition by P will be eliminated by saturation with B and all inhibition by Q eliminated by saturation with A.

In the present study, phosphate which is one of the products of enzyme reaction was used as product inhibitor and the results reported in Table 17 and Figure-9A,B show that with ornithine as varied substrate, phosphate gives a linear-noncompetitive inhibition i.e. both slope and intercept were increased with increase in phosphate concentration (Figure - 9A inset). The increase was statistically significant. The Ki (intercept) was 4.6 and Ki (slope) was 2.5 μmoles.

The results of carbamyl phosphate as the varied substrate are plotted in Figure - 9B and show a linear-competitive inhibition i.e. only slope was affected but not the intercept (Figure - 9B inset). The Ki (slope) was found to be 0.4 μmoles.

In order to confirm the nature of inhibition a separate study was carried out according to the method of Dixon (1953). The results of this study are reported in Table 18 and Figures - 10A,B. The Dixon plots also confirm that the phosphate inhibition is noncompetitive with respect to ornithine and competitive with respect to carbamyl phosphate. Ki values obtained with ornithine and carbamyl phosphate were 4.3 and 0.4 μmoles respectively which agree well with the study reported in preceeding para.

As mentioned earlier in this section, if the mechanism of this enzyme is of Theorell-Chance type with two competitive

Table 17 : Effect of substrate variation on the inhibition of ornithine carbamoyl transferase activity by phosphate.

Varied substrate concentration* (µmoles)	Enzyme activity (citrulline formed, µmoles)			
	Control	in presence of phosphate (µmoles)		
		1	2	4
A - ornithine				
0.50	0.11	0.08	0.06	0.05
0.70	0.15	0.11	0.09	0.06
1.00	0.18	0.13	0.11	0.08
1.25	0.22	0.18	0.13	0.09
1.50	0.26	0.21	0.15	0.11
2.00	0.33	0.21	0.18	0.13
B - carbamyl phosphate				
0.30	0.19	0.07	0.03	
0.50	0.26	0.10	0.06	
0.70	0.30	0.13	0.07	
1.00	0.33	0.18	0.10	
1.25	0.36	0.21	0.10	
1.50	0.38	0.23	0.14	
2.00	0.43	0.26	0.17	

* Concentration of the nonvaried substrate was 2 µmoles.

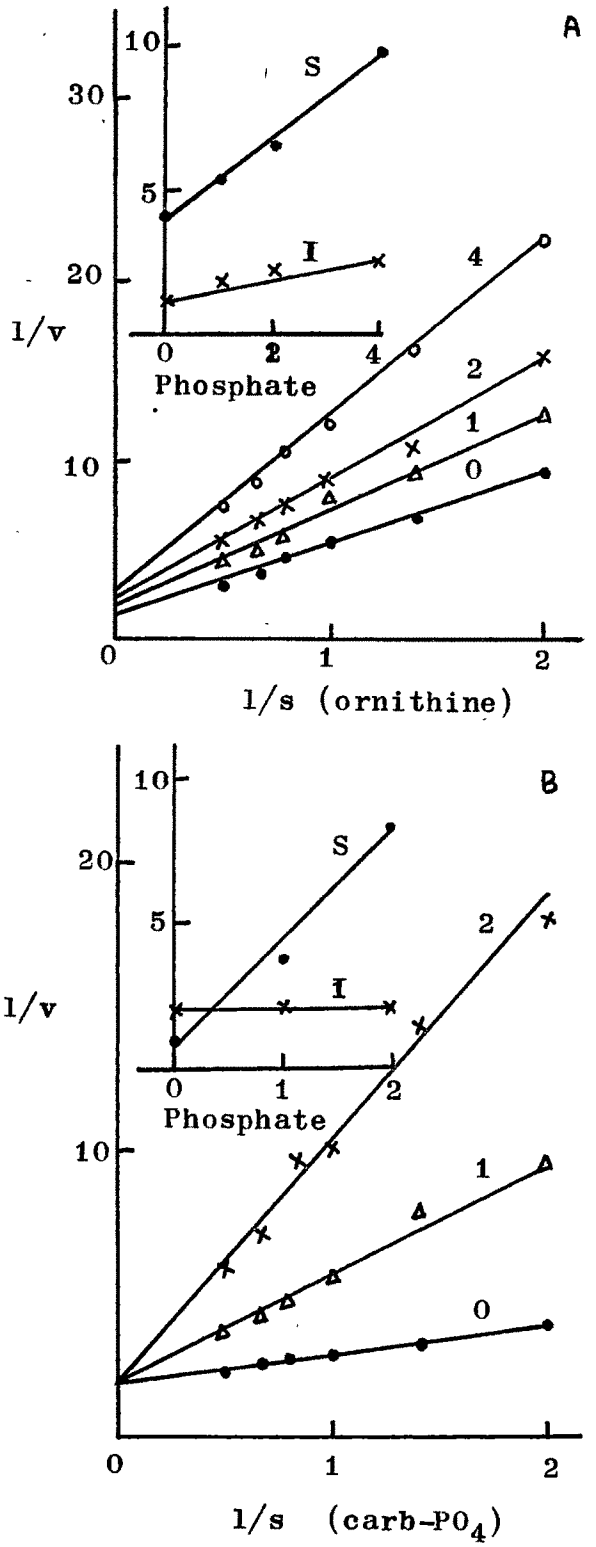


Fig.9: Double reciprocal plots for the inhibition of reaction velocity by phosphate.

Concentrations of phosphate (in μmoles) are shown on the lines.

Table 18 : Effect of phosphate concentration on the inhibition of ornithine carbamoyl transferase activity at various fixed levels of substrate.

Phosphate concentration (µmoles)	Enzyme activity (citrulline formed, µmoles) at ornithine concentration* (µmoles)		
	0.5	1.0	2.0
0	0.13	0.23	0.33
0.5	0.13	0.20	0.34
1.0	0.12	0.18	0.29
1.5	0.10	0.17	0.27
2.0	0.10	0.18	0.24
2.5	0.09	0.16	0.20
3.0	0.09	0.15	0.19
3.5	0.08	0.13	0.18
4.0	0.08	0.12	0.19
	at carbamyl phosphate concentration* (µmoles)		
	0.5	1.0	2.0
0	0.26	0.35	0.39
0.5	0.17	0.26	0.34
1.0	0.15	0.20	0.28
1.5	0.12	0.19	0.28
2.0	0.10	0.18	0.23
2.5	0.08	0.16	0.27
3.0	0.07	0.14	0.24
3.5	0.07	0.13	0.19
4.0	0.06	0.12	0.21

* Concentration of the other substrate was 2 µmoles.

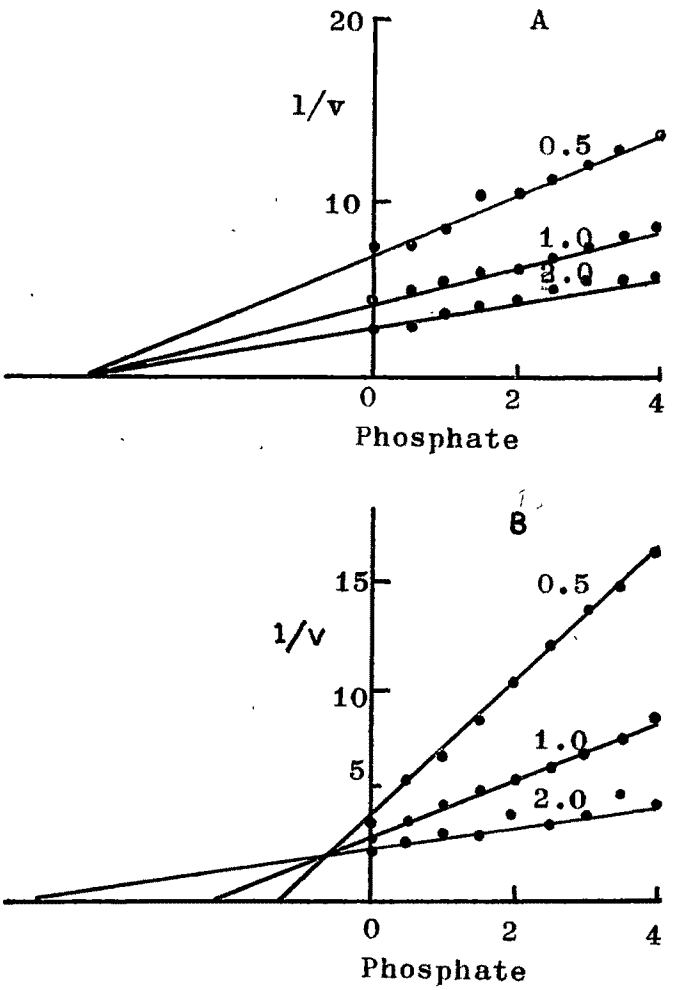


Fig. 10 : Dixon plots for the inhibition of reaction velocity by phosphate at three fixed levels of (A) ornithine (B) Carb-PO_4.

Concentrations of substrates are indicated on the lines.

and two noncompetitive inhibitions, saturation with the substrate would abolish the inhibition if it is competitive. When such a study was made by saturating with carbamyl phosphate (Table 19) it was found that the noncompetitive inhibition of phosphate against ornithine was abolished completely at very high concentrations of carbamyl phosphate. It would have been of interest to study the inhibition patterns of the other product citrulline but since the reaction velocity was measured by estimating the citrulline formed no studies could be made using citrulline as product inhibitor. The other product phosphate could not be used for estimation of reaction velocity because of the interference in the estimation of phosphate due to the lability of carbamyl phosphate.

The studies reported in this section on initial velocity kinetics and kinetics of product inhibition were analysed and compared with the predicted nature of inhibition patterns for various mechanisms (Cleland, 1963). It may be interpreted that the enzyme ornithine carbamoyl transferase follows Theorell-Chance mechanism. However, it is difficult to state from these studies as to which substrate (ornithine or carbamyl phosphate) corresponds to A or B as these studies will not differentiate between A and B. It can only be differentiated by isotope exchange technique.

Table 19 : Effect of phosphate on ornithine carbamoyl transferase activity at nonsaturating and saturating levels of carbamyl phosphate with ornithine as variable substrate.

Ornithine (µmoles)	Enzyme activity (citrulline formed, µmoles)					
	Carbamyl phosphate (2 µmoles)			Carbamyl phosphate (50 µmoles)		
	Phosphate (µmoles)			Phosphate (µmoles)		
	0	2	4	0	2	4
0.50	0.11	0.08	0.06	0.11	0.12	0.12
0.70	0.18	0.11	0.09	0.15	0.16	0.15
1.00	0.23	0.16	0.10	0.20	0.20	0.19
1.25	0.25	0.17	0.11	0.23	0.24	0.24
1.50	0.28	0.19	0.12	0.24	0.27	0.27
2.00	0.29	0.18	0.13	0.29	0.30	0.30

Effect of metal ions

The data reported in Table 20 show that all the metals tested have inhibitory effect to various degrees. Mercury and zinc inhibit the enzyme at very low concentrations; copper, nickel, manganese and cobalt come next in the series.

Effect of various other compounds

The results given in Table 21 show that azide, pCMB and iodoacetate all inhibit the enzyme. Inhibition by pCMB and iodoacetate indicates the involvement of -SH groups for enzyme activity.

The effect of several sugars and sugar phosphates was tried to see whether they may have any influence on the reaction velocity. The results reported in Table 22 show that except fructose,1:6-diphosphate, which inhibits the enzyme activity, no other sugar or sugar phosphate have any significant effect. The kinetics of inhibition of FDP is reported in Table 23 and plotted in Figures - 11A,B. The plots show that FDP with both substrates gives a linear-noncompetitive inhibition.

The effect of TCA cycle intermediates is reported in Table 24. It can be seen that except citrate, succinate, pyruvate and isocitrate which did not have any significant effect, all other compounds tested showed inhibitory effect. The kinetics of inhibition of oxaloacetate, 2-oxoglutarate,

Table 20 : Effect of metal ions on ornithine carbamoyl transferase activity.

Metal salt added	Concentration (µmoles)	Inhibition %
None	-	0
$MgSO_4$	5	38
	10	47
$CaCl_2$	5	18
	10	27
$MnCl_2$	1.5	32
	2.5	95
$CuSO_4$	0.25	24
	1.00	71
$NiCl_3$	1.0	27
	2.5	84
$CoCl_2$	2.0	29
	3.0	52
$FeCl_3$	5	24
	10	84
$AlCl_3$	5	39
	10	73
$ZnCl_2$	0.002	36
	0.006	82
$HgCl_2$	0.001	35
	0.0025	49
$NaMoO_4$	5	22
	10	45

Table 21 : Effect of various compounds as inhibitors of ornithine carbamoyl transferase activity.

Compound	Concentration (µmoles)	Inhibition %
None	-	0
EDTA	5	0
	10	7
NaF	10	6
	20	6
NaN_3	5	100
	10	100
pCMB	0.00010	3
	0.00025	50
	0.00050	100
Iodoacetate	10	38
	20	54

Table 22 : Effect of various sugar and sugar phosphates on ornithine carbamoyl transferase activity.

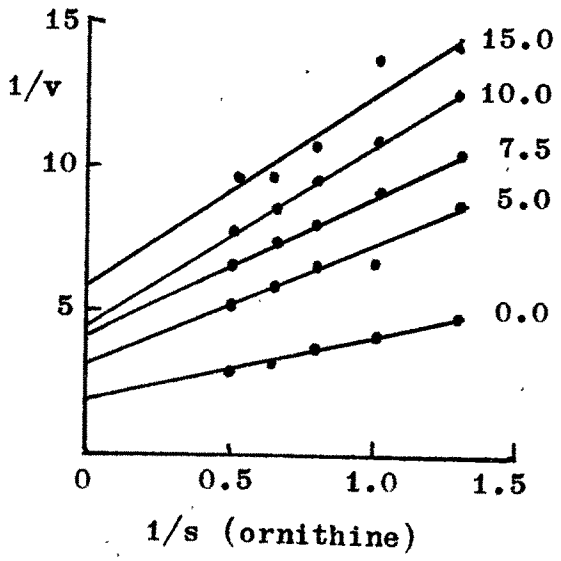
Additions	Concentration (µmoles)	Inhibition %
None	-	0
Ribose	5	2
	10	3
Glucose	5	0
	10	8
Fructose	5	2
	10	10
Sucrose	5	10
	10	14
Glucose-1-phosphate	5	0
	10	0
Glucose-6-phosphate	5	0
	10	17
Fructose-6-phosphate	5	13
	10	20
Fructose-1:6-diphosphate	5	26
	10	45

Table 23 : Effect of substrate variation on the inhibition of ornithine carbamoyl transferase activity by fructose-1:6-diphosphate.

Varied substrate concentration* (µmoles)	Enzyme activity (citrulline formed, µmoles)				
	Control	in presence of fructose-1:6-diphosphate (µmoles)			
		5	7.5	10	15
A - ornithine					
0.75	0.20	0.11	0.10	0.08	0.07
1.00	-	0.15	0.11	0.09	0.07
1.25	0.27	0.15	0.13	0.10	0.09
1.50	0.34	0.17	0.13	0.12	0.10
2.00	0.34	0.19	0.16	0.13	0.10
B - carbamyl phosphate					
0.50	0.26	0.11	0.09	0.06	0.06
0.75	0.27	0.14	0.12	0.09	0.08
1.00	0.30	0.16	0.13	0.10	0.08
1.25	0.33	0.16	0.16	0.11	0.09
1.50	0.34	0.19	0.17	0.12	0.10

* Concentration of the nonvaried substrate was 2 µmoles.

A



B

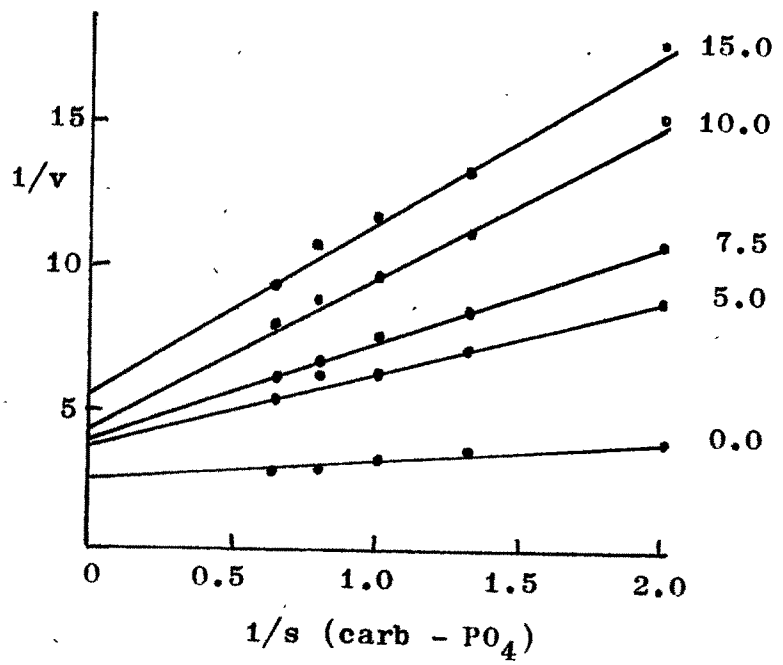


Fig. 11 : Double reciprocal plots for the inhibition of reaction velocity by Fructose-1:6-diphosphate.

Concentrations of fructose-1:6-diphosphate (in μmóles) are shown on the lines.

Table 24 : Effect of Krebs cycle intermediates on ornithine carbamoyl transferase activity.

Addition	Concentration (µmoles)	Inhibition %
None	-	0
Citrate	1	6
	3	7
	5	17
Isocitrate	1	29
	3	20
	5	25
Cis-aconitate	1	36
	3	69
	5	77
Succinate	1	10
	3	10
	5	12
Malate	1	25
	3	45
	5	57
Fumarate	5	17
	10	39
	20	78
Pyruvate	1	16
	3	0
	5	0
Oxaloacetate	1	35
	3	51
	5	71
2-oxoglutarate	1	25
	3	48
	5	70

cis-aconitate and malate are reported in Table 25 and Figures - 12 and 13. All these compounds show a linear competitive inhibition with ornithine as the varied substrate and linear-noncompetitive with carbamyl phosphate as the varied substrate.

However, further analysis of inhibition kinetics of FDP or organic acids did not indicate any effect on the cooperativity and thus may not be involved as allosteric inhibitors.

The results reported in Table 26 show the effect of various purine and pyrimidine nucleotides on enzyme activity. It can be seen that AMP, XMP, and IMP inhibit the enzyme activity whereas other nucleotides have no effect. The inhibition of XMP and IMP was less compared to AMP. Table 27 shows the effect of adenine and various derivatives of adenine. Eventhough, adenine itself inhibits but the inhibition was less compared to adenosine or AMP.

NAD and NADP were also tested as inhibitors since they also contain adenine moiety. Both were found to inhibit enzyme activity. NMN, however, did not give any inhibition indicating that the inhibition of NAD and NADP was due to the adenine moiety only.

The inhibition by adenine derivatives was quite unexpected. It was expected that any of the pyrimidine derivatives may have some influence since carbamyl phosphate

Table 25 : Effect of substrate variation on the inhibition of ornithine carbamoyl transferase activity by organic acids.

Varied substrate concentration* (µmoles)	Control	Enzyme activity (citrulline formed, µmoles) with							
		Oxaloacetate (µmoles)		2-oxoglutarate (µmoles)		Cis-aconitate (µmoles)		Malate (µmoles)	
		2.5	5.0	2.5	5.0	2.5	5.0	2.5	5.0
A - ornithine									
0.75	0.28	0.20	0.15	0.16	0.10	0.18	0.12	0.17	0.10
1.00	0.34	0.25	0.19	0.20	0.13	0.24	0.16	0.20	0.13
1.25	0.41	0.29	0.25	0.24	0.15	0.28	0.19	0.24	0.16
1.50	0.43	0.32	0.26	0.27	0.18	0.30	0.22	0.27	0.18
2.00	0.45	0.37	0.30	0.31	0.22	0.35	0.27	0.35	0.24
B - carbamyl phosphate									
0.75	0.35	0.27	0.24	0.22	0.15	0.24	0.20	0.16	0.11
1.00	0.39	0.31	0.27	0.25	0.17	0.27	0.20	0.18	0.12
1.25	0.41	0.34	0.28	0.26	0.18	0.30	0.23	0.20	0.13
1.50	0.45	0.36	0.31	0.28	0.20	0.31	0.24	0.21	0.14
2.00	0.47	0.38	0.34	0.32	0.21	0.33	0.26	0.23	0.15

* Concentration of nonvaried substrate was 2 µmoles.

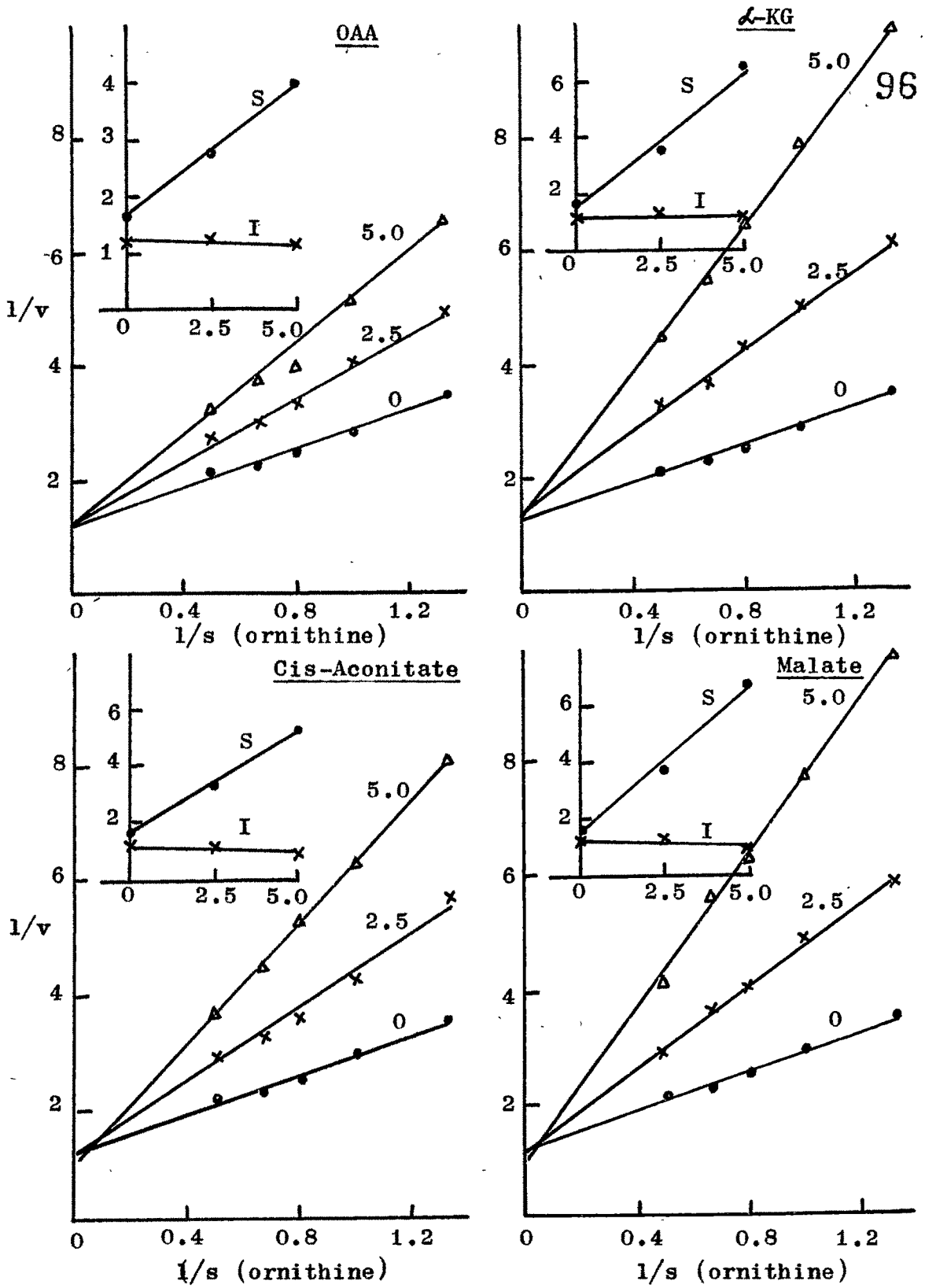


Fig.12: Double reciprocal plots for the inhibition of reaction velocity by organic acids.

Concentrations of organic acid (in μmoles) are shown on the lines.

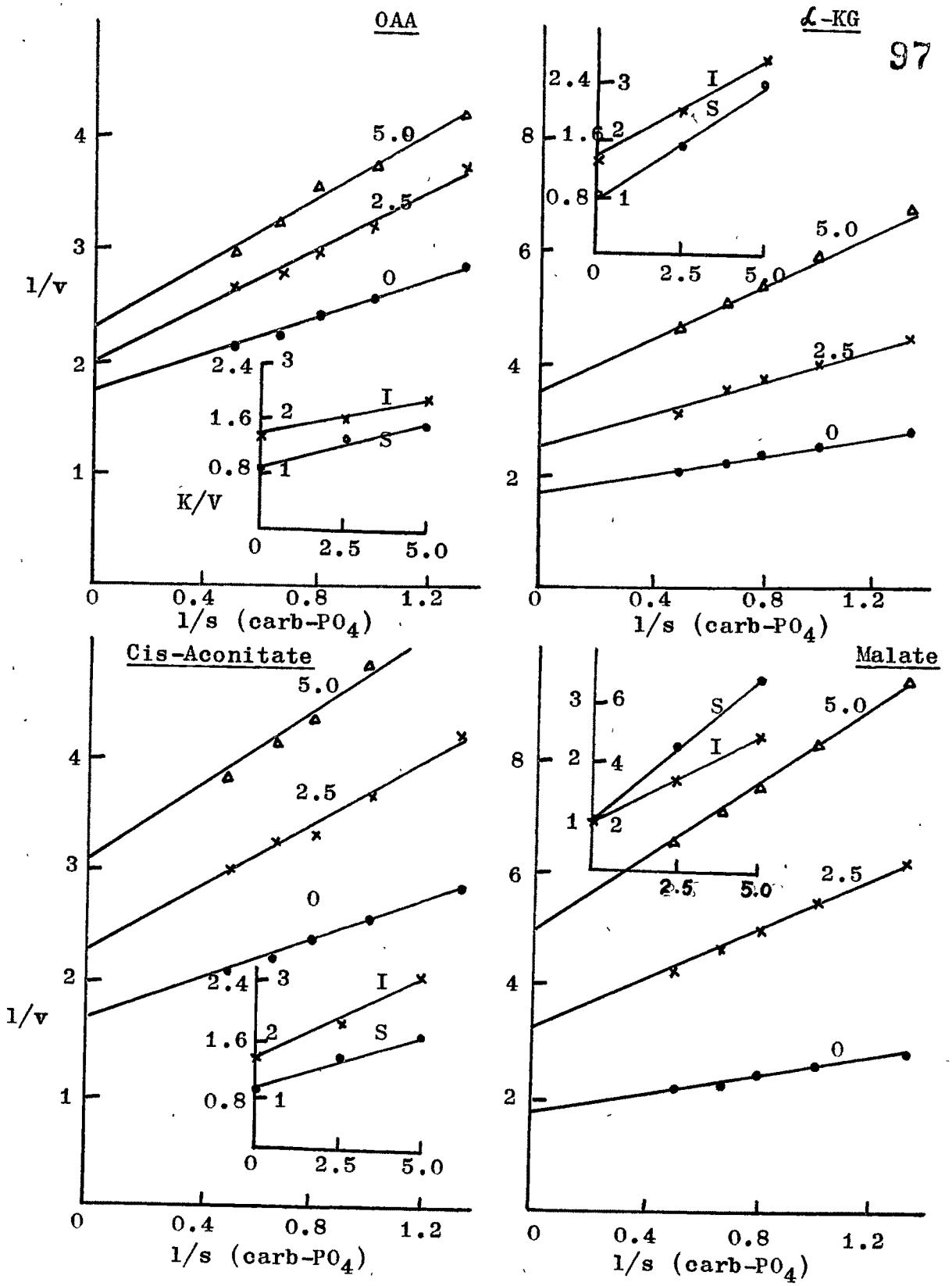


Fig. 13: Double reciprocal plots for the inhibition of reaction velocity by organic acids.

Concentrations of organic acid (in μmoles) are shown on the lines.

Table 26 : Effect of purine and pyrimidine nucleotides on ornithine carbamoyl transferase activity.

Additions	Concentration (µmoles)	Inhibition %
None	-	0
IMP	5	17
	10	29
GMP	5	18
	10	20
AMP	5	50
	10	69
XMP	5	10
	10	31
UMP	5	2
	10	2
CMP	5	6
	10	6
TMP	5	0
	10	8

Table 27 : Effect of various adenine derivatives on ornithine carbamoyl transferase activity.

Additions	Concentration (µmoles)	Inhibition %
None	-	0
Adenine	5	35
	10	36
Adenosine	5	55
	10	68
AMP	5	56
	10	75
ADP	5	71
	10	87
ATP	5	22
	10	46
NAD	2	36
	4	58
	5	61
NADP	0.5	59
	1.0	74
	2.0	100
NMN	5	11
	10	4

is the common substrate for this enzyme as well as aspartate transcarbamylase involved in pyrimidine biosynthesis.

The AMP inhibition was studied further and the results reported in Figure - 14 show the effect of varying substrate concentration at several fixed levels of AMP. Unexpectedly the velocity versus ornithine as variable substrate plots for AMP became sigmoidal as the AMP concentration was increased. These observations clearly point to the possibility that AMP is an allosteric inhibitor. However, no such response was obtained when carbamyl phosphate was the varied substrate.

This data of Table 28 when plotted in double reciprocal form (Figure - 15) showed that AMP inhibition is competitive for ornithine and noncompetitive for carbamyl phosphate. With ornithine also the linearity was not good at high AMP concentrations. The Ki (slope) with ornithine was 2.2 µmoles whereas with carbamyl phosphate the Ki (slope) and Ki (intercept) were 3.1 and 4.2 µmoles respectively.

The Dixon plots (Table 29 and Figure - 16A,B) gave Ki values as 2.2 and 3.0 µmoles respectively with ornithine and carbamyl phosphate.

Attempts to desensitize enzyme

Since much of the kinetic evidence suggests that the enzyme may be an allosteric one and that AMP has a regulatory

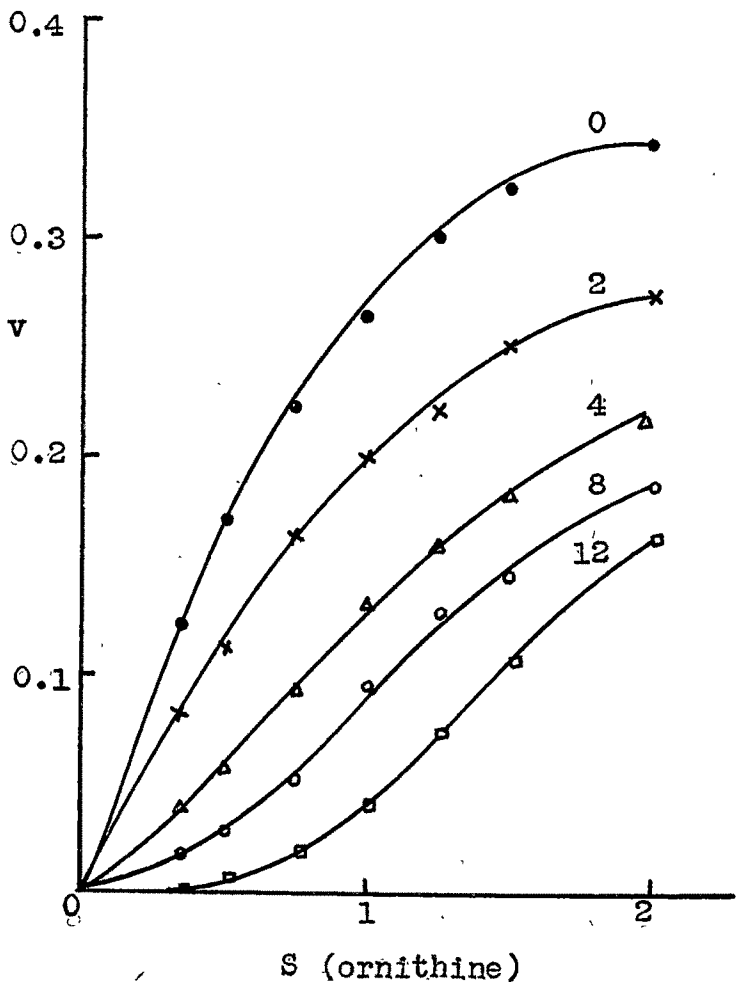


Fig.14 : Effect of AMP on reaction velocity with ornithine as varied substrate.

Concentrations of AMP (in μmoles) are shown on the lines.

Table 28 : Effect of substrate variation on the inhibition of ornithine carbamoyl transferase by AMP.

Varied substrate concentration* (µmoles)	Enzyme activity (citrulline formed, µmoles)			
	Control	in presence of AMP (µmoles)		
		2	4	8
A - ornithine				
0.50	0.17	0.11	0.07	0.05
0.75	0.22	0.18	0.10	0.07
1.00	0.26	0.20	0.14	0.11
1.25	0.30	0.22	0.16	0.13
1.50	0.32	0.25	0.17	0.13
2.00	0.34	0.27	0.22	0.15
B - carbamyl phosphate				
0.25	0.21	0.13	0.11	0.07
0.50	0.27	0.18	0.13	0.09
0.75	0.37	0.21	0.19	0.10
1.00	0.36	0.25	0.17	0.12
1.25	0.36	0.26	0.19	0.12
1.50	0.39	0.28	0.21	0.12

* Concentration of the nonvaried substrate was 2 µmoles.

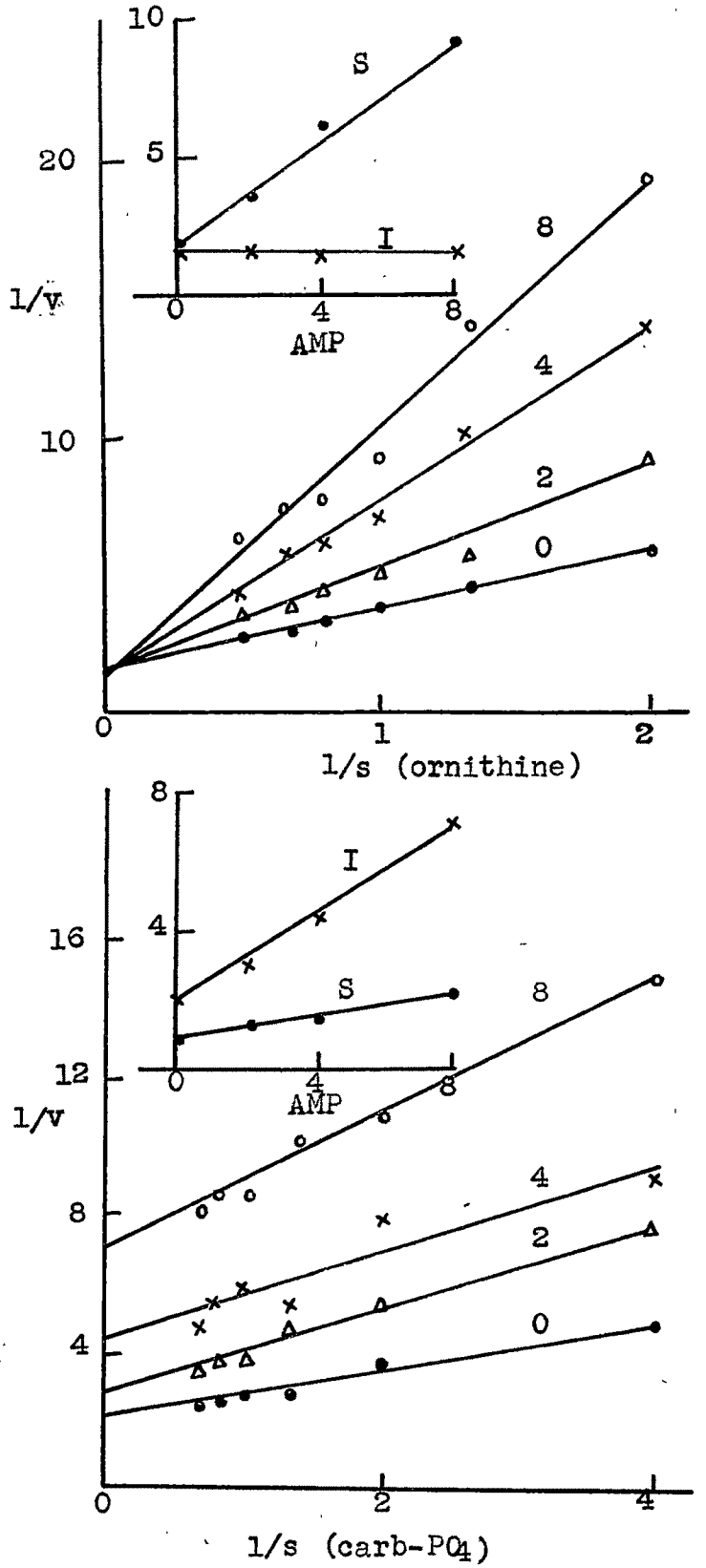


Fig.15: Double reciprocal plots for the inhibition of reaction velocity by AMP.

Concentrations of AMP. (in µmoles) are shown on the lines.

Table 29 : Effect of AMP concentration on the inhibition of ornithine carbamoyl transferase activity at various fixed levels of substrate.

AMP concentration (µmoles)	Enzyme activity (citrulline formed, µmoles)		
	at ornithine concentration* (µmoles)		
	0.5	1.0	2.0
0	0.24	0.32	0.50
2	0.15	0.24	0.32
4	0.11	0.16	0.23
6	0.09	0.13	0.18
8	0.07	0.11	0.16
	at carbamyl phosphate concentration* (µmoles)		
	0.25	0.50	1.00
0	0.20	0.25	0.32
2	0.11	0.13	0.23
4	0.08	0.11	0.19
6	0.06	0.10	0.11
8	0.05	0.07	0.10

* Concentration of the other substrate was 2 µmoles.

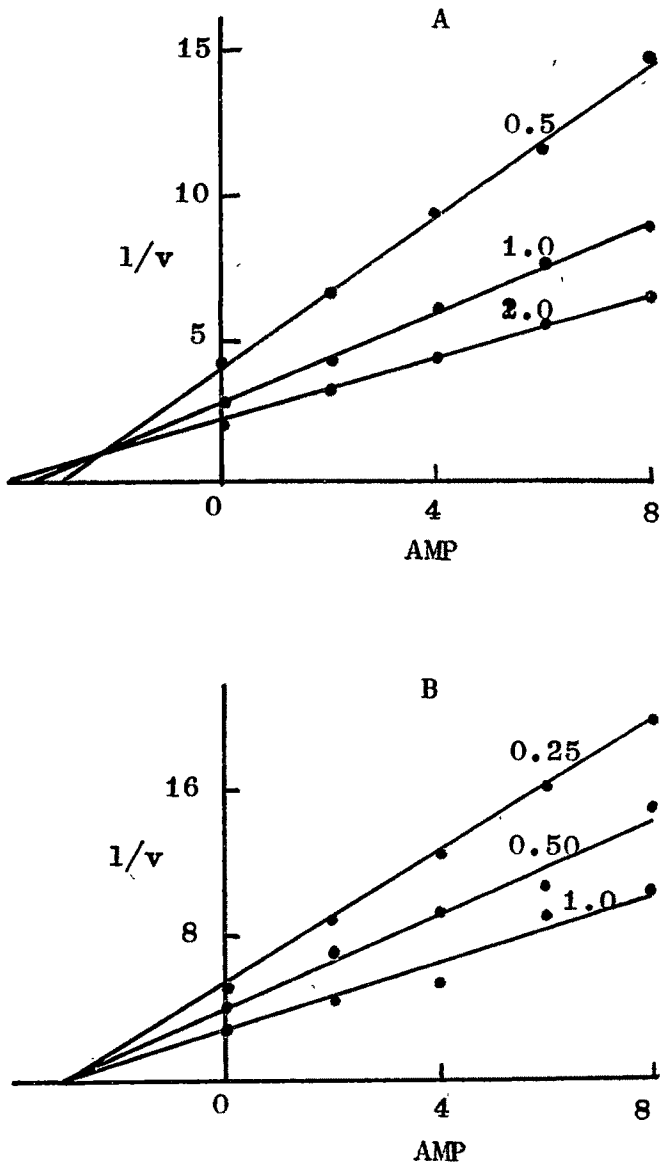


Fig.16: Dixon plots for the inhibition of reaction velocity by AMP at three fixed levels of (A) ornithine (B) carb-PO_4.

Concentrations of substrates are indicated on the lines.

site distinct from the substrate binding sites, attempts were made to desensitize the enzyme by heat treatment, urea, pCMB and chelating agents. However, all attempts failed to bring about desensitization.