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: RESULTS :  
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## RESULTS

### Dry weight and protein content of tissues :

The dry weight and protein content of the tissues cultivated for 30 days is given in Table 5. It can be seen that the dry weight varies from 3-10 percent in normal and tumour tissues. The protein content does not show any regular pattern in various tissues. In case of Rumex and tobacco tissues the protein content is practically same in normal and tumour tissues whereas in case of Parthenocissus the protein content is about four times higher in normal than in the tumour tissue.

### Free amino acid composition of normal and tumour tissues :

The data reported in Table 6 show that glutamic acid, aspartic acid, serine, glycine, alanine and arginine are low in tumour than in the corresponding normal tissue in case of all the three plant tissues studied. Lysine content was found to be low in all the tissues. Histidine was practically absent in all the tissues except Rumex tumour tissue.  $\gamma$ -Aminobutyric acid was found to be considerably high in Parthenocissus and tobacco normal tissues compared to their corresponding tumour tissues. However, there was no difference in  $\gamma$ -aminobutyrate content between

Table 5. Dry weight and protein content of normal and tumour tissues of plants cultivated in vitro

Tissue	Dry weight (%)	Protein (mg/g fresh tissue)
<u>Rumex</u>		
Normal	2.9	6.67
Tumour	5.2	6.33
<u>Parthenocissus</u>		
Normal	9.0	13.67
Crown-gall tumour	6.6	3.67
<u>Tobacco</u>		
Normal	9.4	21.33
Crown-gall tumour	11.6	18.00

Table 6. Free amino acid composition of normal and tumour tissues of plants cultivated in vitro

Amino acid	Rumex acetosa		Parthenocissus tricuspidata		Tobacco	
	Normal	Tumour	Normal	Crown-gall tumour	Normal	Crown-gall tumour
Glutamic acid	126.0	95.8	129.4	30.5	17.6	4.7
Aspartic acid	48.7	34.8	115.8	42.0	52.5	6.9
Serine	76.6	60.4	45.9	8.9	17.8	5.8
Glycine	25.5	23.8	12.4	8.6	3.4	0.0
Lysine	14.8	19.8	25.7	17.4	0.0	0.0
Histidine	0.0	134.1	0.0	0.0	0.0	0.0
Arginine	114.8	88.1	211.0	46.2	126.9	94.8
Glutamine	13.9	34.8	36.2	7.0	28.0	0.0
Alanine	214.3	144.2	80.5	26.3	15.4	2.7
$\gamma$ -Amino butyric acid	68.9	61.3	845.8	44.3	625.0	30.3
Threonine	59.9	10.0	20.8	traces	traces	traces
Methionine + valine	65.0	19.2	9.3	14.1	traces	traces
Isoleucine + Leucine + Phenylalanine	19.8	traces	0.0	0.0	0.0	0.0

normal and tumour tissues of Rumex. Other amino acids were either absent or present only in traces. The data thus show that in general the free amino acid content is more in normal than in tumour tissues.

Activities of enzymes involved in arginine, glutamate and aspartate metabolism of normal and tumour tissues :

A comparative study carried out on the enzymes involved in glutamate, aspartate and arginine metabolism (Tables 7 and 8) show that the specific activity of arginase, ornithine transaminase and the enzyme systems involved in conversion of glutamate to pyrroline-5-carboxylic acid and proline to pyrroline-5-carboxylic acid and glutamate decarboxylase are higher in tumour tissues. The specific activity in case of other enzymes either did not show any difference or any regular pattern between various normal and tumour tissues.

In the present case arginase activity is found to be higher in tumour tissues. In the case of Scorsonera crown-gall tissue (Menage and Morel, 1964) no arginase activity has been detected and arginine has been shown to be metabolised by a different pathway involving various guanidine compounds. As the arginase activity was very low in Parthenocissus and tobacco tissues compared to Rumex tissue attempts were made to see whether the arginine in the former tissues is metabolised by a different pathway than that of Rumex tissue. The enzymes such as transamidinase,

Table 7. Enzymes of arginine and glutamate metabolism in normal and tumour tissues of plants cultivated in vitro

Enzymes	Units/g fresh tissue							
	Rumex		Parthenocissus		Tobacco			
	Normal	Tumour	Normal	Crown-gall tumour	Normal	Crown-gall tumour	Normal	Crown-gall tumour
1. Arginase	41.30	61.70	0.05	0.14	0.01	0.01	0.04	
2. Ornithine transcarbamylase	5.71	4.85	1.50	0.71	0.86	0.86	0.37	
3. Ornithine amino transferase	7.00	19.10	0.75	1.15	3.50	3.50	6.65	
4. Glutamic acid → PCA	1.00	2.75	0.00	1.40	0.60	0.60	1.60	
5. Proline → PCA	1.00	2.00	2.40	0.75	0.00	0.00	2.15	
6. Glutamate dehydrogenase	33.20	38.00	-	-	-	-	-	
7. Glutamate decarboxylase	3.66	5.97	0.13	2.80	2.89	2.89	2.17	
8. Aspartate amino transferase	41.60	41.62	2.15	11.08	34.12	34.12	22.00	
9. Alanine amino transferase	22.50	27.68	4.80	6.52	7.68	7.68	0.79	
10. γ-Amino butyric acid amino transferase	2.43	0.79	0.00	0.27	1.18	1.18	0.29	

Table 8. Enzymes of arginine and glutamate metabolism in normal and tumour tissues of plants cultivated in vitro

Enzymes	Specific activity $\times 10^{-3}$							
	Rumex		Parthenocissus		Tobacco			
	Normal	Tumour	Normal	Crown-gall tumour	Normal	Crown-gall tumour	Normal	Crown-gall tumour
1. Arginase	6191.9	10537.12	3.66	39.2	0.60	2.73		
2. Ornithine transcarbamylase	856.0	766.20	110.00	193.5	40.30	20.60		
3. Ornithine aminotransferase	1049.4	3017.40	54.80	313.3	175.80	370.00		
4. Glutamic acid $\rightarrow$ PCA	150.0	442.50	0.00	381.5	28.10	88.80		
5. Proline $\rightarrow$ PCA	149.9	316.00	175.60	204.4	0.00	120.00		
6. Glutamate dehydrogenase	5.0	5.90	-	-	-	-		
7. Glutamate decarboxylase	549.2	946.00	9.80	762.1	135.10	120.70		
8. Aspartate amino transferase	6233.9	6575.00	157.30	3019.1	1600.00	1222.20		
9. Alanine amino transferase	3373.3	4360.20	350.40	1776.6	360.10	43.90		
10. $\gamma$ -Amino butyric acid aminotransferase	364.3	124.3	0.00	73.6	55.30	16.10		

desimidase, decarboxylase or oxidase involved in the breakdown of arginine were tried but could not be detected in any of these tissues. Attempts to detect any guanidino acetic acid or  $\gamma$ -guanidino butyric acid in these tissues by chromatography were also not successful.

Effect of omission of trace elements from the cultivation medium on arginase activity :

The data reported in Table 9 show that omission of trace elements increases arginase activity in all the tissues though the effect was more pronounced in the case of Parthenocissus and tobacco tissues which show very little activity when grown in presence of trace elements compared to that of Rumex tissue.

The data reported in Table 10 show that in Rumex tumour tissue the omission of not only trace elements but Fe-EDTA which is normally used in the cultivation medium causes an increase in arginase activity.

Studies carried out on parthenocissus normal tissue (Table 11) also show that of the various microelements used in the cultivation medium Zn, Cu and Ni are mainly responsible for the decrease in arginase activity.

Presence of inhibitor of Rumex tumour arginase in other tissues:

The possibility that the normal and tumour tissues of Parthenocissus and tobacco have some inhibitory substance was checked by adding the homogenate of the latter two tissues

Table 9. Arginase activity of normal and tumour tissues of Rumex, Parthenocissus and tobacco grown in presence and absence of trace elements

Tissue	Units/g fresh tissue x 10 <sup>-3</sup>		Specific activity x 10 <sup>-3</sup>	
	With trace elements	Without trace elements	With trace elements	Without trace elements
<u>Rumex</u>				
Normal	41300.0	49560.0	6191.9	7436.2
Tumour	61700.0	81400.0	10537.1	12859.4
<u>Parthenocissus</u>				
Normal	50.0	237.5	3.7	17.4
Crown-gall tumour	143.8	306.6	39.2	83.4
<u>Tobacco</u>				
Normal	12.5	87.5	0.6	4.1
Crown-gall tumour	43.8	300.0	2.4	16.7

Table 10. Effect of omission of trace elements and Fe - EDTA from the medium on arginase activity of Rumex tumour tissue

System*	Enzyme activity % on	
	15th day	30th day
Control	100	100
Control - trace elements	122	135
Control - Fe - EDTA	115	132
Control - Fe - EDTA - Trace elements	133	154

\* Control medium contains trace elements and Fe-EDTA  
Five flasks from each group were pooled together  
for analysis on 15th and 30th day of cultivation.

Table 11. Effect of addition of individual metal ions to the medium devoid of microelements on arginase activity of parenthenocissus normal tissue\*\*

Medium	Metal salt added to the medium	Concentration p.p.m.	Enzyme activity %
Control	-	-	43* (100)
Control - microelements	-	-	100 (233)
" "	MnSO <sub>4</sub>	0.50	105 (244)
" "	ZnSO <sub>4</sub>	1.00	27 ( 63)
" "	CuSO <sub>4</sub>	0.03	43 (100)
" "	NiCl <sub>3</sub>	0.03	43 (100)
" "	AlCl <sub>3</sub>	0.03	98 (228)
" "	KI	0.01	86 (200)
" "	FeCl <sub>3</sub>	1.00	86 (200)
" "	H <sub>3</sub> BO <sub>3</sub>	1.00	98 (228)

\* Values are percent of the control - microelements value taken as 100 percent. Values given in the parentheses are the percent values taking control as 100 percent.

\*\* The tissue used for the cultivation in this experiment was grown on medium devoid of microelements for two months.

Five flasks were pooled together for analysis after 2 months of cultivation.

to Rumex tumour tissue homogenate. It was found that the homogenates of these tissues inhibit the arginase activity of Rumex tumour tissue to various degrees. The effect was maximum with Parthenocissus normal tissue. Attempts were, therefore, made to study the nature of the inhibitor present in Parthenocissus normal tissue. The results reported in Table 12 show that both supernatant and residue fractions of homogenate inhibit the activity of Rumex tumour arginase. The inhibitory material in the supernatant was found to be some metal ion. The residual inhibitor could not be solubilized by any of the procedures tried. The inhibitor always remained associated with the residue fraction.

Studies on arginase of tumour tissue of Rumex acetosa :

As the tumour tissue contained only arginase and none of the other enzymes involved in arginine breakdown in tissue, arginase was studied in detail to find whether the characteristics of this enzyme isolated from Rumex tissue are different from that isolated from other sources.

Localization :

Studies reported in Table 13 show that the enzyme activity is localized almost equally in the fractions separating at 480 x g, 5090 x g and supernatant. This suggests the possibility of the enzyme being present in particulate as well as non-particulate fractions.

Table 12. Presence of an inhibitor of Rumex tumour tissue arginase in parthenocissus normal tissue

			: Inhibition : % :
1.	RT-homogenate		-
2.	"	+ PN-homogenate	74
3.	"	+ PN-supernatant	60
4.	"	+ PN-residue	60
-----			
5.	"	+ boiled PN-supernatant	65
6.	"	+ PN-supernatant dialysed against water for 24 hrs.	42
7.	"	+ PN-supernatant dialysed against 0.0001 M EDTA for 72 hrs. and then dialysed against water for 72 hrs.	5
8.	"	+ ashed PN-supernatant	75

RT = Rumex tumour

PN = Parthenocissus normal

Table 13. Localization of arginase activity in Rumex tumour tissue

Fractions	Enzyme activity (units/20 ml homogenate)*	Activity %
Homogenate	79.3	100
480 x g, 10 min.	21.3	26
5090 x g, 20 min.	22.8	28
20000 x g, 30 min.	1.3	2
Supernatant	26.3	33

Medium used for grinding was 0.25 M Sucrose.

\* One unit = 1 micromole of ornithine formed per hour under the assay conditions.

#### Purification of arginase from tumour tissue :

The data reported in Table 14 show that in presence of added  $MnCl_2$  the enzyme could be purified 81 fold without any loss. The slight increase in activity during purification may be due to the removal of inhibitory metal ions from the crude extract. It has been found that if  $MnCl_2$  is not added the enzyme loses 50 percent of the activity. The  $MnCl_2$  thus seems to give a partial protection towards inactivation during purification.

The partially purified enzyme is found to be essentially free from ornithine transaminase and ornithine transcarbamylase activity.

#### Stability of the enzyme on storage :

The enzyme preparation is found to be very unstable. It loses its complete activity in 6-8 hours even when stored at  $0^\circ$ . The enzyme cannot be further stabilized by the addition of  $MnCl_2$  or bovine albumin. Due to the unstability and low protein content of the partially purified fraction further attempts made for purification were not successful and the purifications were carried out in batches. Any batch differing more than  $\pm 15$  percent in specific activity was not used for studies.

#### Effect of pH :

The data reported in Table 15 and Figure 1 show that the pH optima depends on the type of the buffer used. It was not possible to obtain an optimum pH with tris-HCl buffer as the

Table 14. Partial purification of arginase from Rumex tumour tissue

Procedure	Volume (ml)	Total units*	Total Protein (mg)	Specific activity (units/mg protein)	Purification (fold)	Recovery (%)
Homogenate	40	95.0	75.00	1.26	-	100
Supernatant	53	108.6	15.20	7.14	5.6	114
Calcium phosphate gel eluate						
(i) with 0.02 M phosphate buffer pH 7.5	20	8.3	7.85	1.05	0.8	8
(ii) with 0.05 M phosphate buffer pH 7.5	20	114.6	1.12	102.30	81.0	120

\* One unit = 1 micromole of ornithine formed per hour under the assay conditions.

Table 15. Effect of pH on arginase activity

pH	Ornithine formed (micromoles)		
	Tris-HCl buffer	Carbonate-bicarbonate buffer	Glycine-NaOH buffer
7.5	0.00	-	-
8.0	0.14	-	-
8.5	0.41	-	0.01
9.0	0.58	0.07	0.04
9.5	0.66	0.27	0.19
10.0	-	0.94	0.52
10.5	-	1.10	0.70
11.0	-	1.05	0.62
11.5	-	-	0.69
12.0	-	-	0.88
12.5	-	-	1.08

50 micromoles of buffer was used.

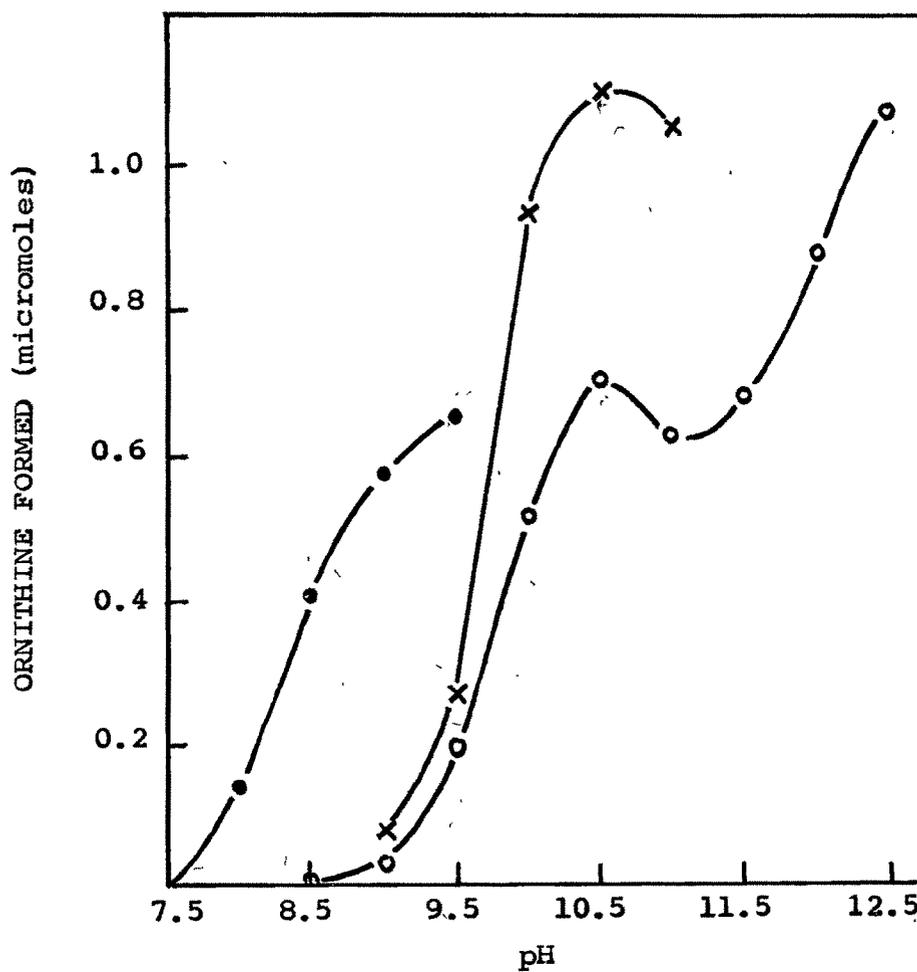


Figure 1. pH activity relationship for arginase activity (-●-), Tris-HCl buffer; (-x-), Carbonate-bicarbonate buffer; (-○-), Glycine - NaOH buffer

activity went on increasing with increase in pH. In the case of carbonate-bicarbonate buffer the optimum pH was found to be 10.5. The activity showed a major peak at 12.5 with a minor peak at 10.5 in the case of glycine-NaOH buffer. The activity also seems to depend on the nature of the buffer at any particular pH. This suggests that the formation of enzyme-substrate complex requires a specific ionic state of the enzyme or substrate which will depend on the pH as well as the nature of the buffer used.

Effect of buffer concentration :

The data reported in Table 16 show that enzyme activity increases upto about 40-50 micromoles of carbonate-bicarbonate buffer at pH 10.5 and decreases with further increase in the concentration of the buffer. At pH 9.5 the activity of the enzyme is only one third at a buffer concentration of 50 micromoles and becomes normal at a concentration of 100 micromoles. Similar observations were made in case of other buffers. This would again indicate that not only the pH and nature of the buffer but also its concentration plays an important role in the formation of enzyme-substrate complex. The decrease of enzyme activity at higher concentration of buffer could be due to the inactivation of the enzyme.

For all the studies reported hereafter carbonate-bicarbonate buffer of pH 10.5 was used at a concentration of 50 micromoles.

Table 16. Effect of buffer concentration on arginase activity

Buffer concentration (micromoles)	Ornithine formed (micromoles)					
	Tris-HCl buffer at pH 9.5	Carbonate-bicarbonate buffer at pH 9.5	Carbonate-bicarbonate buffer at pH 10.5	9.5	10.5	Glycine-NaOH buffer at pH 12.5
10	0.08	0.00	0.38	0.00	0.00	0.00
20	0.17	0.07	0.92	0.00	0.18	0.47
30	0.21	0.18	1.10	0.07	0.45	0.68
40	0.47	0.21	1.20	0.15	0.60	0.96
50	0.60	0.37	1.15	0.20	0.75	1.10
60	0.78	0.69	0.84	0.30	0.85	1.20
80	0.89	0.88	0.64	0.46	0.90	0.85
100	0.80	1.08	0.45	0.54	0.94	0.20
120	0.65	0.90	0.26	0.54	0.92	0.00

Effect of sequence of addition of components of the assay system on enzyme activity :

In order to study further the role of buffer ions, the sequence of addition of components of the assay system was studied. Normally the components are added in the order - buffer,  $MnCl_2$ , substrate and water and reaction started by adding enzyme. However, if the reaction was started by adding buffer (Table 17) the activity was only 38 percent of that obtained when the reaction was started by adding the enzyme. This suggests that eventhough the enzyme and substrate can combine in the absence of buffer it does not form a very active complex. The active complex is perhaps formed from a certain ionic form of either arginine or enzyme in combination with Mn.

In another experiment the two components of the assay system were combined into six possible combinations and the remaining two components were added in the order shown in Table 18. The data reported in the table can be explained by making a tentative model of the enzyme substrate combination with the following assumptions :

- (a) The enzyme molecule has three sites A, B and C and substrate has atleast two binding sites X and Y. (Figure 2a).
- (b) For active enzyme-substrate complex formation site A of the enzyme must combine with site X of substrate through Mn. Site C of the enzyme combines with site Y of the substrate.

Table 17. Effect of sequence of addition of various components of the assay system on arginase activity

Reaction started by the addition of	:	Enzyme activity %
1. Enzyme	:	100
2. Substrate	:	90
3. $MnCl_2$	:	80
4. Buffer	:	38

The components of the assay system are normally added in the following order :

Buffer,  $MnCl_2$ , substrate and water to make up the volume and the reaction started by adding enzyme.

Table 18. Effect of sequence of addition of other components to a combination of two components of the assay system on arginase activity

Sequence of components added					Enzyme activity %	
1	:	2	:	3	Expt. I	Expt. II
1. Buf. - Mn	(a)	Sub.		Enz.	100	100
	(b)	Enz.		Sub.	94	98
2. Buf. - Sub.	(a)	Enz.		Mn.	81	80
	(b)	Mn.		Enz.	91	95
3. Buf. - Enz.	(a)	Mn.		Sub.	83	85
	(b)	Sub.		Mn.	75	75
4. Enz. - Sub.	(a)	Buf.		Mn.	71	70
	(b)	Mn.		Buf.	49	40
5. Enz. - Mn.	(a)	Sub.		Buf.	39	40
	(b)	Buf.		Sub.	76	80
6. Sub. - Mn.	(a)	Enz.		Buf.	42	38
	(b)	Buf.		Enz.	86	89

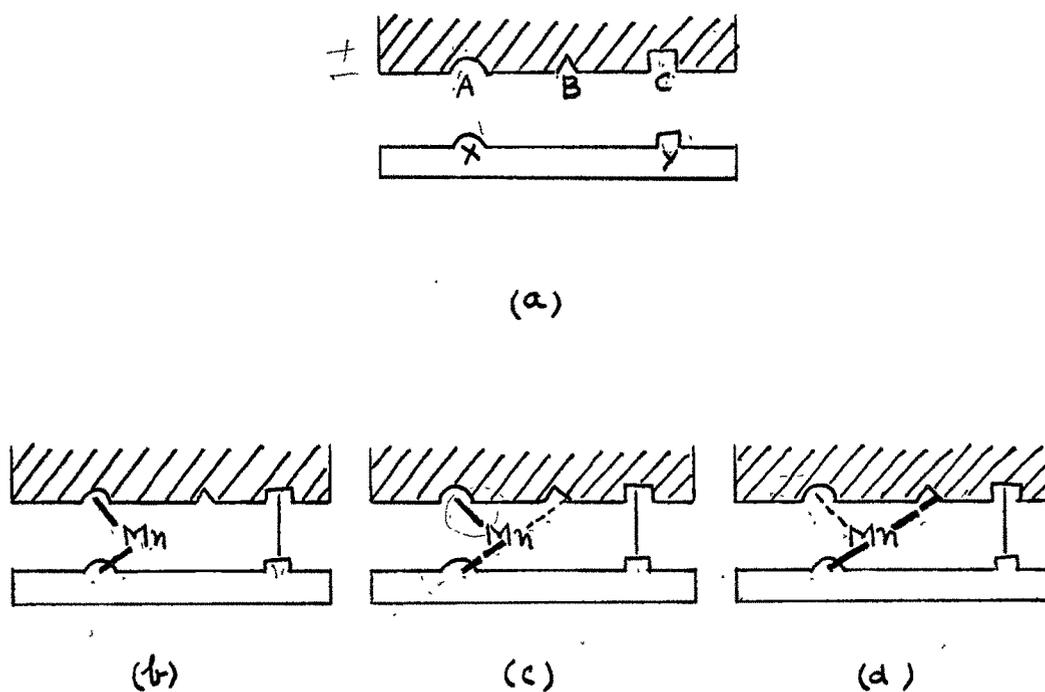


Figure 2. Plausible model for the enzyme-substrate complex formation through Mn

- (c) The Mn can combine with sites A, B or X. Site A of the enzyme can combine with Mn only when it is in a certain ionic form, the ionization being brought about by the buffer. The affinity of Mn is more for site A in the ionized form and for site X than for site B.
- (d) Site C of the enzyme when not in combination with site Y of the substrate keeps site B masked in such a way that B cannot combine with Mn. However, When site C combines with site Y, B becomes available for combination with Mn.

Thus from these results and assumptions three types of combinations can be visualised as given in Figure 2(b,c,d).

- (i) Fully active complex giving 90-100 percent activity.
- (ii) Partially active complex giving 70-85 percent activity, the extent varying with the sequence of additions.
- (iii) Ineffective complex giving 40 percent activity.

The site A of the enzyme seems to be the active site and buffer seems to play an important role in the formation of active enzyme-substrate complex through Mn.

Effect of enzyme concentration and period of incubation :

The enzyme activity was found to increase proportionately upto 0.2 ml enzyme concentration (Table 19 and Figure 3) and a period of incubation of 60 minutes (Table 20 and Figure 4).

Effect of substrate concentration :

The data reported in Table 21 and Figure 5 show that

Table 19. Effect of enzyme concentration on arginase activity

Enzyme * (ml)	Ornithine formed (micromoles)
0.05	0.27
0.10	0.55
0.15	0.82
0.20	1.06
0.25	1.21
0.30	1.34
0.35	1.52
0.40	1.63

\* 0.052 mg protein/ml.

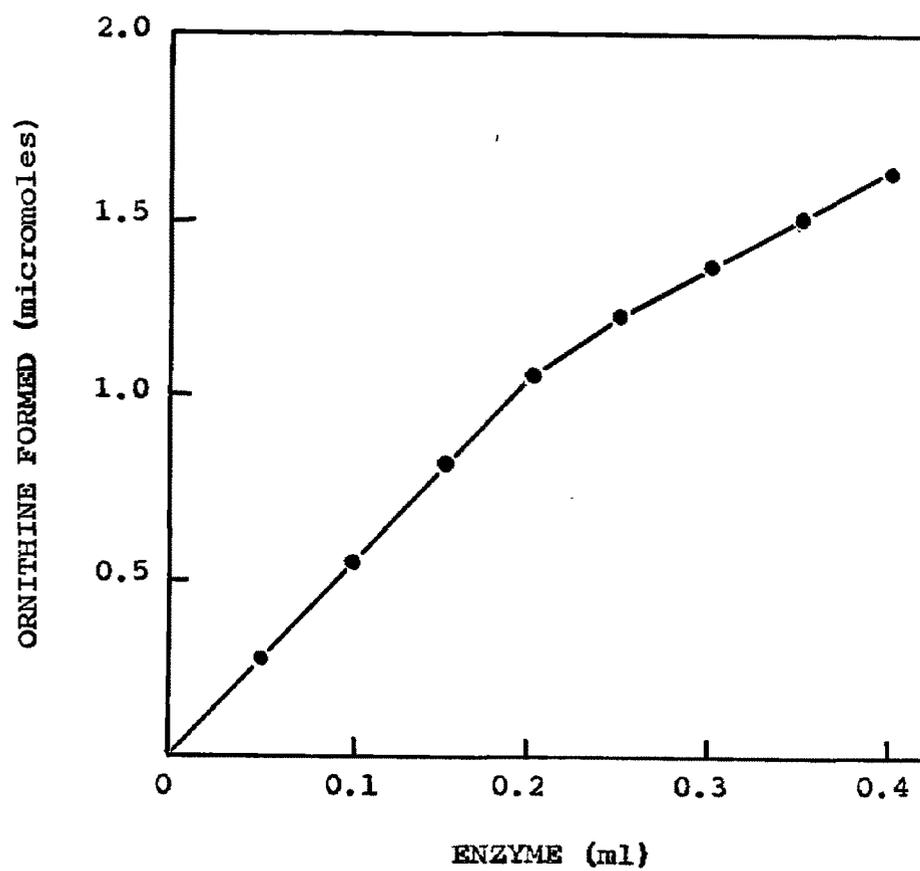


Figure 3. Effect of enzyme concentration on arginase activity.

Table 20. Effect of period of incubation on arginase activity

Period of incubation (minutes)	Ornithine formed (micromoles)
15	0.33
30	0.61
45	0.90
60	1.23
75	1.32
90	1.42

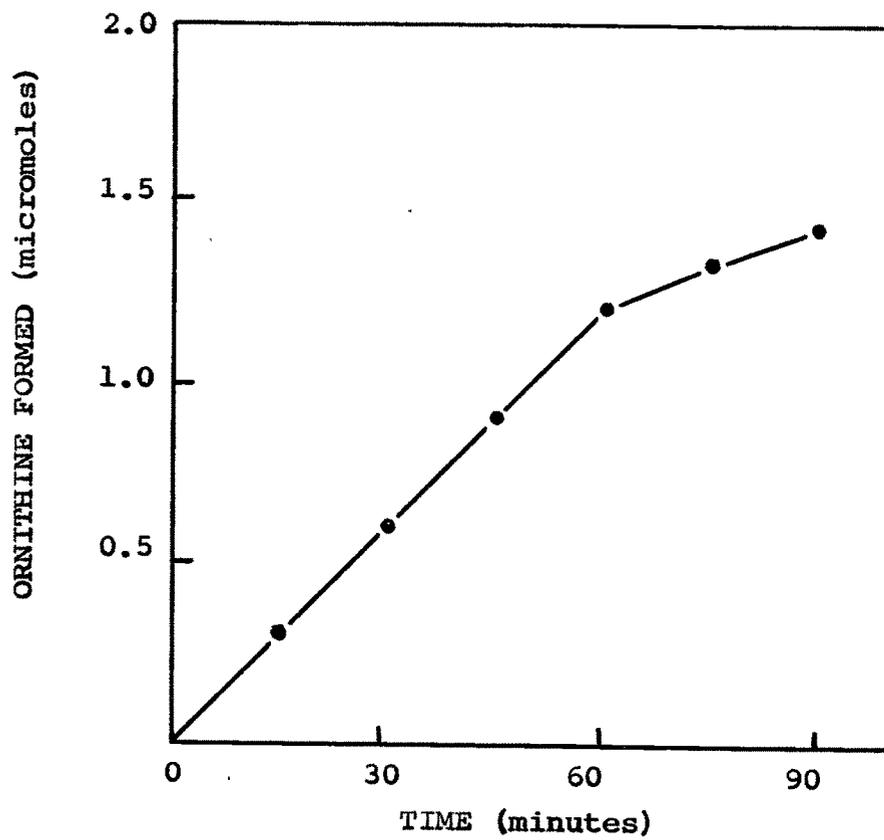


Figure 4. Effect of period of incubation on arginase activity

Table 21. Effect of substrate concentration on arginase activity

Arginine added (micromoles)	Ornithine formed (micromoles)
1	0.07
2	0.10
3	0.16
4	0.25
5	0.30
6	0.36
7	0.43
8	0.51
9	0.60
10	0.70
11	0.77
12	0.86
13	0.95
14	1.02
15	1.09
16	1.12
17	1.18
18	1.22
19	1.24
20	1.26

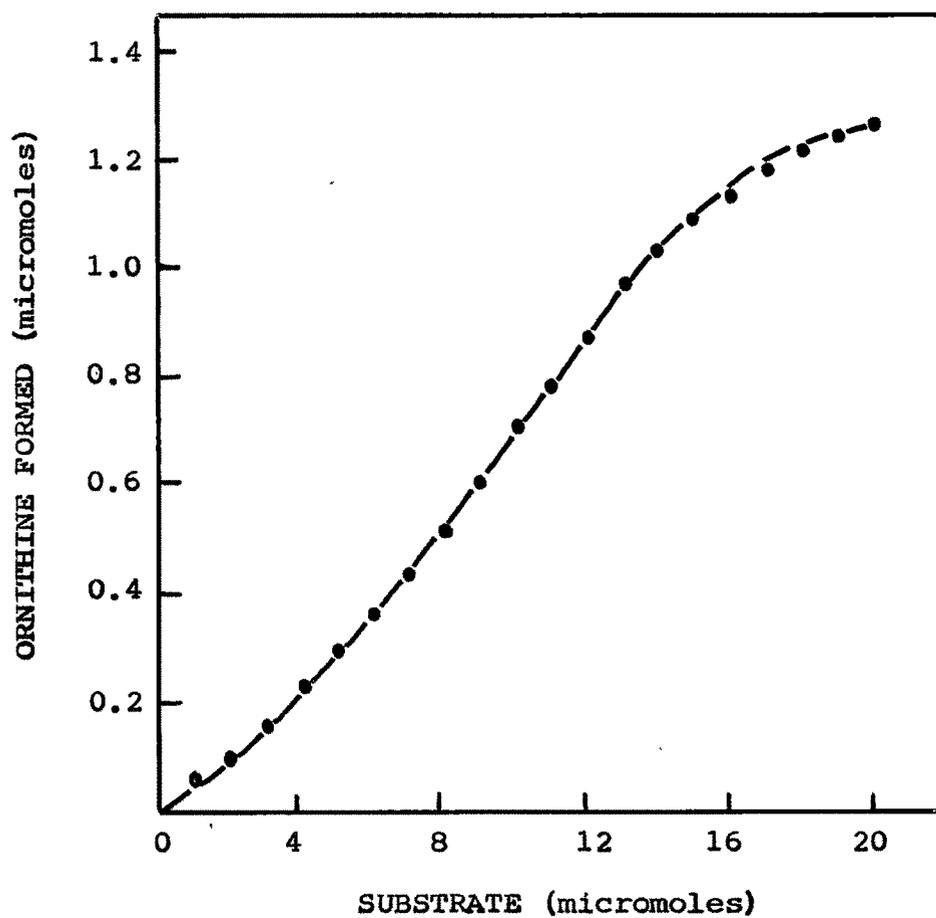


Figure 5. Effect of substrate concentration on arginase activity

substrate concentration curve is sigmoidal or 'S' shaped. The double reciprocal Lineweaver - Burk plot did not give a straight line and thus the  $K_m$  was not calculated.

Substrate specificity :

The enzyme was found to act on l-arginine but not on canavanine, p-tosyl arginine methyl ester,  $\gamma$ -guanidino-butyric acid, guanidino acetic acid, agmatine and putamine.

Effect of  $MnCl_2$  :

The enzyme activity was found to increase with increase in concentration of  $MnCl_2$  in the assay system (Table 22 and Figure 6) showing the absolute requirement of Mn for the enzyme activity. It also seems to protect the enzyme from inactivation since the enzyme purified in absence of added  $MnCl_2$  showed only 60 percent activity.

Effect of temperature of incubation :

The data reported in Table 23 and Figure 7 show that the activity increases with increase in temperature upto  $40^\circ$  and then decreases giving a characteristic bell shaped curve. The arrhenius plot (Figure 8) gave a discontinuity of slope and approximates to two straight lines meeting at an angle. The energy of activation was found to be 6585 and 1464 calories/mole with a transition temperature of  $23.5^\circ$  at pH 10.5

Table 22. Effect of  $\text{MnCl}_2$  concentration on the activity of arginase purified with and without addition of  $\text{MnCl}_2$

MnCl <sub>2</sub> added (micromoles)	Ornithine formed (micromoles) in the case of enzyme purified	
	without Mn	with Mn
0	0.00	0.15
1	0.13	0.25
2	0.23	0.38
3	0.31	0.50
4	0.42	0.68
5	0.45	0.78
6	0.49	0.84
7	0.57	0.95
8	0.61	0.98
9	0.63	1.08
10	0.66	1.10

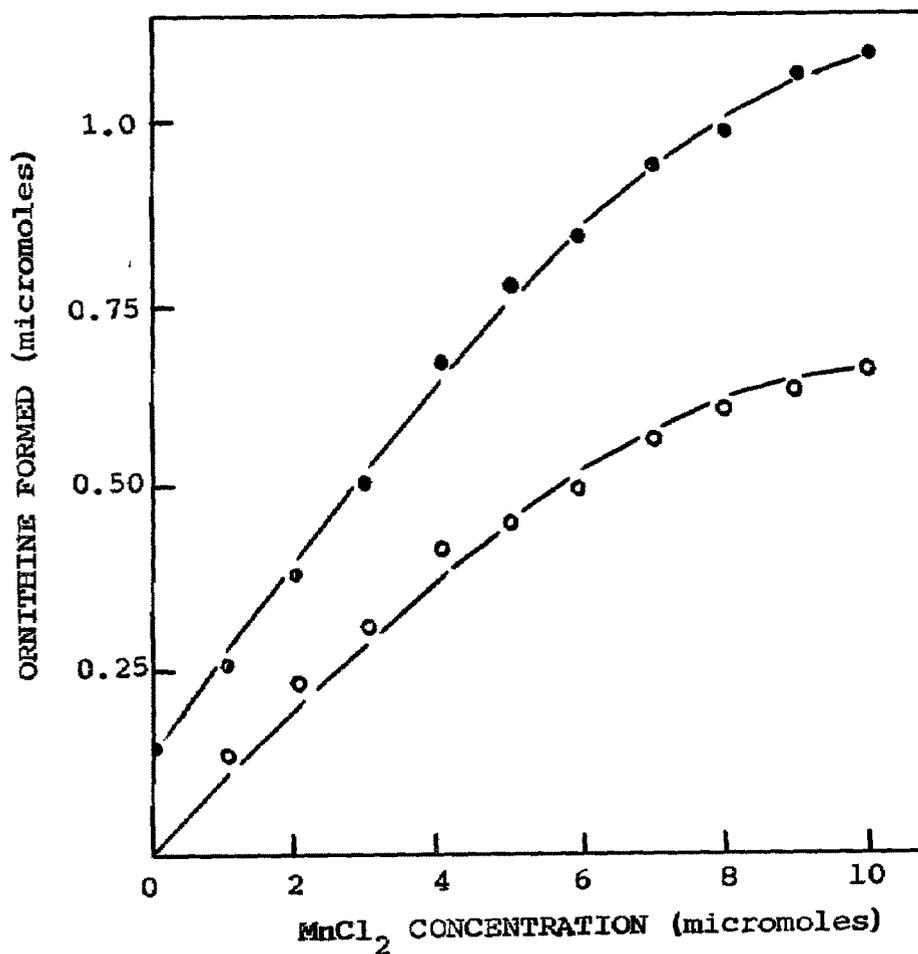


Figure 6. Effect of  $\text{MnCl}_2$  concentration on arginase activity  
(-o-), enzyme purified without addition of  $\text{MnCl}_2$   
(-●-), enzyme purified with addition of  $\text{MnCl}_2$

Table 23. Effect of temperature of incubation on arginase activity

Temperature of incubation	:	Ornithine formed (micromoles)
0	:	0.38
10	:	0.57
20	:	0.87
30	:	1.05
37	:	1.09
40	:	1.11
50	:	1.09
60	:	0.33
70	:	0.11

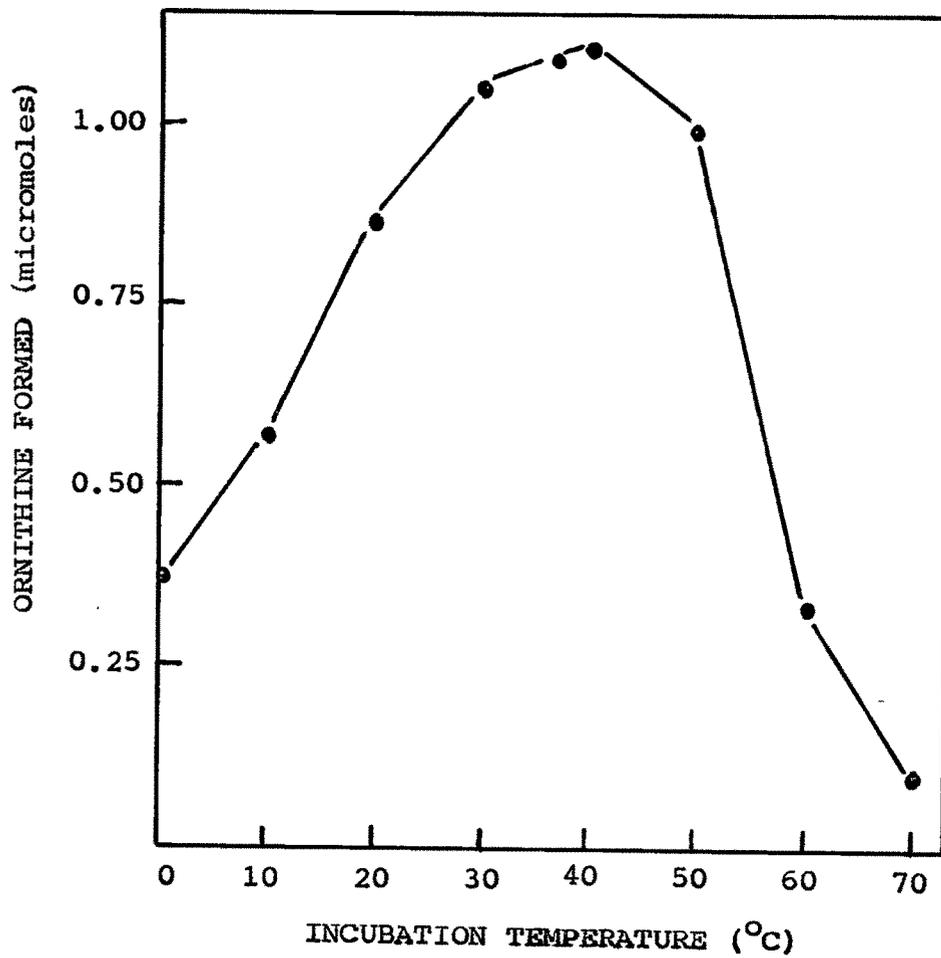


Figure 7. Effect of temperature of incubation on arginase activity

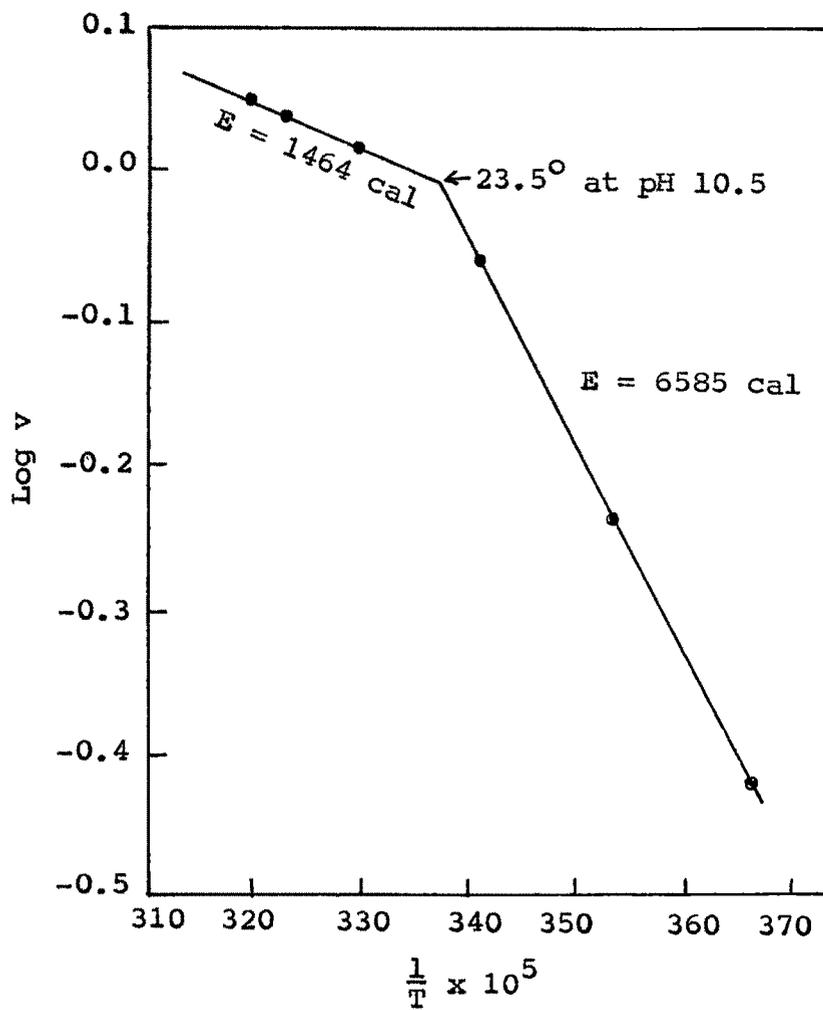


Figure 8. Arrhenius plot of arginase activity

#### Effect of heat inactivation :

The data reported in Table 24 and Figure 9 show that if the enzyme purified with and without  $MnCl_2$  was kept at different temperatures for 10 minutes, cooled and then assayed at  $37^\circ$ , the enzyme purified in presence of  $MnCl_2$  was quite stable upto  $50^\circ$  whereas the enzyme purified in the absence of  $MnCl_2$  lost 50 percent of the activity at this temperature.

#### Effect of other metal ions :

It can be seen from Table 25 that without addition of Mn ions there was no activity and none of the other metals could replace Mn. However, in presence of Mn ions Cu, Ni, Co, Zn and Hg ions were found to be inhibitory. Fe and Ca also show slight inhibition.

The data reported in Table 26 and 27 show the effect of Cu, Zn, Ni and Co ion concentrations on the enzyme activity. Almost all the activity was lost at a concentration of 0.06 micromoles of Cu and Zn. The inhibition with Ni and Co ions was less compared to Cu and Zn ions and even at a concentration of 10 micromoles there was some activity.

#### Effect of amino acids and their derivatives :

The data reported in Table 28 show that ornithine, lysine, agmatine,  $\gamma$ -guanidino butyric acid and guanidino acetic acid which have a structure similar to that of

Table 24. Effect of heat inactivation on arginase activity

Temperature of inactivation	: Ornithine formed		: Residual enzyme activity	
	:(micromoles) in case		%	
	: of enzyme purified		: in case of enzyme purified	
	: With Mn	: Without Mn:	With Mn	: Without Mn
0	0.98	0.60	100	59 (100)*
40	0.96	0.39	98	40 ( 65)
45	0.94	0.35	96	35 ( 58)
50	0.86	0.32	87	32 ( 53)
55	0.78	0.27	80	27 ( 45)
60	0.55	0.15	56	15 ( 25)
65	0.40	0.14	45	14 ( 23)
70	0.20	0.02	25	2 ( 3)
75	0.10	0.02	11	2 ( 3)
80	0.00	0.00	0	0 ( 0)

The enzyme was kept at various temperatures for 10 min., cooled and assayed at 37°.

\* The value of without Mn enzyme at 0° is taken as 100%.

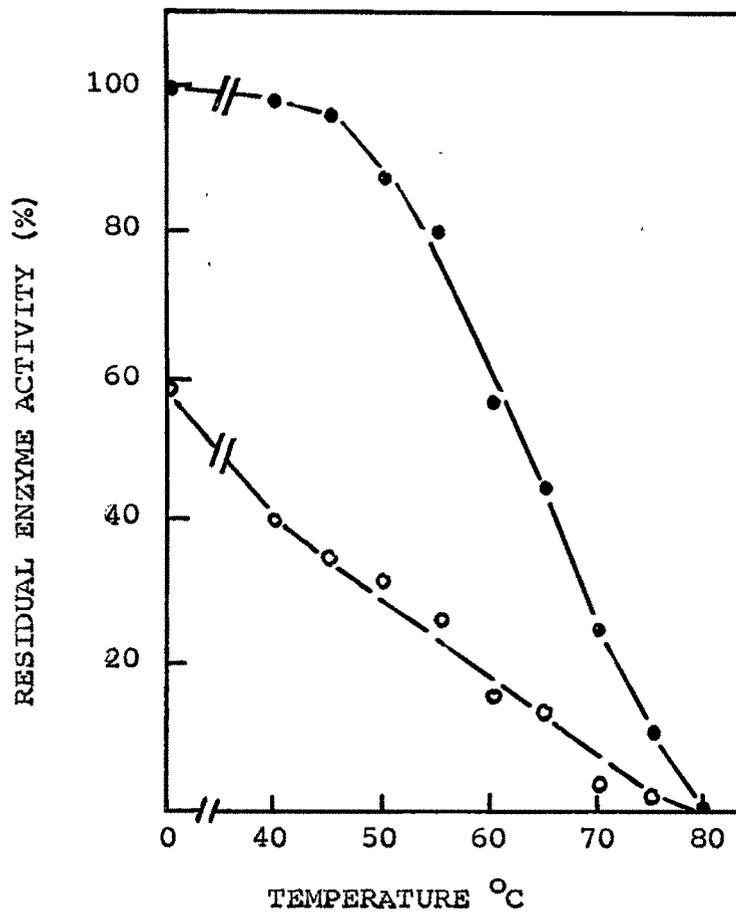


Figure 9. Effect of heat inactivation on arginase activity  
(—●—), enzyme purified with addition of  $\text{MnCl}_2$ ;  
(—○—), enzyme purified without addition of  $\text{MnCl}_2$

Table 25. Effect of various metal ions on arginase activity in presence and absence of  $Mn^{++}$  ions\*

Metal salt added	Enzyme activity %	
	Without $Mn^{++}$	With $Mn^{++}$
-	0	100
$CuCl_2$	0	0
$NiCl_2$	0	12
$CaCl_2$	0	72
$FeCl_3$	0	69
$HgCl_2$	0	0
$ZnCl_2$	0	0
$Na_2MoO_4$	0	84
$CoCl_2$	0	23
$MgCl_2$	0	88
$AlCl_3$	0	100

\* 10 micromoles of metal ions were used.

The enzyme used for this experiment was purified without the addition of  $MnCl_2$ .

Table 26. Effect of  $\text{Cu}^{++}$  and  $\text{Zn}^{++}$  concentration on arginase activity in presence of  $\text{Mn}^{++}$  ions\*

Concentration (micromoles)	Enzyme activity%	
	With $\text{Cu}^{++}$	With $\text{Zn}^{++}$
0.00	100	100
0.01	34	45
0.02	25	26
0.03	18	15
0.04	10	12
0.05	9	7
0.06	4	0
0.07	0	0
0.08	0	0

\* 10 micromoles of  $\text{MnCl}_2$  was used.

Table 27. Effect of  $\text{Ni}^{+++}$  and  $\text{Co}^{++}$  concentration on arginase activity in presence of  $\text{Mn}^{++}$  ions\*

Concentration (micromoles)	Enzyme activity %	
	With $\text{Ni}^{+++}$	With $\text{Co}^{++}$
0.00	100	100
1.25	60	53
2.50	47	50
3.75	43	45
5.00	35	39
6.25	28	37
7.50	24	29
8.75	19	26
10.00	10	20

\*10 Micromoles of  $\text{MnCl}_2$  was used.

Table 28. Effect of various amino acids and derivatives  
on arginase activity

Additions	Concentration (micromoles)	Enzyme activity %
-	-	100
L - Aspartic acid	10	103
L - Glutamic acid	10	103
L - Asparagine	10	103
L - Glutamine	10	121
	20	130
DL - Alanine	10	101
Glycine	10	103
DL - Serine	10	103
L - Tryptophan	10	94
L - Tyrosine	10	97
L - Leucine	10	99
DL - Isoleucine	10	91
DL - Phenylalanine	10	103
L - Valine	10	105
DL - Methionine	10	105
L - Cystine	10	84
DL - Threonine	10	103

continued..

Table 28 (continued)

Additions	Concentration (micromoles)	Enzyme activity %
DL - Histidine	10	118
γ - Aminobutyric acid	10	101
L - Proline	10	107*
L - Hydroxyproline	10	113*
L - Lysine	10	4*
DL - Ornithine	10	66*
Putrescine	10	105
Agmatine	10	71
γ-Guanidino butyric acid	5 10	60 11
Guanidino acetic acid	5 10	38 8
2 - Oxoglutaric acid	10	108
Pyruvic acid	10	117
Glyoxalic acid	10	105

\* Urea was estimated due to the interference in ornithine estimation by these compounds.

arginine inhibit enzyme activity significantly whereas glutamine, histidine, hydroxyproline and pyruvate show some activation.

Effect of various compounds as inhibitors :

The data reported in Table 29 show that EDTA, iodoacetate and p-CMB inhibit the enzyme activity significantly. Ascorbic acid shows some inhibition while glutathione shows some activation. The inhibition by p-CMB could be reversed by the addition of cysteine showing the presence of -SH groups in the enzyme.

Effect of Drugs :

Addition of terramycin, aureomycin, chloramphenicol, sulphaguanidine and phenobarbitone to the assay system was found to inhibit the enzyme activity completely (Table 30). Streptomycin also causes slight inhibition but penicillin had no effect.

Effect of purines, pyrimidines and their derivatives :

The data reported in Table 31 show that all the purines and pyrimidines tested except hypoxanthine, allantoin and orotic acid inhibit enzyme activity significantly. Inhibition with adenine and thymine was less compared to others. All the derivatives of purines except guanosine and 2-acetylamino-6-hydroxy purine inhibited the enzyme.

Table 29. Effect of various compounds as inhibitors of arginase activity

Compound added	Concentration (micromoles)	Enzyme activity %
-	-	100
NaN <sub>3</sub>	10	91
NaF	10	97
Na <sub>3</sub> AsO <sub>4</sub>	10	95
EDTA	2.5	106
	5.0	101
	7.5	69
	10.0	43
Iodoacetate	5	69
	10	32
p-CMB	0.25	12
	0.50	10
	0.75	8
	1.00	5
Ascorbic acid	5	63
	10	69
Glutathione	5	121
	10	118
Cysteine	10	100
Cysteine + p-CMB	10 + 0.25	108
Cysteine + p-CMB	10 + 0.50	104
Urea	25	108
	50	108
	75	92
	100	92

Table 30. Effect of various drugs on arginase activity

Addition	Concentration (mgs)	Enzyme activity %
-	-	100
Procaine penicillin	5	102
	10	105
Penicillin G.Sodium	5	100
	10	117
Terramycin	5	0
	10	0
Streptomycin sulphate	5	89
	10	78
Aureomycin	5	0
	10	0
Chloramphenicol	5	0
	10	0
Sulphaguanidine	5	0
	10	0
Phenobarbitone	5	0
	10	0

Table 31. Effect of various purines and pyrimidines and their derivatives on arginase activity

Compound added	Concentration (micromoles)	Enzyme activity %
-	-	100
Adenine	10	84
Guanine	10	33
Hypoxanthine	10	91
Xanthine	10	31
Uric acid	10	37
Allantoin	10	100
Uracil	10	29
Thymine	10	60
Cytosine	10	53
Orotic acid	5	110
	10	111
2-Thio-xanthine	5	13
	10	8
6-Thio-xanthine	5	35
	10	32
2,6-Dithio-xanthine	5	24
	10	21
6-Hydrazinopurine	5	41
	10	40
2-Amino-6-mercaptapurine	5	21
	10	19
2-Acetylamino-6-hydroxypurine	5	112
	10	106
Guanosine	10	97
AMP	10	100
ADP	10	100
ATP	10	100
RNA	5	66
	10	50
DNA	5	50
	10	32

RNA and DNA were also found to inhibit though AMP, ADP and ATP did not.

It is interesting to note that guanine inhibits whereas guanosine which has ribose in the 9th position or acetylated guanine in which the amino group of guanine in 2nd position is changed into acetyl amino group or hypoxanthine which has no substituent group in the 2nd position or allantoin which has no ring system do not inhibit. Also uracil inhibits whereas orotic acid which has a carboxyl group in the 4th position does not. These studies suggest that a ring system and a polar group in the 2nd position is essential for the inhibition.

Kinetics of the inhibition by agmatine, ornithine, lysine, guanidino acetic acid and  $\gamma$ -guanidino butyric acid :

As reported earlier these compounds were found to inhibit the enzyme activity significantly. It can be seen that all these compounds resemble arginine in structure and they might thus be inhibiting competitively. In order to confirm the competitive nature of these inhibitors agmatine, ornithine, lysine and guanidino acetic acid were chosen for further studies.

The results reported in Tables 32, 33, 34 and 35 show the effect of concentration of inhibitors on enzyme activity.

Table 32. Effect of agmatine concentration on the inhibition of arginase activity

Inhibitor concentration (micro-moles)	Ornithine formed (micromoles) when substrate added was		Inhibition % when substrate added was	
	5 micro-moles	15 micro-moles	5 micro-moles	15 micro-moles
0	0.39	1.18	0	0
2	0.39	1.14	0	4
4	0.34	1.10	14	7
6	0.29	1.09	26	8
8	0.27	1.08	32	9
10	0.26	1.06	33	10
12	0.25	0.99	36	17
14	0.23	0.96	42	18
16	0.20	0.94	49	20
18	0.19	0.93	51	22
20	0.18	0.91	54	23

Table 33. Effect of ornithine concentration on the inhibition of arginase activity

Inhibitor concentra- tion (micro- moles)	Urea formed (micromoles) when substrate added was		Inhibition % when substrate added was	
	5 micro- moles	15 micro- moles	5 micro- moles	15 micro- moles
0	0.387	1.149	0	0
2	0.182	1.113	53	3
4	0.121	1.041	69	9
6	0.100	1.016	74	12
8	0.075	0.968	81	16
10	0.067	0.811	83	29
12	0.059	0.641	85	44
14	0.048	0.557	88	52
16	0.045	0.484	88	59
18	0.036	0.448	91	61
20	0.024	0.424	94	63

Table 34. Effect of lysine concentration on the inhibition of arginase activity

Inhibitor concentra- tion (micro- moles)	Urea formed (micromoles) when substrate added was		Inhibition % when substrate added was	
	5 micro- moles	15 micro- moles	5 micro- moles	15 micro- moles
0	0.387	1.149	0	0
2	0.073	0.363	81	68
4	0.048	0.182	88	83
6	0.036	0.109	91	90
8	0.028	0.085	93	83
10	0	0	100	100
12	0	0	100	100

Table 35. Effect of guanidino acetic acid concentration on the inhibition of arginase activity

Inhibitor concentration (micro- moles	Ornithine formed (micromoles) when substrate added was		Inhibition % when substrate added was	
	5 micro- moles	15 micro- moles	5 micro- moles	15 micro- moles
0.0	0.48	1.09	-	-
0.5	0.48	1.08	0	1
1.0	0.37	1.10	33	0
1.5	0.27	0.99	44	10
2.0	0.21	0.97	57	11
2.5	0.18	0.84	63	23
3.0	0.15	0.74	69	32
3.5	0.13	0.70	73	36
4.0	0.12	0.58	75	46

Guanidino acetic acid and lysine were found to be highly inhibitory followed by ornithine and agmatine. The inhibition was also found to increase by increase in inhibitor concentration. When the data of reciprocals of velocity were plotted against inhibitor concentration (Dixon, 1953), the Dixon plots (Figures 10, 11, 12 and 13) gave a  $K_i$  value of 10, 2.8, 0.9 and 0.2 micromoles respectively for agmatine, ornithine, lysine and guanidino acetic acid.

Studies were also made using two concentrations of inhibitors at various arginine concentrations. The results reported in Tables 36, 37, 38 and 39 show that inhibition was more at lower substrate concentrations and it could be reversed by adding more substrate indicating the competitive nature of inhibition. The double reciprocal plots (Figures 14, 15, 16 and 17) of velocity against substrate concentration did not follow the normal Michaelis-Menten pattern but showed an upward curvature at low substrate concentrations, a characteristic of allosteric proteins. (Monod, Wyman and Changeux, 1965).

The competitive nature of these inhibitors was confirmed by calculating the values of Tables 36, 37, 38 and 39 as  $i \frac{V_i}{V - V_i}$  against substrate concentration according to the method of Hunter and Downs (1945) where -

- i = inhibitor concentration
- V = velocity in the absence of inhibitor
- $V_i$  = Velocity in the presence of inhibitor

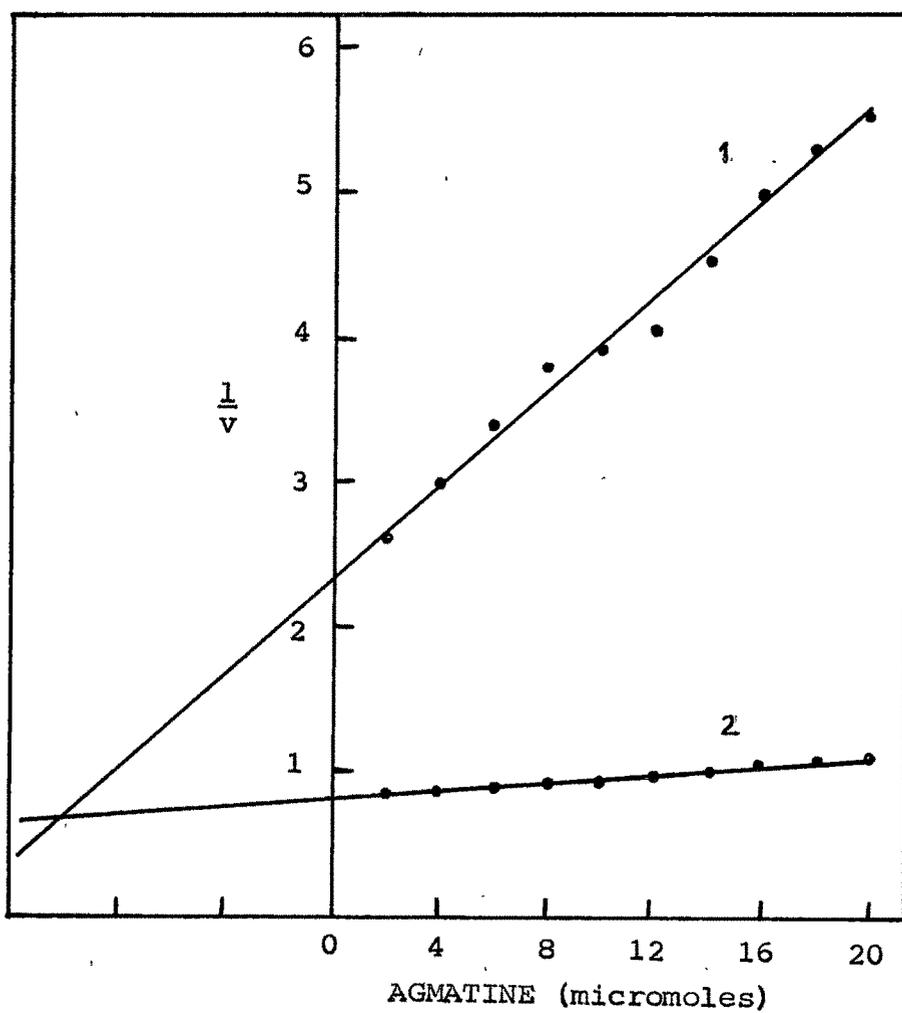


Figure 10. Dixon plot for the inhibition of arginase by agmatine ; 1 = 5 micromoles of substrate; 2 = 15 micromoles of substrate

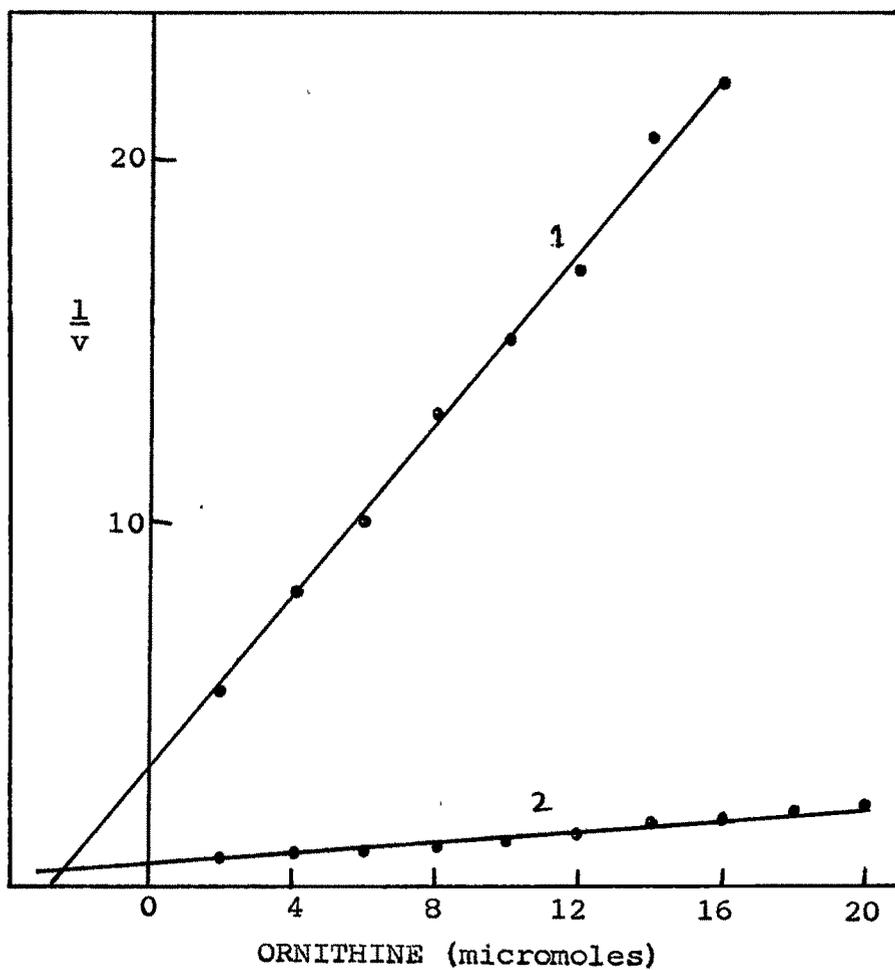


Figure 11. Dixon plot for the inhibition of arginase by ornithine; 1=5 micromoles of substrate; 2=15 micromoles of substrate

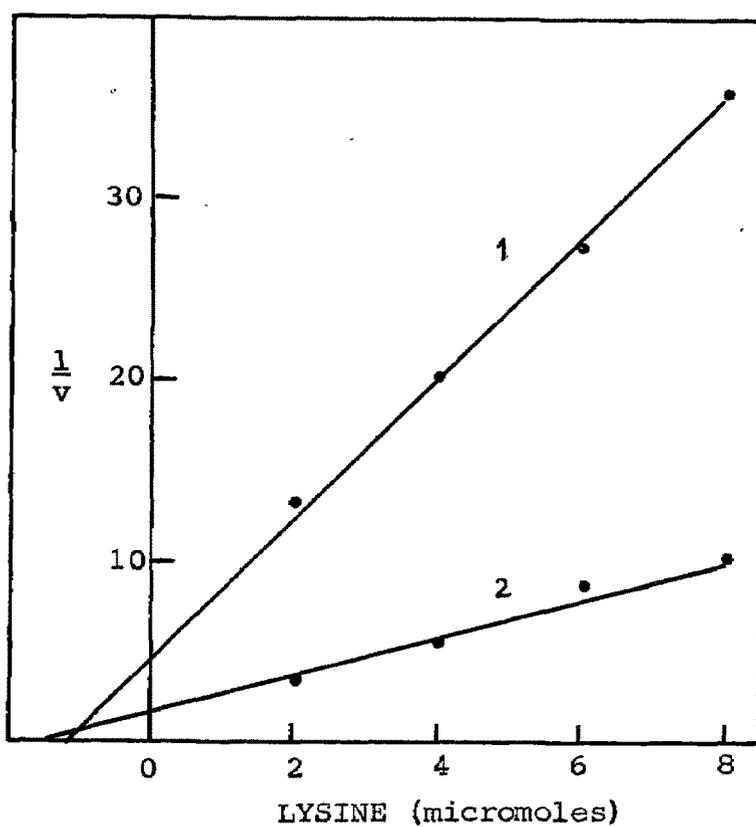


Figure 12. Dixon plot for the inhibition of arginase by lysine;  
1=5 micromoles of substrate;  
2=15 micromoles of substrate

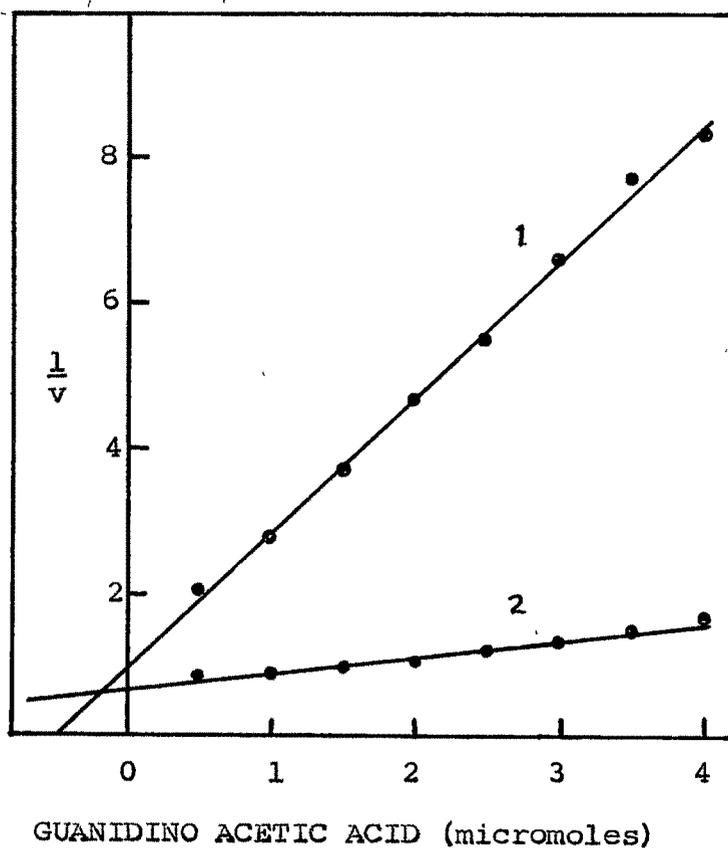


Figure 13. Dixon plot for the inhibition of arginase activity by guanidino acetic acid; 1=5 micromoles of substrate; 2=15 micromoles of substrate

Table 36. Effect of substrate concentration on the inhibition of arginase activity by agmatine

Substrate concentration (micromoles)	Ornithine formed (micromoles)		
	Control	with agmatine (micromoles)	
		5	10
2	0.130	0.080	0.056
4	0.270	0.180	0.125
6	0.370	0.255	0.180
8	0.490	0.350	0.255
10	0.610	0.455	0.340
12	0.770	0.580	0.440
14	0.900	0.680	0.540
16	1.020	0.800	0.640
18	1.180	0.930	0.750
20	1.250	1.010	0.815

Table 37. Effect of substrate concentration on the inhibition of arginase activity by ornithine

Substrate concentration (micromoles)	Urea formed (micromoles)		
	Control	with ornithine (micromoles)	
		2	4
2	0.109	0	0
4	0.254	0	0
6	0.339	0.221	0.133
8	0.436	0.330	0.218
10	0.557	0.460	0.290
12	0.678	0.569	0.420
14	0.811	0.699	0.600
16	0.944	0.830	0.724
18	1.041	0.928	0.824
20	1.210	1.089	0.944

Table 38. Effect of substrate concentration on the inhibition of arginase activity by lysine

Substrate concentration (micromoles)	Urea formed (micromoles)		
	Control	with lysine (micromoles)	
		2	4
2	0.109	0	0
4	0.254	0	0
6	0.339	0	0
8	0.436	0.123	0.070
10	0.557	0.194	0.105
12	0.678	0.303	0.157
14	0.811	0.389	0.213
16	0.944	0.520	0.303
18	1.041	0.601	0.351
20	1.210	0.702	0.436

Table 39. Effect of substrate concentration on the inhibition of arginase activity by guanidino acetic acid

Substrate concentration (micromoles)	Ornithine formed (micromoles)		
	Control	with guanidino acetic acid (micromoles)	
		1	2
2	0.19	0.12	0.07
4	0.38	0.26	0.16
6	0.51	0.38	0.22
8	0.71	0.58	0.42
10	0.81	0.69	0.53
12	0.93	0.82	0.68
14	1.04	0.94	0.78
16	1.12	1.02	0.90
18	1.11	1.11	1.00
20	1.25	1.13	1.15

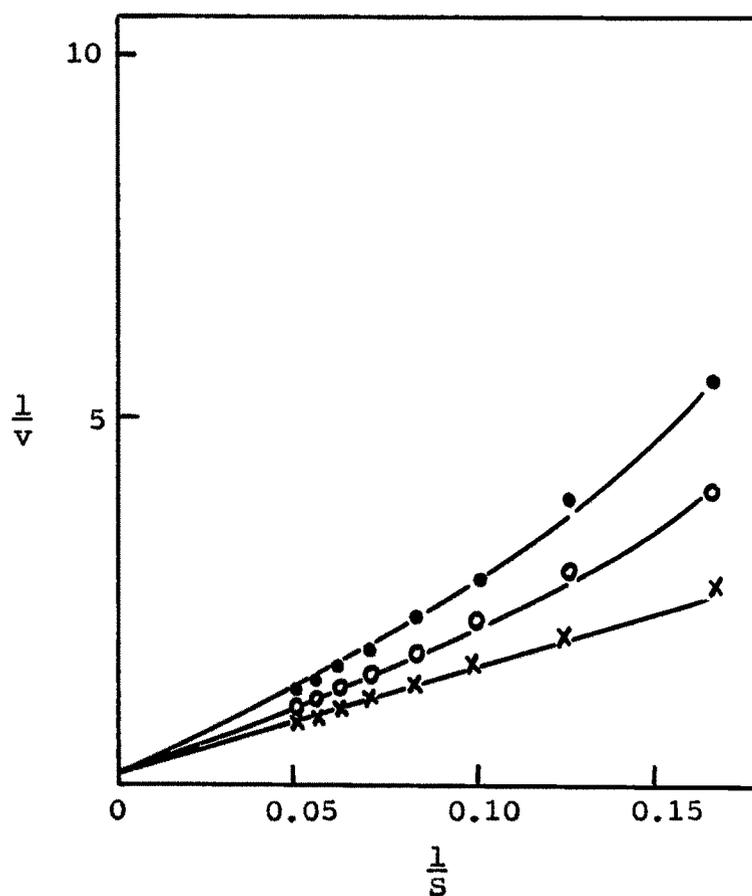


Figure 14. Double reciprocal plots of activity against arginine concentration in the presence of two fixed levels of agmatine  
(-x-), without agmatine;  
(-o-), with 5 micromoles of agmatine  
(-●-), with 10 micromoles of agmatine

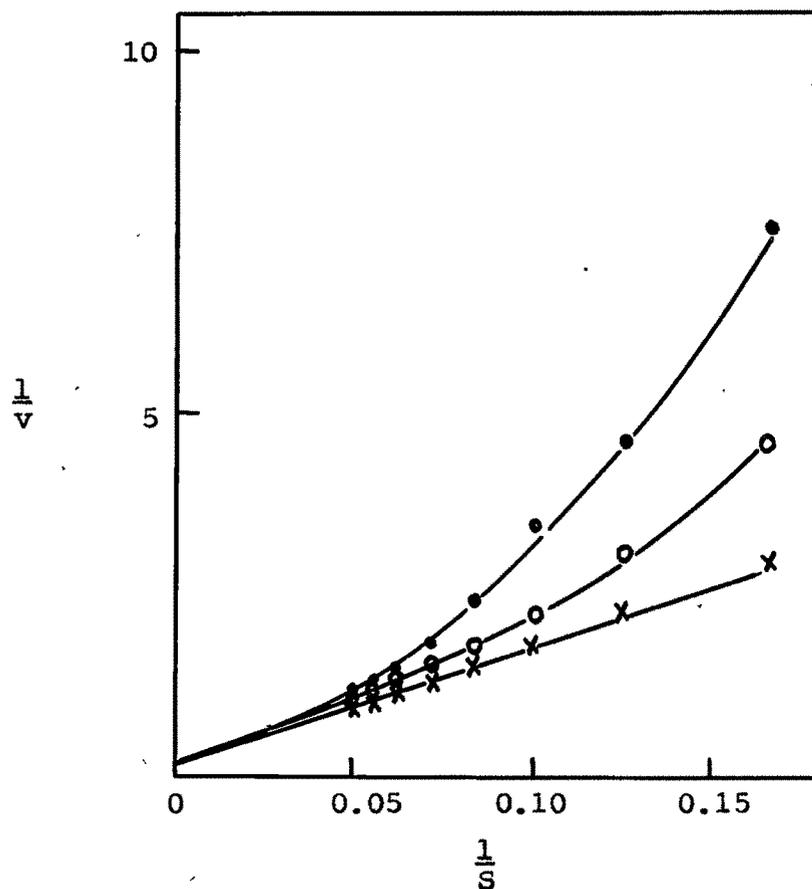


Figure 15. Double reciprocal plots of activity against arginine concentration at two fixed levels of ornithine.

(—x—), without ornithine;

(—o—), with 2 micromoles of ornithine

(—•—), with 4 micromoles of ornithine

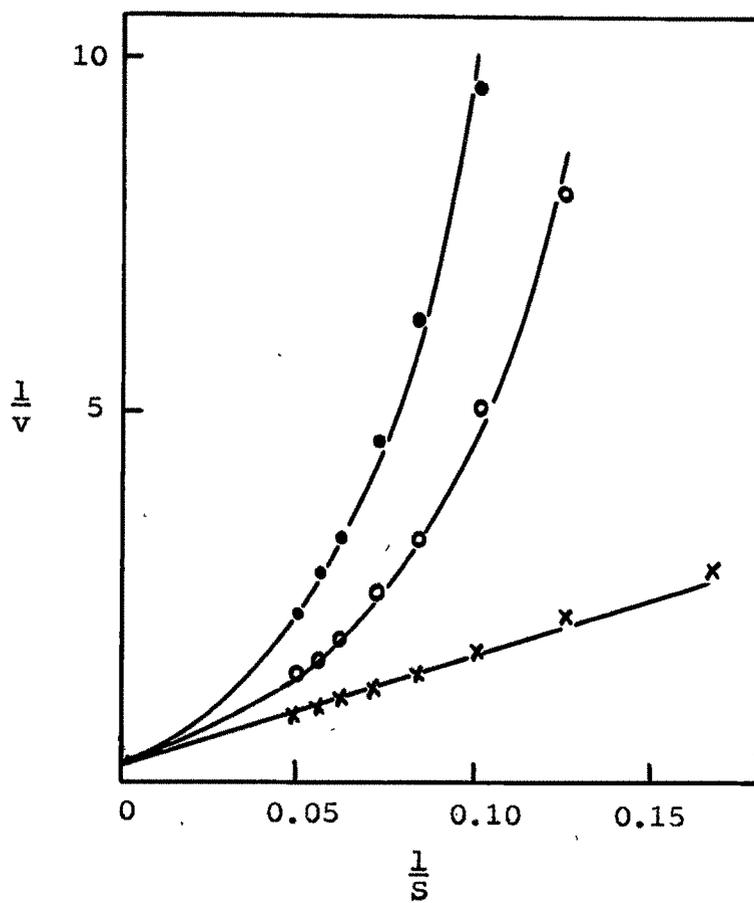


Figure 16. Double reciprocal plots of activity against arginine concentration at two fixed levels of lysine  
 (-x-), without lysine  
 (-o-), with 2 micromoles of lysine  
 (-●-), with 4 micromoles of lysine

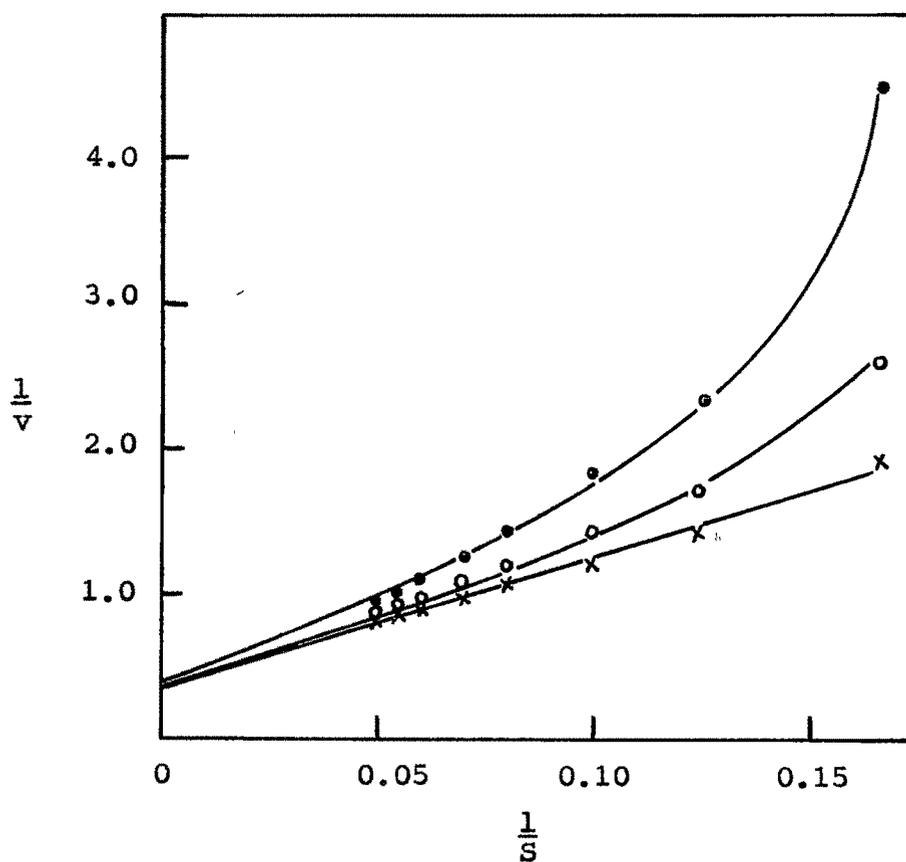


Figure 17. Double reciprocal plots of activity against arginine concentration at two fixed levels of guanidino acetic acid (GAA)

(-x-), without GAA

(-o-), with 1 micromole of GAA

(-●-), with 2 micromoles of GAA

The values given in Table 40 and Figures 18,19,20 and 21 show a dependence on substrate concentration confirming the competitive nature of these inhibitors.

The values of Tables 36, 37, 38 and 39 were also calculated according to Hill reaction ( $\log \frac{V}{V_{\max}-V} = n \log(S) - \log K$ ) and when  $\log \frac{V}{V_{\max}-V}$  was plotted against  $\log(S)$  the slope of the line (Figures 22,23,24 and 25) was found to be significantly increased in presence of inhibitors showing a negative cooperative effect of these compounds and involvement of more than one binding site on the enzyme molecule.

#### Kinetics of the inhibition by xanthine, guanine and uracil :

The data reported in Tables 41, 42 and 43 show that all the three compounds tested inhibited the enzyme activity and the inhibition increases with increase in inhibitor concentration. The  $K_i$  values calculated from Dixon plots (Figures 26, 27 and 28 ) were found to be 1.8, 2.4 and 3.8 micromoles respectively. The plots were also found to be curved upwards.

Studies were also made using two concentrations of inhibitor at various arginine concentrations. The results reported in Tables 44, 45 and 46 show that the inhibition was more at lower substrate concentrations and it could be reversed by adding more substrate indicating the competitive nature of inhibition. The double reciprocal plots (Figures

Table 40. Competitive effect of agmatine, ornithine, lysine and guanidino acetic acid on arginase activity as determined by the method of Hunter and Downs (1945)

Substrate Concentration (micromoles)	$i \frac{V_i}{V - V_i}$							
	Agmatine (micromoles)		Ornithine (micromoles)		Lysine (micromoles)		Guanidino acetic acid (micromoles)	
	5	10	2	4	2	4	1	2
2	8.0	7.6	-	-	-	-	1.7	1.2
4	10.0	8.6	-	-	-	-	2.2	1.4
6	11.1	9.4	3.8	2.6	-	-	2.9	1.5
8	12.5	10.8	6.2	4.0	0.8	0.7	4.5	2.9
10	14.7	12.6	9.4	4.3	1.1	0.9	5.8	3.8
12	15.3	13.3	10.4	8.0	1.7	1.2	7.5	5.4
14	15.5	15.0	12.4	11.2	1.8	1.4	9.4	6.0
16	18.0	17.0	14.4	12.8	2.4	1.9	10.2	8.1
18	18.6	17.2	16.4	15.2	2.6	2.0	-	-
20	21.0	18.0	18.0	14.4	2.8	2.2	-	-

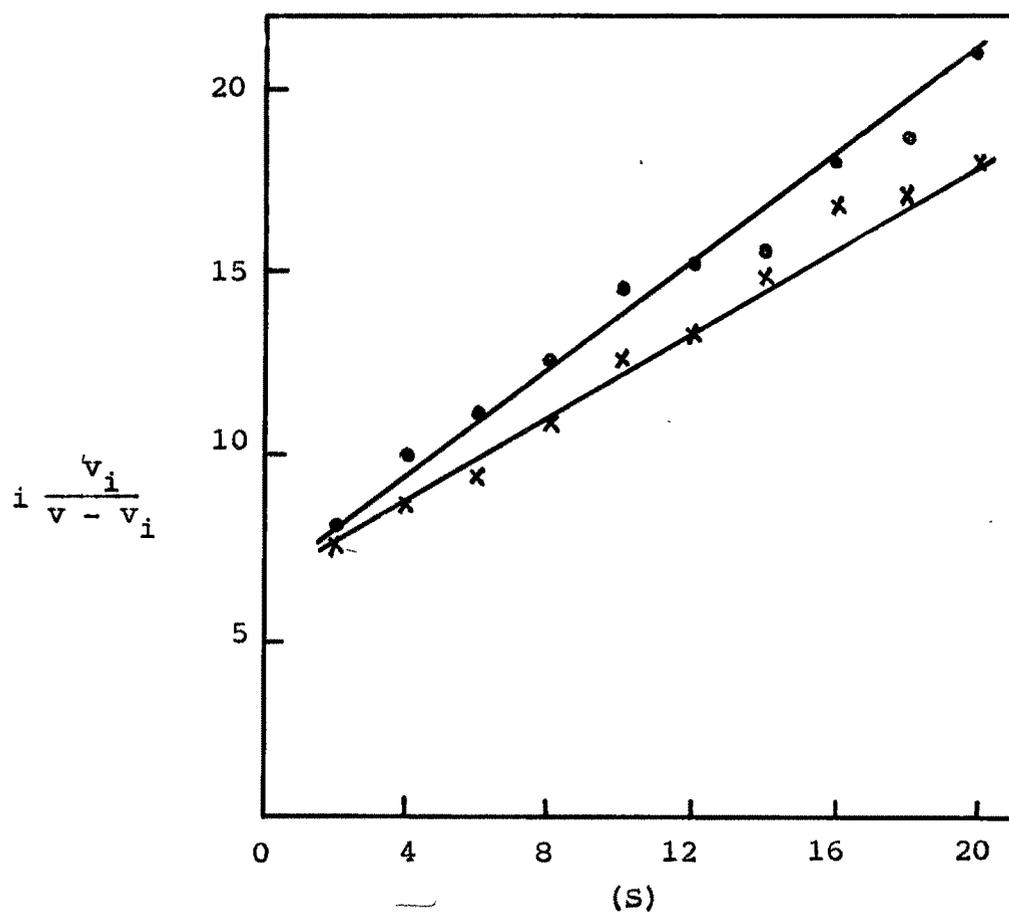


Figure 18. Plots showing the competitive nature of the inhibition by agmatine  
(—●—), with 5 micromoles of agmatine  
(—x—), with 10 micromoles of agmatine

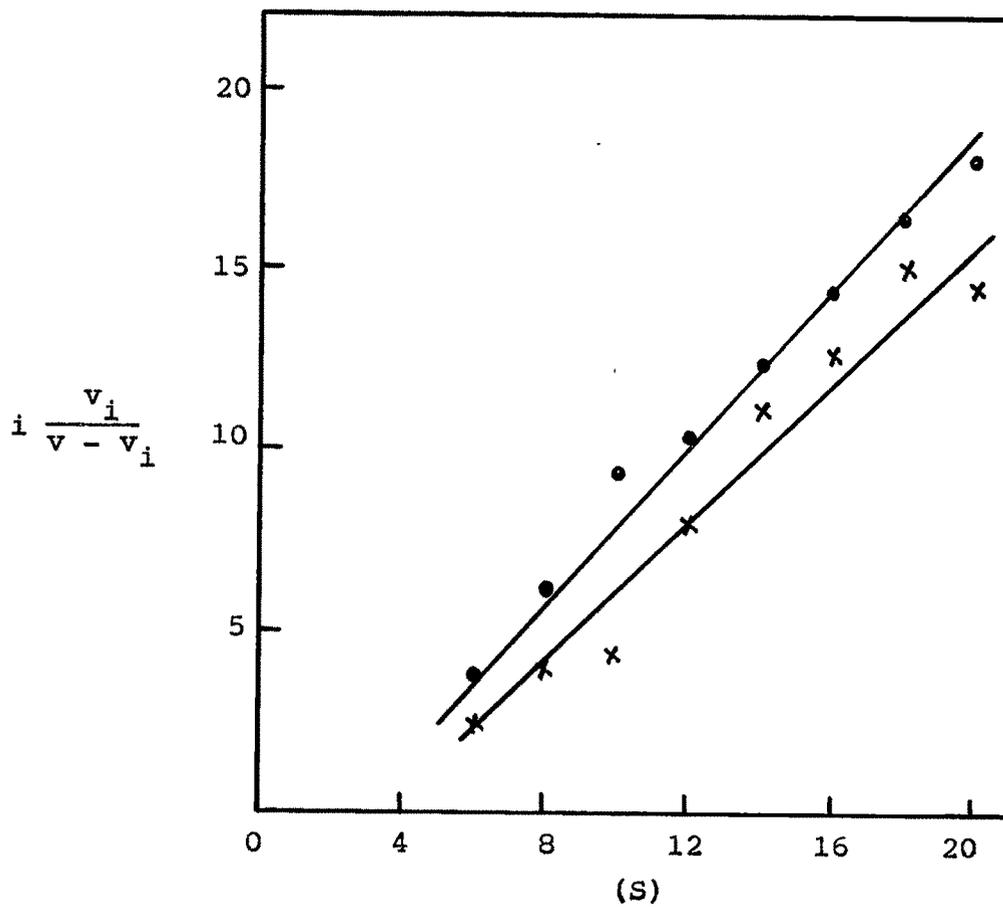


Figure 19. Plots showing the competitive nature of the inhibition by ornithine

(—●—) with 2 micromoles of ornithine

(—x—) with 4 micromoles of ornithine

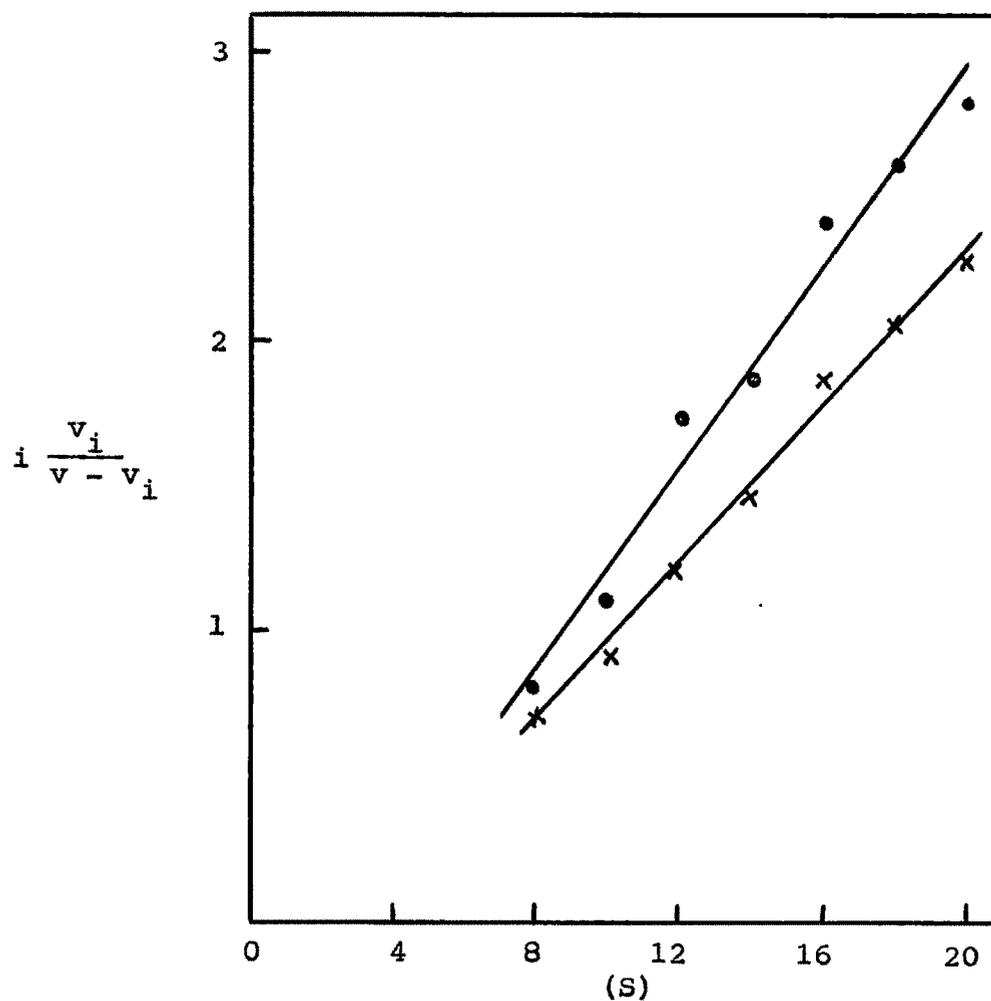


Figure 20. Plots showing the competitive nature of the inhibition by lysine  
(—●—) with 2 micromoles of lysine  
(—x—) with 4 micromoles of lysine

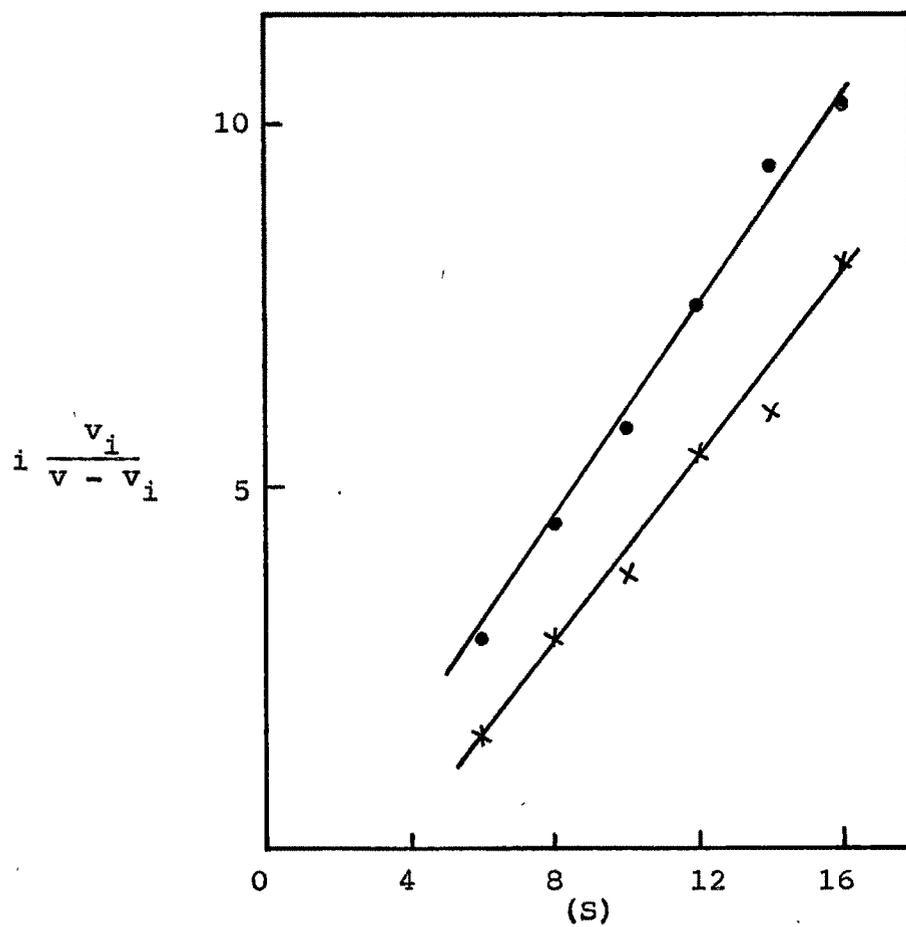


Figure 21. Plots showing the competitive nature of the inhibition by guanidino acetic acid (GAA)  
(—●—) with 1 micromole of GAA  
(—×—) with 2 micromoles of GAA

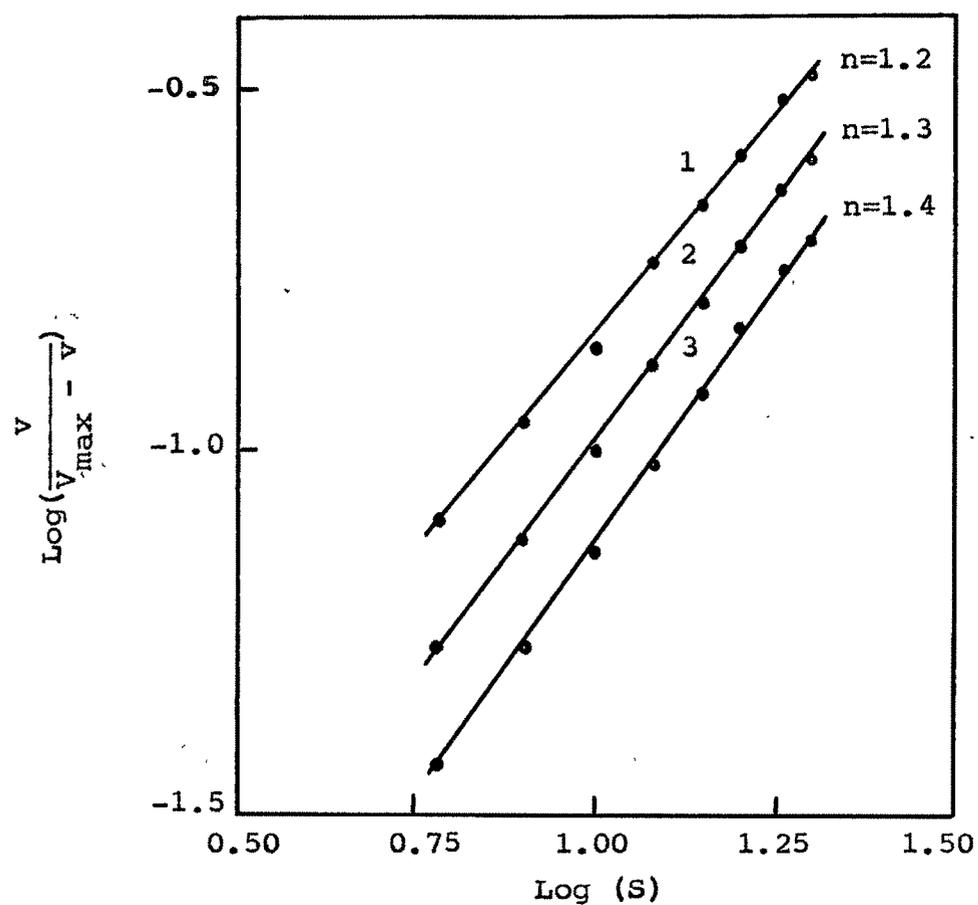


Figure 22. Hill plot of the reaction velocity as a function of substrate concentration at different agmatine concentrations. The  $n$  values represent the slopes of the corresponding lines.

1 = no agmatine

2 = 5 micromoles of agmatine

3 = 10 micromoles of agmatine

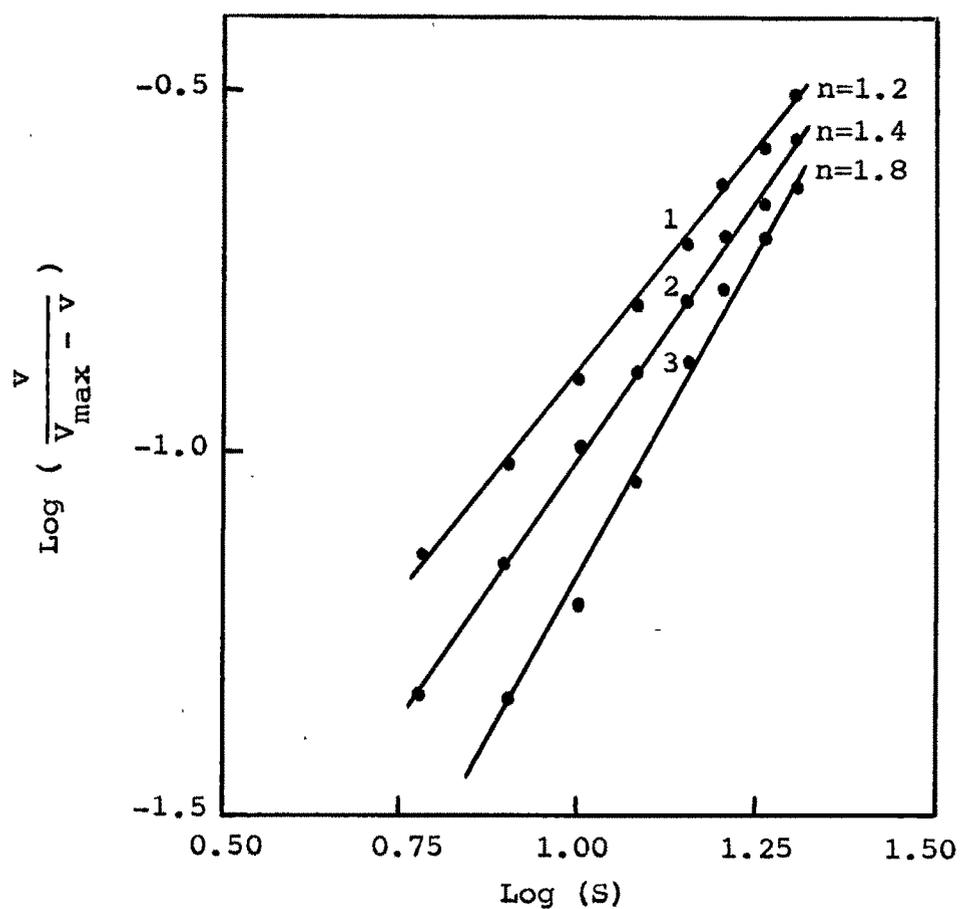


Figure 23. Hill plot of the reaction velocity as a function of substrate concentration at different ornithine concentrations. The  $n$  values represent the slopes of the corresponding lines.

1 = no ornithine

2 = 2 micromoles of ornithine

3 = 4 micromoles of ornithine

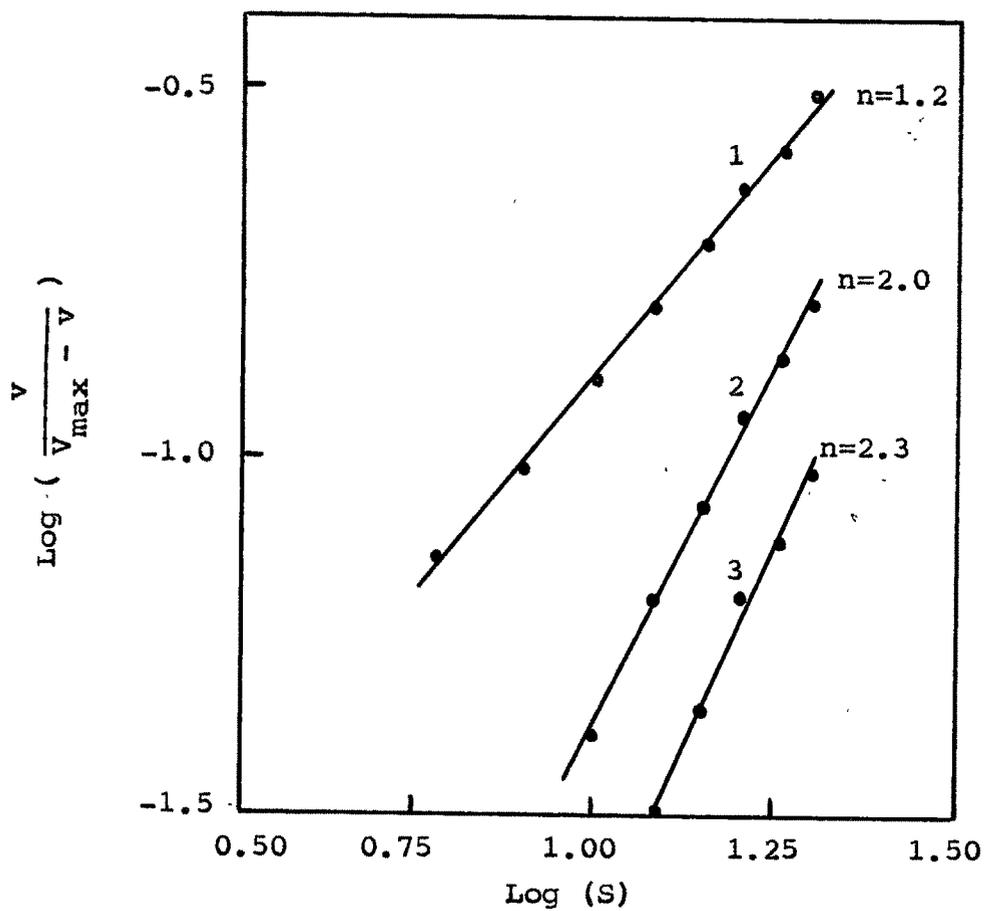


Figure 24. Hill plot of the reaction velocity as a function of substrate concentration at different lysine concentrations. The  $n$  values represent the slopes of the corresponding lines.

1 = no lysine

2 = 2 micromoles of lysine

3 = 4 micromoles of lysine

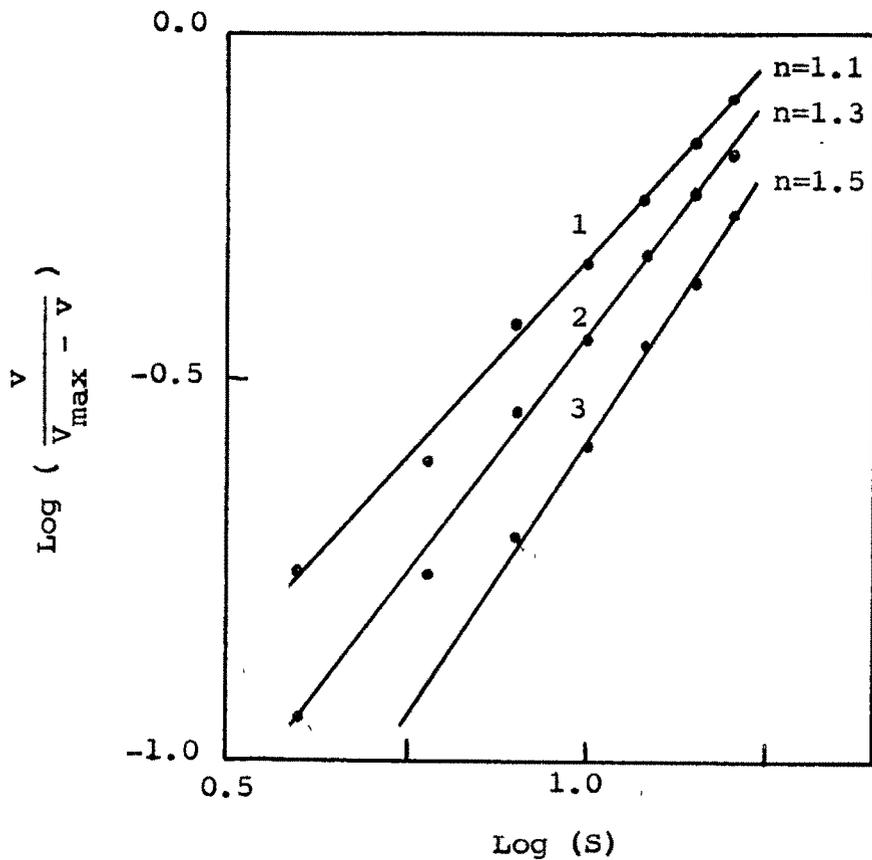


Figure 25. Hill plot of the reaction velocity as a function of substrate concentration at different guanidino acetic acid (GAA) concentrations. The  $n$  values represent the slopes of the corresponding lines.

1 = no GAA

2 = 1 micromole of GAA

3 = 2 micromoles of GAA

Table 41. Effect of xanthine concentration on the inhibition of arginase activity

Inhibitor concentra- tion (micro- moles)	Ornithine formed (micromoles) when substrate added was		Inhibition % when substrate added was	
	10 micro- moles	20 micro- moles	10 micro- moles	20 micro- moles
0	0.93	1.59	0	0
2	0.55	1.27	41	20
4	0.38	1.06	59	34
6	0.26	0.79	72	50
8	0.13	0.59	86	63
10	0.09	0.37	90	77
12	0.08	0.24	92	85
14	0.07	0.15	93	91
16	0.06	0.12	94	92
18	0.01	0.07	99	96
20	0.00	0.05	100	97

Table 42. Effect of guanine concentration on the inhibition of arginase activity

Inhibitor concentration (micro- moles)	Ornithine formed (micromoles) when substrate added was		Inhibition % when substrate added was	
	10 micro- moles	20 micro- moles	10 micro- moles	20 micro- moles
0	0.93	1.59	0	0
2	0.55	1.25	61	22
4	0.44	1.02	69	36
6	0.36	0.83	75	49
8	0.25	0.68	82	58
10	0.15	0.53	90	67
12	0.14	0.34	91	79
14	0.10	0.23	93	86
16	0.08	0.20	94	88
18	0.04	0.18	96	89
20	0.02	0.14	98	91

Table 43. Effect of uracil concentration on the inhibition of arginase activity

Inhibitor concentration (micromoles)	Ornithine formed (micromoles) when substrate added was		Inhibition % when substrate added was	
	10 micro-moles	20 micro-moles	10 micro-moles	20 micro-moles
0	0.93	1.59	0	0
2	0.52	1.34	44	16
4	0.44	1.11	53	30
6	0.32	0.88	66	45
8	0.21	0.82	77	48
10	0.17	0.56	82	65
12	0.12	0.47	87	71
14	0.11	0.42	88	74
16	0.09	0.25	90	84
18	0.06	0.18	94	89
20	0.04	0.11	96	93

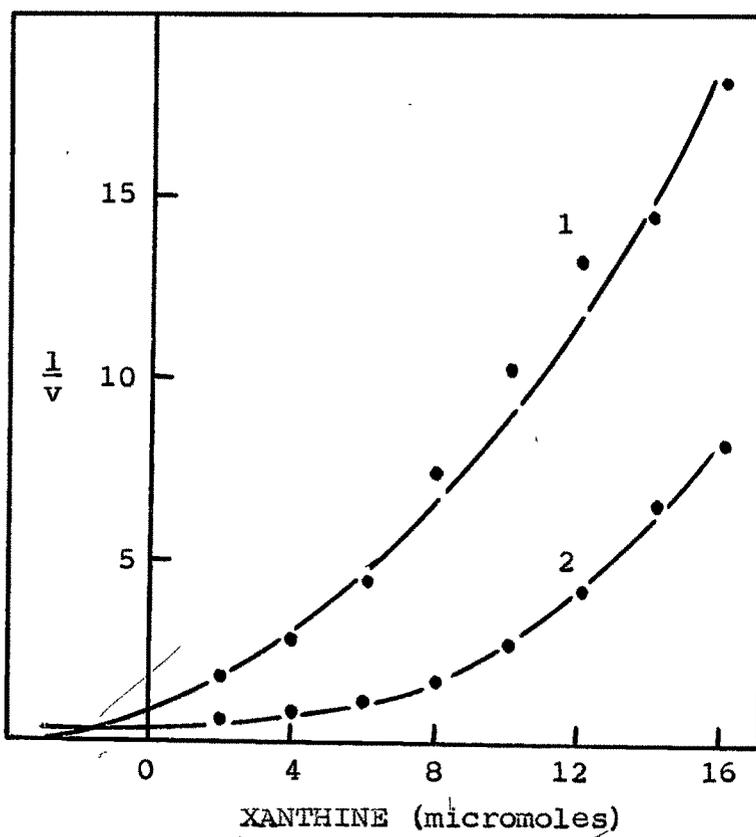


Figure 26. Dixon plot for the inhibition of arginase by xanthine.

1 = 10 micromoles of substrate

2 = 20 micromoles of substrate

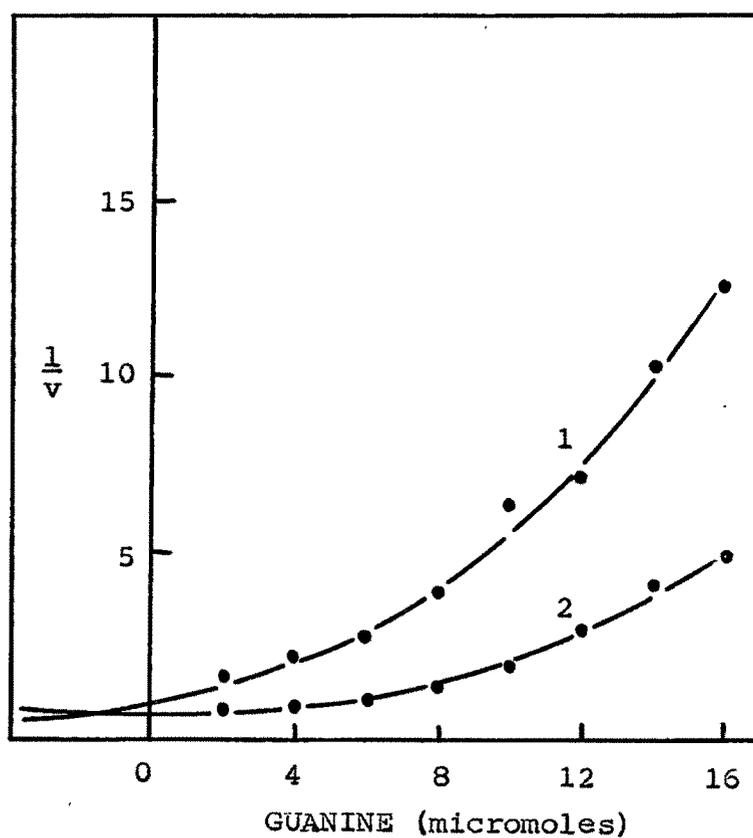


Figure 27. Dixon plot for the inhibition of arginase by guanine.

1 = 10 micromoles of substrate

2 = 20 micromoles of substrate

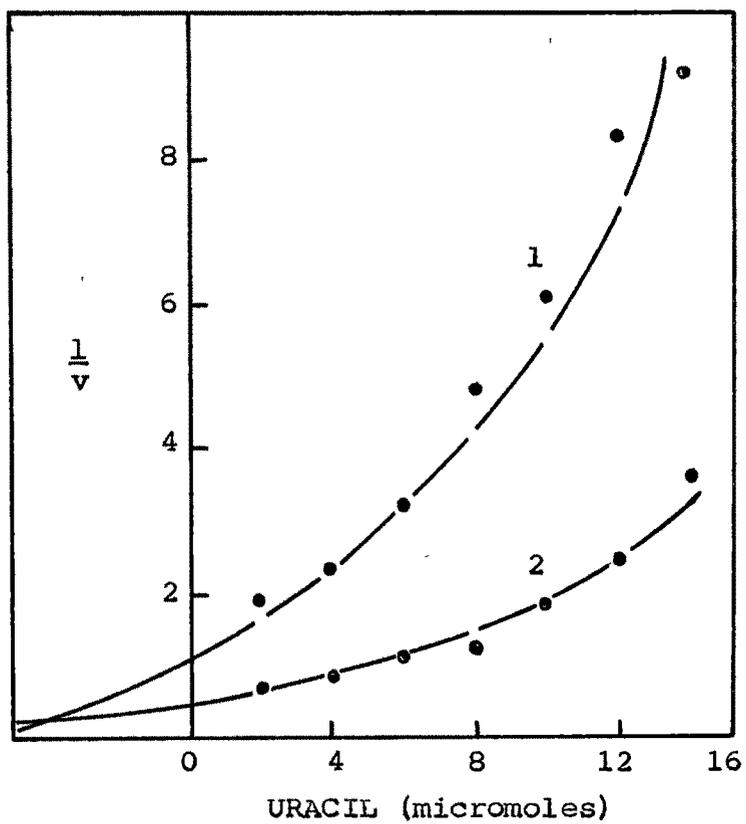


Figure 28. Dixon plot for the inhibition of arginase by uracil.

1 = 10 micromoles of substrate

2 = 20 micromoles of substrate

Table 44. Effect of substrate concentration on the inhibition of arginase by xanthine

Substrate concentration (micromoles)	Ornithine formed (micromoles)		
	Control	With xanthine (micromoles)	
		5	10
2	0.140	0.085	0.055
4	0.310	0.200	0.120
6	0.465	0.360	0.200
8	0.500	0.400	0.275
10	0.685	0.565	0.390
12	0.825	0.700	0.480
14	0.915	0.800	0.550
16	1.000	0.895	0.690

Table 45. Effect of substrate concentration on the inhibition of arginase by guanine

Substrate concentration (micromoles)	Ornithine formed (micromoles)		
	Control	with guanine (micromoles)	
		5	10
2	0.140	0.070	0.060
4	0.310	0.170	0.085
6	0.465	0.325	0.165
8	0.500	0.360	0.195
10	0.685	0.544	0.285
12	0.825	0.660	0.390
14	0.915	0.750	0.420
16	1.000	0.825	0.490

Table 46. Effect of substrate concentration on the inhibition of arginase by uracil

Substrate Concentration (micromoles)	Ornithine formed (micromoles)		
	Control	with uracil (micromoles)	
		5	10
2	0.140	0.070	0.050
4	0.310	0.185	0.105
6	0.465	0.300	0.185
8	0.500	0.365	0.245
10	0.685	0.535	0.360
12	0.825	0.680	0.435
14	0.915	0.755	0.545
16	1.000	0.855	0.600

29, 30 and 31) of velocity against substrate concentration also showed upward curvature like that reported above for agmatine, ornithine etc.

The competitive nature of these inhibitors was also confirmed by calculating the values of Tables 44, 45 and 46 by the method of Hunter and Downs. The values are given in Table 47 and are plotted in Figures 32, 33 and 34. It can be seen that the plots show a dependence on substrate concentration a characteristic of competitive inhibition. The hill plots (Figures 35, 36 and 37) also showed a significant increase in the slope in presence of inhibitors indicating more than one binding site.

It can also be seen that in all the cases the 'n' value obtained from Hill plots without inhibitor which should have been 1.0 varied from 1.1 to 1.2. The reason for this may be that the ornithine, which is an inhibitor increasing the n value, was formed during the reaction itself and thereby giving a slight increase in n value over 1.0 even in the absence of any added inhibitor.

The curved Dixon plots and double reciprocal plots along with the increase in n value indicate the allosteric nature of the enzyme. However, it is not clear from these studies whether the site of binding of agmatine, ornithine, lysine and guanidino acetic acid is same as that of

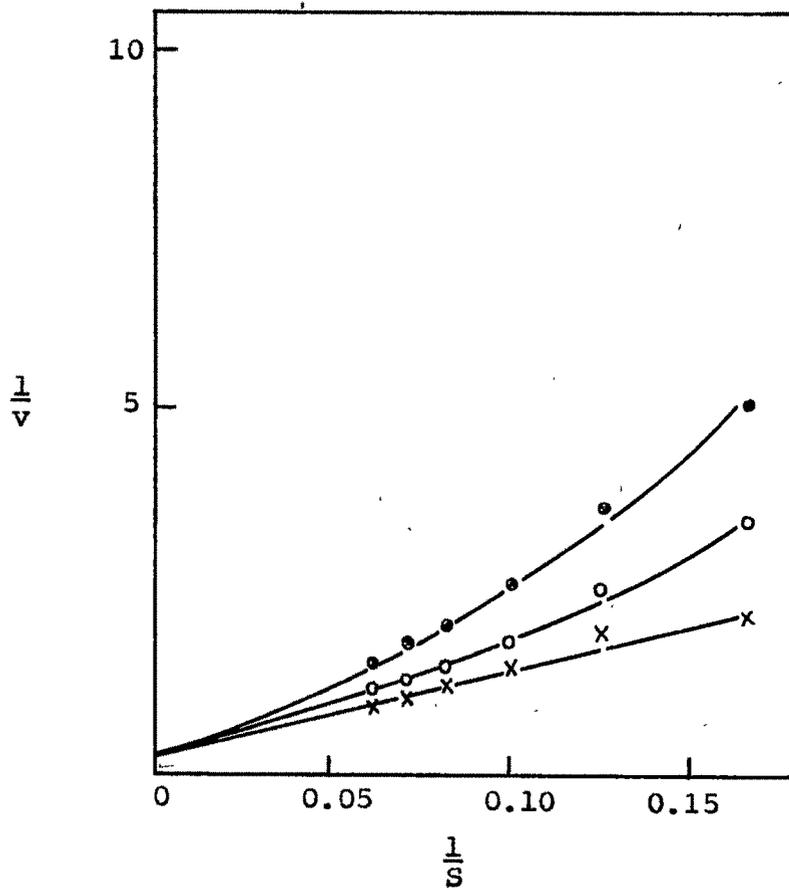


Figure 29. Double reciprocal plot of velocity against arginase concentration at two fixed levels of xanthine.

(-x-), without xanthine

(-o-), with 5 micromoles of xanthine

(-•-), with 10 micromoles of xanthine

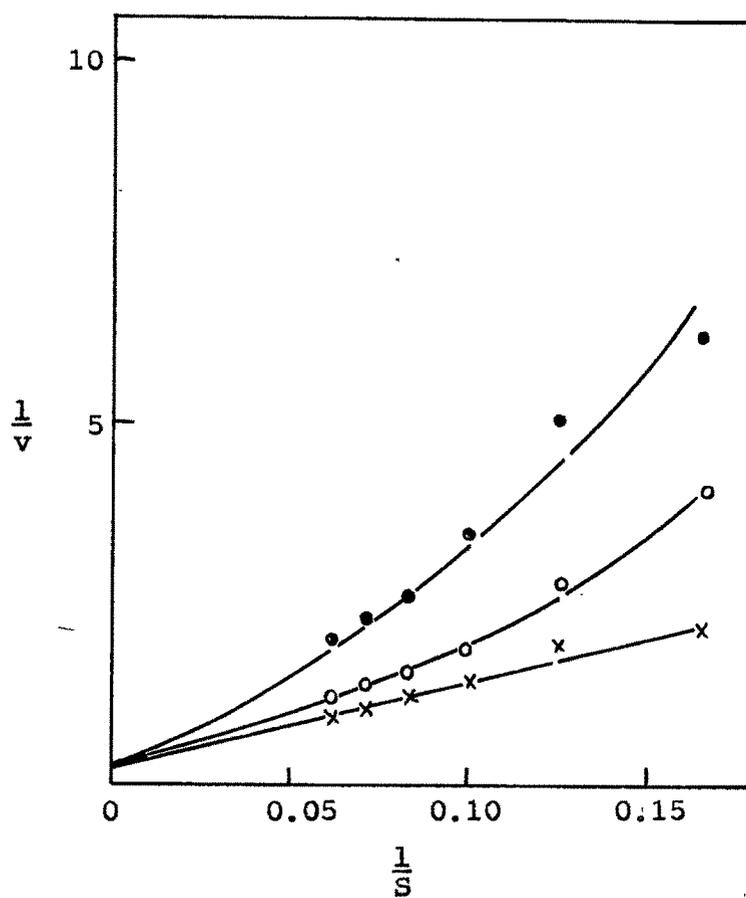


Figure 30. Double reciprocal plot of velocity against arginine concentration at two fixed levels of guanine.

(—x—), without guanine

(—o—), with 5 micromoles of guanine

(—•—), with 10 micromoles of guanine

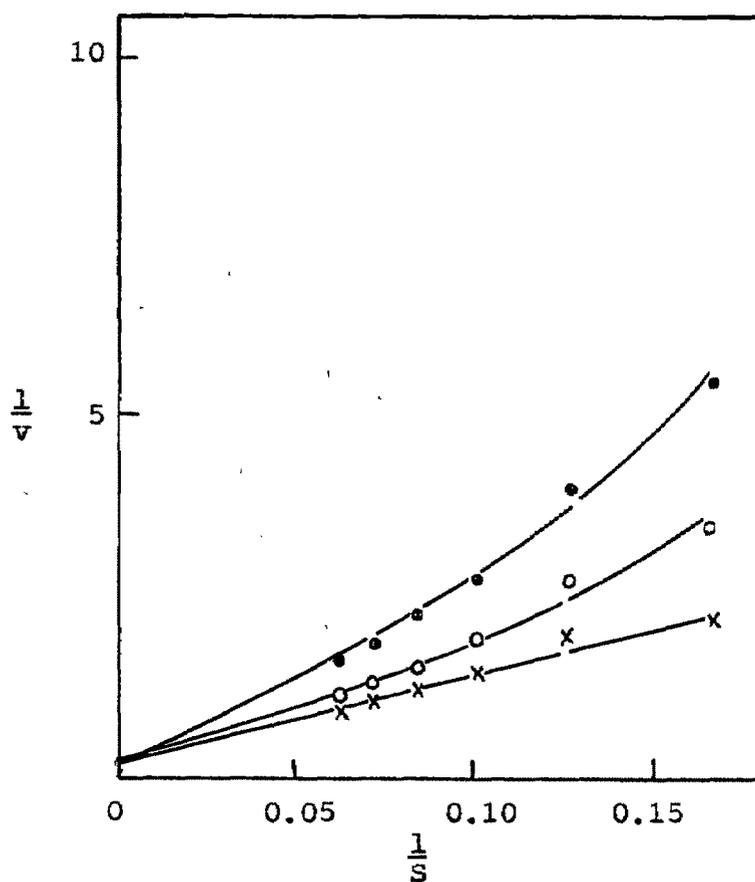


Figure 31. Double reciprocal plot of velocity against arginine concentration at two fixed levels of uracil.

(—x—), without uracil

(—o—), with 5 micromoles of uracil

(—•—), with 10 micromoles of uracil

Table 47. Competitive effect of xanthine, guanine and uracil on arginase activity as determined by the method of Hunter and Downs (1945)

Substrate concentration (micro- moles)	$i \frac{V_i}{V - V_i}$					
	Xanthine (micromoles)		Guanine (micromoles)		Uracil (micromoles)	
	5	10	5	10	5	10
2	7.0	5.8	5.0	7.5	5.0	5.5
4	9.0	6.3	6.0	3.8	7.0	5.1
6	17.0	7.5	11.6	5.5	9.0	7.0
8	20.0	12.2	13.0	6.4	13.5	9.6
10	23.5	13.1	19.5	7.1	18.0	11.0
12	28.0	14.0	20.0	8.9	20.0	11.2
14	35.0	15.0	22.5	8.4	23.5	14.7
16	42.5	23.0	23.5	9.6	28.0	15.0

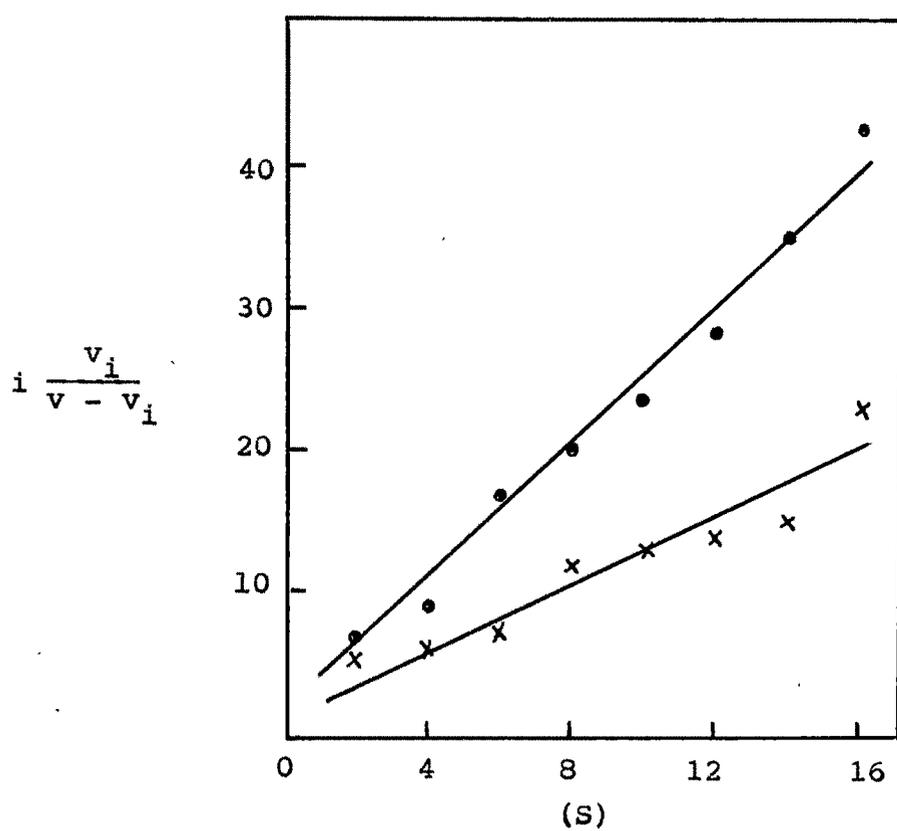


Figure 32. Plots showing the competitive nature of the inhibition by xanthine.

(—●—) with 5 micromoles of xanthine

(—x—) with 10 micromoles of xanthine

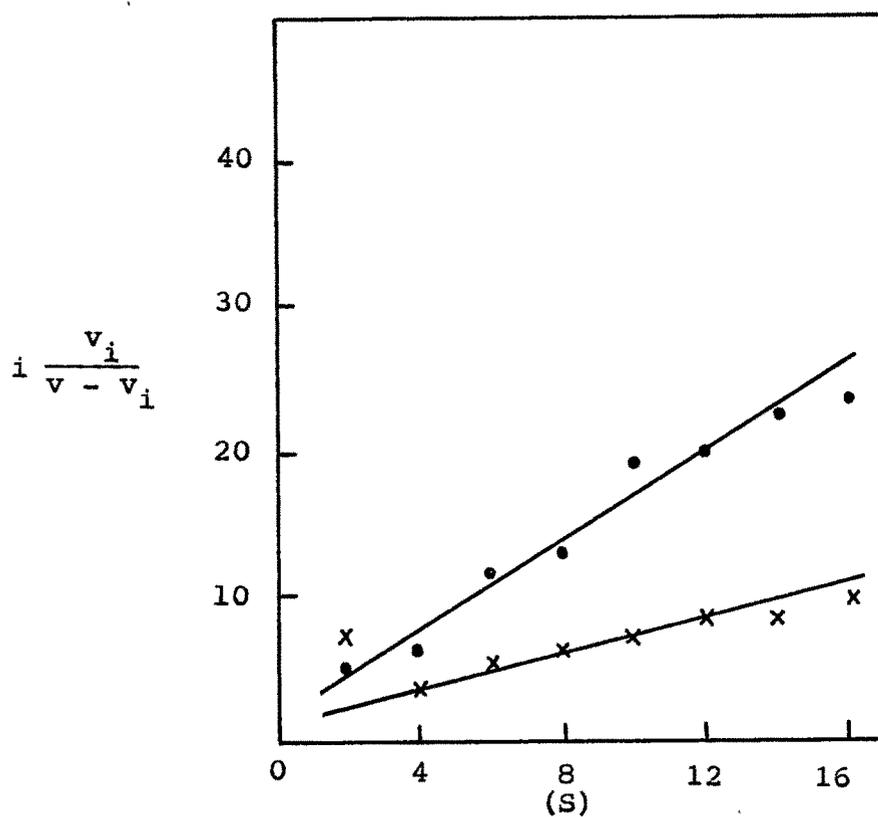


Figure 33. Plots showing the competitive nature of the inhibition by guanine  
(—●—), with 5 micromoles of guanine  
(—x—), with 10 micromoles of guanine

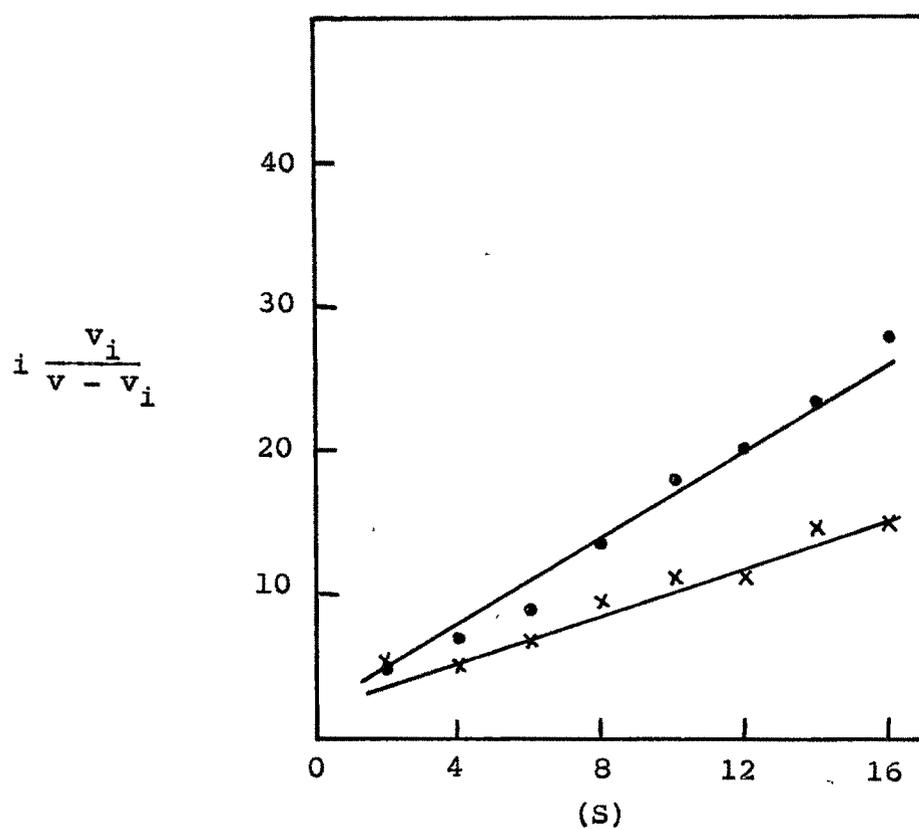


Figure 34. Plots showing the competitive nature of the inhibition by uracil.  
(—●—), with 5 micromoles of uracil  
(—x—), with 10 micromoles of uracil

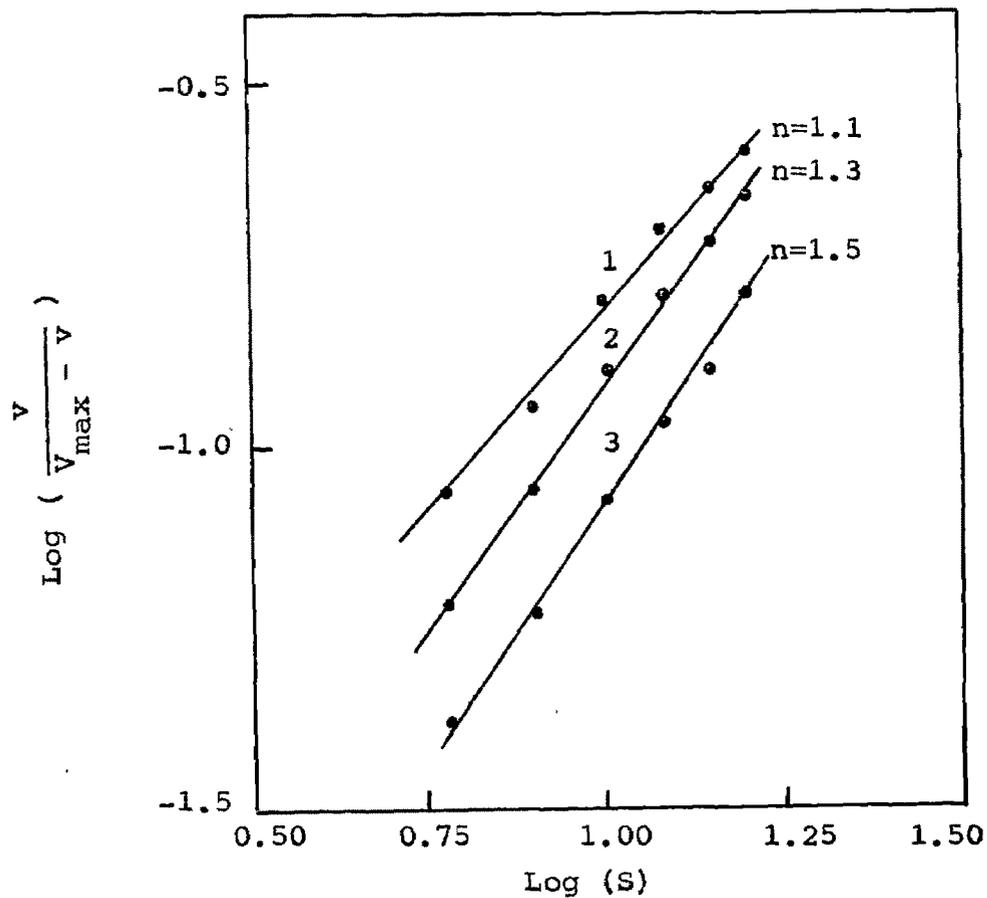


Figure 35. Hill plot of the reaction velocity as a function of substrate concentration at different xanthine concentrations. The  $n$  values represent the slopes of the corresponding lines.

1 = No xanthine

2 = 5 micromoles of xanthine

3 = 10 micromoles of xanthine

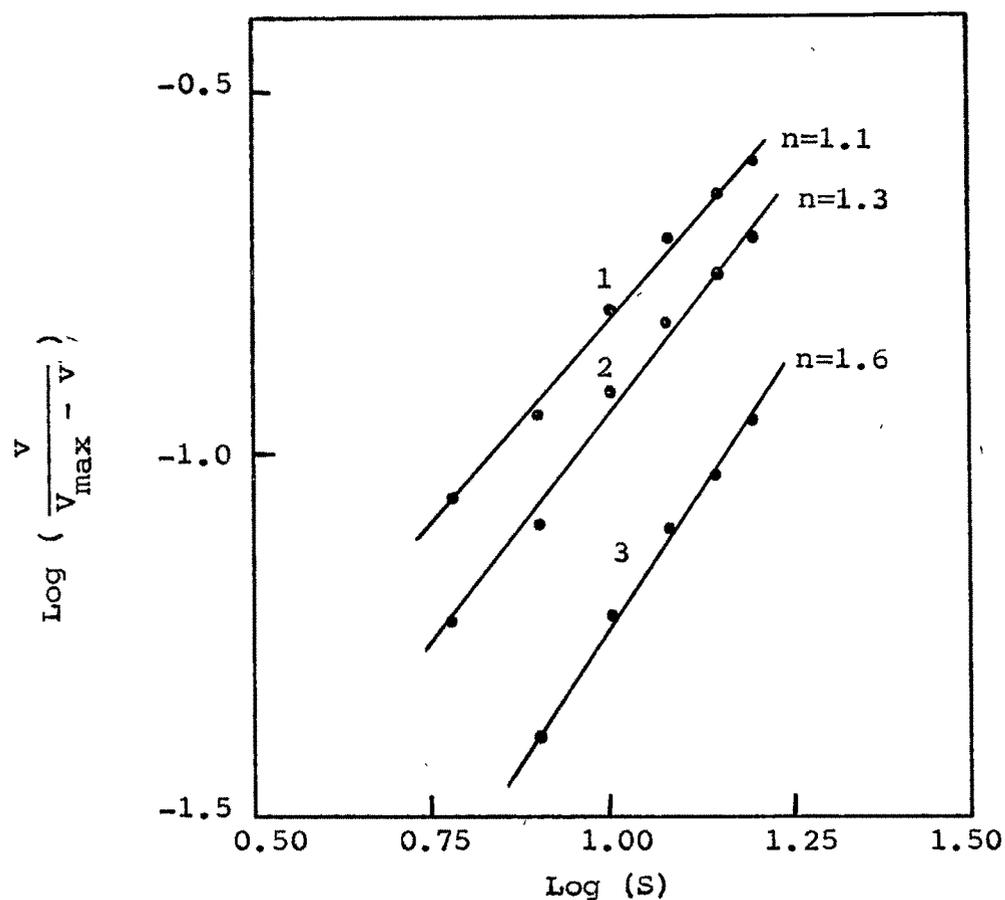


Figure 36. Hill plot of the reaction velocity as a function of substrate concentration at different guanine concentrations. The  $n$  values represent the slopes of the corresponding lines.

1 = No guanine

2 = 5 micromoles of guanine

3 = 10 micromoles of guanine

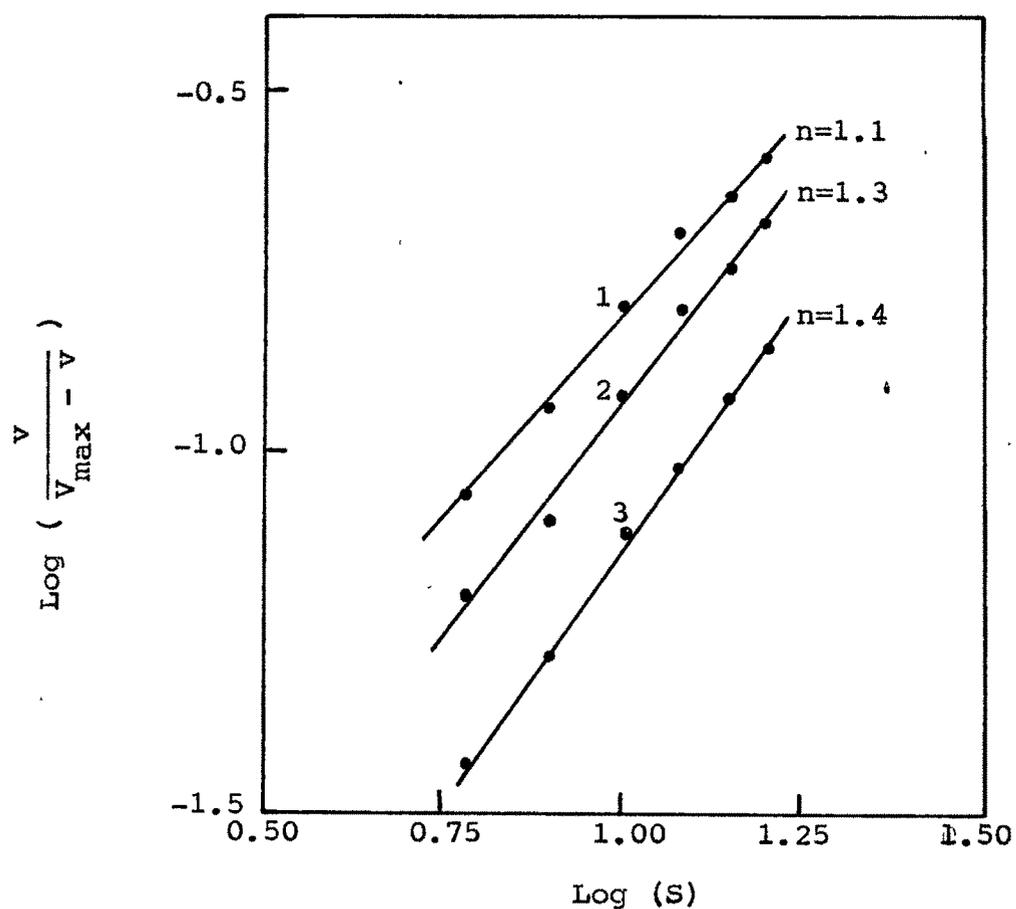


Figure 37. Hill plot of the reaction velocity as a function of substrate concentration at different uracil concentrations. The  $n$  values represent the slopes of the corresponding lines.

1 = No uracil

2 = 5 micromoles of uracil

3 = 10 micromoles of uracil



purines and pyrimidines or different. When the asymptote slopes ( when  $\frac{1}{S}$  approached zero) of the two groups of inhibitors were plotted from Figures 14-17 and 29-31 against inhibitor concentrations, it was found that except in case of agmatine all others show curved lines (Figures 38 and 39) indicative of the cooperative interaction.

Addition of xanthine, guanine, uracil, ornithine and lysine in the cultivation medium on arginase activity :

The fact that purines and pyrimidines which have no structural relationship to arginine inhibit arginase competitively and the kinetics of these inhibitors showed that they may affect arginase by allosteric interaction. If it is the case the arginase activity of the tissue will be affected when cultivated in a medium containing these compounds. The data reported in Table 48 show that xanthine guanine and uracil were able to decrease the activity by about 30 percent on the 15th day of cultivation whereas ornithine and lysine did not affect the enzyme activity. This was further confirmed by growing the tissue in presence of xanthine, guanine and uracil and assaying the activity every 5th day of cultivation. It can be seen from the Table 49 and Figure 40 that optimum growth of the tissue was reached in 25-30 days. The arginase activity in the control group increased with period of cultivation and was about 160 percent on the 15th day of cultivation and then decreased to original level on the 30th day. In case of xanthine, guanine and uracil groups

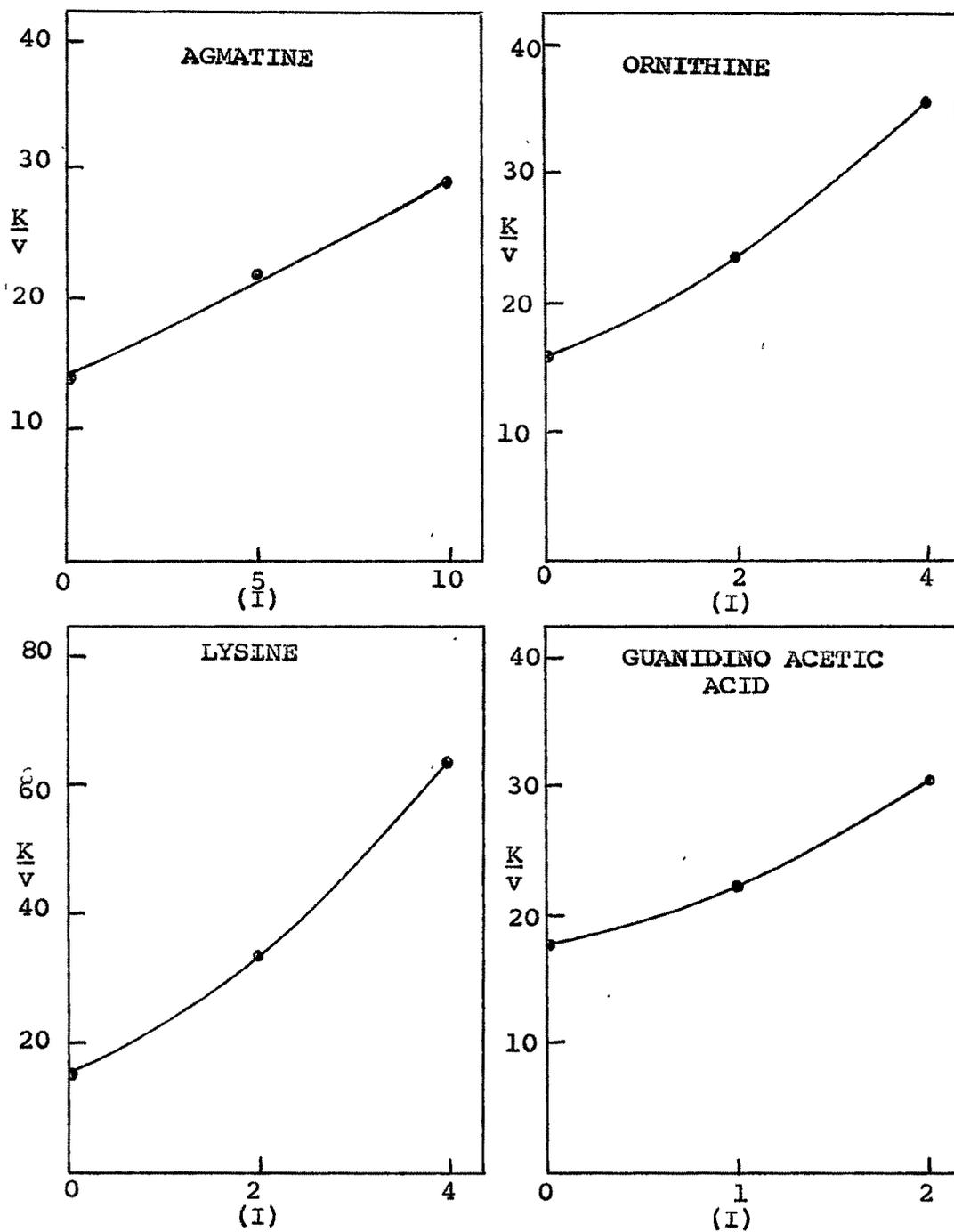


Figure 38. Plots of asymptotes of the curves of substrate (Figs. 14-17) against inhibitor concentration

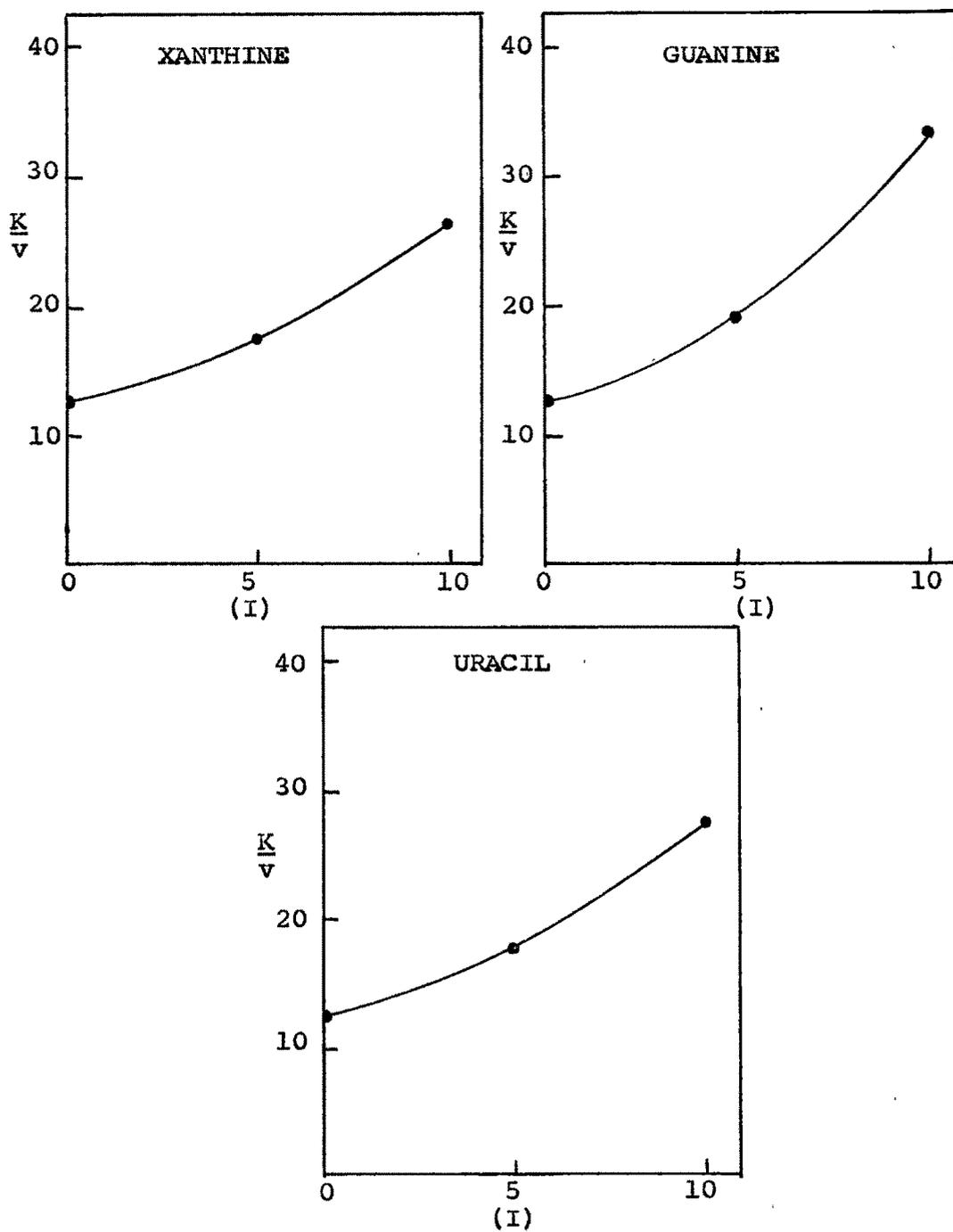


Figure 39. Plots of asymptotes of the curves of substrate (Figs. 29-31) against inhibitor concentration

Table 48. Effect of addition of xanthine, guanine, uracil, ornithine and lysine on arginase activity on different days of cultivation\*

	Enzyme activity % on		
	15th day	30th day	45th day
1. Control	100	100	100
2. " + xanthine	72	96	92
3. " + guanine	74	92	100
4. " + uracil	70	90	100
5. " + ornithine	100	98	100
6. " + lysine	100	99	98

\* The compounds were added in a concentration of 80 p.p.m. Five flasks from each group were pooled together for analysis.

Table 49. Effect of period of cultivation on arginase activity of *Rumex* tumour tissue grown in presence of xanthine, guanine and uracil<sup>†</sup>

Period of cultivation (days)	Average wet weight of tissue/flask* (g)	Enzyme activity %			
		Control	Xanthine	Guanine	Uracil
0	0.16	100	-	-	-
5	0.30	150 <sup>**</sup> (100)	142 (93)	132 (86)	144 (94)
10	0.48	156 (100)	138 (88)	112 (72)	112 (71)
15	0.65	162 (100)	120 (74)	126 (78)	102 (63)
20	0.90	94 (100)	60 (64)	72 (76)	58 (62)
25	1.28	88 (100)	72 (80)	62 (70)	70 (79)
30	1.40	96 (100)	91 (94)	87 (90)	84 (87)
35	1.48	96 (100)	88 (92)	92 (96)	84 (90)
40	1.52	93 (100)	90 (96)	87 (94)	89 (95)
45	1.62	90 (100)	83 (92)	91 (105)	88 (97)

Five flasks from each group were pooled together for analysis.

\* The values given are for the control group. However, no significant difference was found in case of xanthine, guanine and uracil group.

\*\* The values are the percent of zero day control taken as 100 percent whereas the values in the parentheses represent the percent values taking all the corresponding control values as 100 percent.

† The concentration of compounds used was 80 p.p.m.

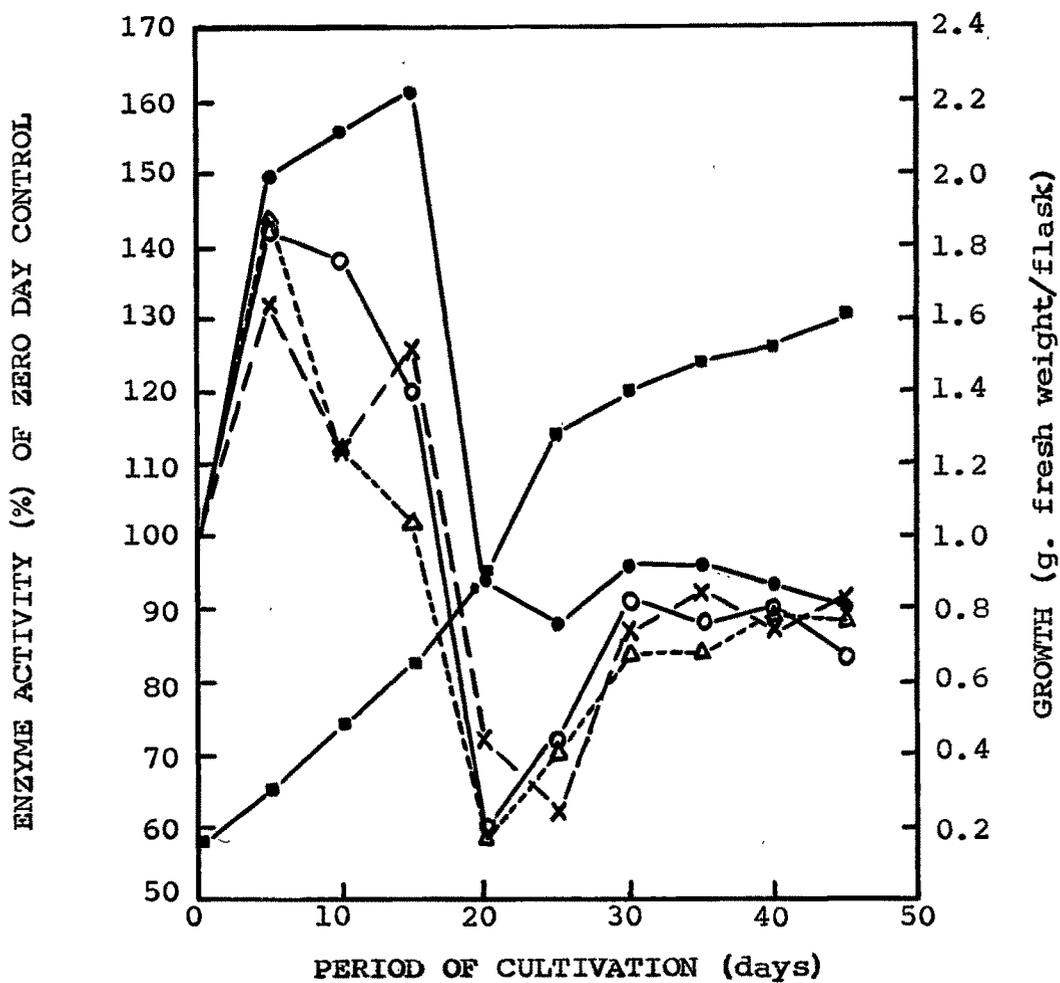


Figure 40. Arginase activity in the absence and presence of xanthine, guanine and uracil on different days of cultivation.

(-■-) Growth : (-●-) Control  
 (-○-) Xanthine : (-X-) Guanine  
 (-Δ-) Uracil

the enzyme activity follows the same pattern but the increase is less due to the inhibition by these compounds during the early period of growth. This can be seen better by taking all the control values as 100 percent and comparing the values of other groups (Figure 41). The activity in xanthine, guanine and uracil group decreased to about 30-40 percent by 20th day. It was also found that addition of boiled tissue homogenate from these groups to the fresh homogenate of control group caused about 40 percent inhibition in enzyme activity showing the presence of inhibitory material in these groups. These results indicate that when the level of purines and pyrimidines is high in tissues the arginase activity is decreased. This might be one of the mechanisms to regulate the level of urea in the tissues.

The data reported in Table 50 and Figure 42 show that maximum inhibition was observed when the concentration of these compounds added to the medium was 80 p.p.m. Higher concentrations could not be tried since they got precipitated when the pH of the medium was adjusted to 5.2.

Effect of addition of arginine in the cultivation medium containing xanthine, guanine and uracil on arginase activity and urea content of Rumex tumour tissue :

In order to see whether the addition of arginine in the medium along with purines and pyrimidines could protect the

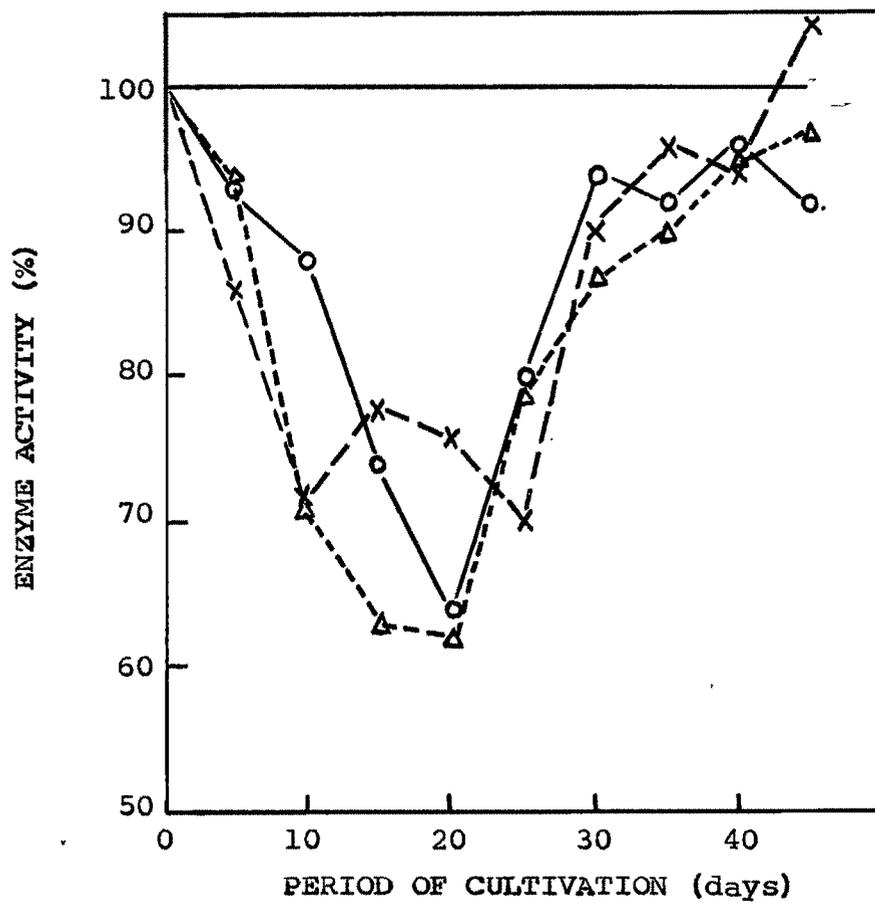


Figure 41. Replot of Fig. 40 taking all the values of control group as 100 percent

( — ) Control : (—○—) Xanthine  
 (—×—) Guanine : (—△—) Uracil

Table 50. Effect of different concentrations of xanthine, guanine and uracil on arginase activity

Concentration (p.p.m)	Enzyme activity % with		
	Xanthine	Guanine	Uracil
0	100	100	100
20	85	92	96
40	82	89	91
60	80	81	72
80	75	77	66
100	78	76	66
120	75	79	68
140	77	77	64
160	76	76	65

Five flasks from each group were pooled together for analysis on 15th day of cultivation.

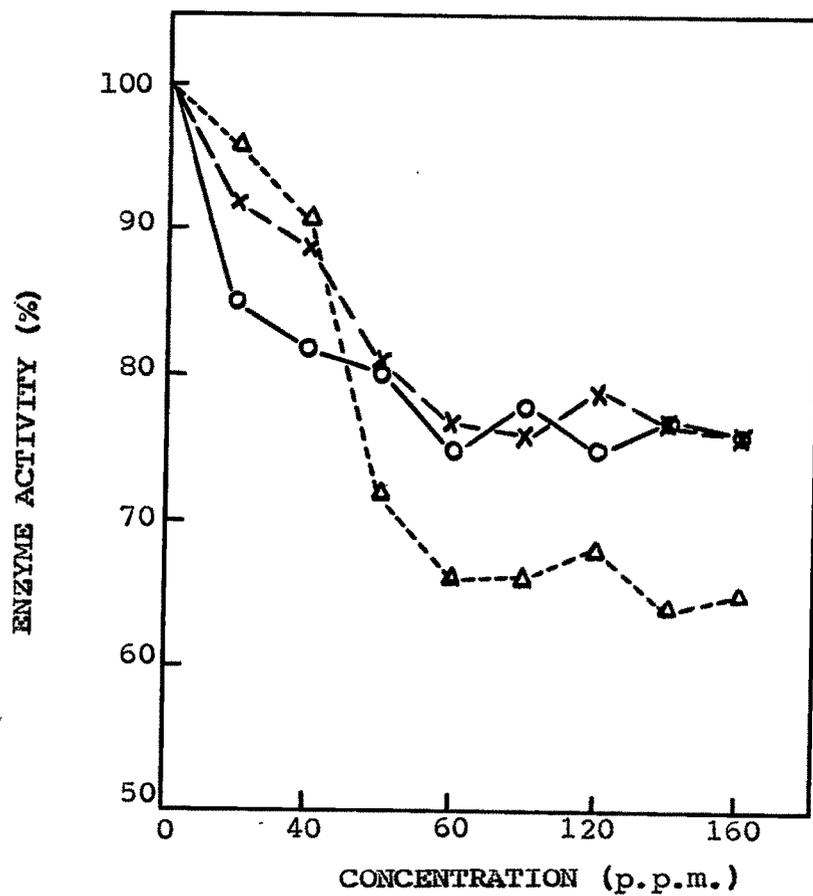


Figure 42. Plot showing the effect of concentration of xanthine (-o-), guanine (-X-) and uracil (-Δ-) on arginase activity

Table 51. Effect of addition of arginine in the cultivation medium containing xanthine, guanine and uracil on arginase activity and urea content of Rumex tumour tissue \*

Period of cultivation (Days)	Arginine	Xanthine	Guanine	Uracil	Arginine + Xanthine	Arginine + Guanine	Arginine + Uracil	Arginine + Xanthine + Guanine	Arginine + Xanthine + Uracil	Arginine + Guanine + Uracil	Arginine + Xanthine + Guanine + Uracil	Urea content % **		
5	95	90	89	95	92	84	90	138	145	129	113	136	119	103
10	97	83	75	81	85	76	78	142	150	138	133	145	139	129
15	103	70	78	66	77	72	73	139	145	133	139	137	140	140
20	99	69	75	67	74	70	70	144	156	125	126	140	126	130
25	95	85	73	75	88	75	85	122	129	118	125	119	105	117
30	105	95	92	91	107	94	91	91	95	114	93	100	104	105

\* Concentration of arginine was 500 p.p.m. while that of xanthine, guanine and uracil was 80 p.p.m.

\*\* The values are percent of the same day's control value taken as 100 percent.

enzyme from inhibition, the tissue was grown in presence of these compounds. The arginase activity and urea content of the tissues was analysed every 5th day of cultivation. The results reported in Table 51 show that arginine alone had no effect on arginase activity whereas xanthine, guanine and uracil were able to decrease the activity by about 30-40 percent upto 20th of cultivation as described earlier. However, addition of arginine to xanthine, guanine or uracil group did not afford any protection towards inhibition by these compounds. The urea content increased in all the groups upto about 20th day and then it started decreasing. However, the urea content in the groups containing both arginine and xanthine, guanine or uracil was not increased more than what is obtained in these groups separately. These results would thus indicate that even though in purified arginase preparation in vitro the arginine can reverse the inhibition of arginase by purines and pyrimidines, it cannot reverse the inhibition obtained in vivo experiments. The level of urea is increased by arginine as well as by purines and pyrimidines but the effect is not additive when the two are present together showing the effect of purines and pyrimidines in the control of urea level in the tissue.

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