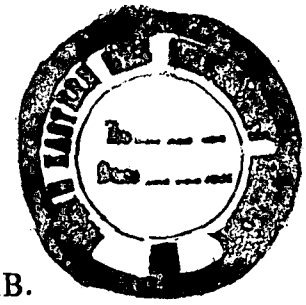
INDUCTION OF UREA CYCLE ENZYMES AND CHARACTERIZATION OF ARGINASE IN A FRESHWATER AIR-BREATHING TELEOST, *Heteropneustes fossilis* (Bloch)



JACQUELINE DKHAR

BIOCHEMICAL ADAPTATION LAB.
DEPARTMENT OF ZOOLOGY
SCHOOL OF LIFE SCIENCES
NORTH-EASTERN HILL UNIVERSITY
SHILLONG-793 022 (INDIA)

THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENT OF THE DEGREE OF

DOCTOR OF PHILOSOPHY IN ZOOLOGY

TO

NORTH-EASTERN HILL UNIVERSITY SHILLONG

DECEMBER,1994

C E R T I F I C A T E

This is to certify that the thesis entitled
"**Induction of urea cycle enzymes and characterization of arginase in a freshwater air-breathing teleost,** *Heteropneustes fossilis* (Bloch)"
submitted by
Ms. Jacqueline Dkhar
for the degree of
Doctor of Philosophy
in Zoologyof the North-Eastern Hill University, Shillong
embodies the record of original investigations
carried out by her under our supervision.

The thesis presented is worthy of being considered for the award of the Ph. D. degree.
This work has not been submittted for any degree of any other university.

(N. Saha)
Supervisor
Biochemical Adaptation Lab.
Department of Zoology
North-Eastern Hill University
Shillong 793022 (India)

(B. K. Ratha)
Supervisor
Environmental Biochemistry Lab.
Centre of Advance Study in Zoology
Banaras Hindu University
Varanasi 221005 (India)

forwarded
V Tandon
22.12.94

Head
Department of Zoology
School of Life Sciences
North Eastern Hill University
Shillong

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. N. Saha, Lecturer, Department of Zoology, North-Eastern Hill University, Shillong and Professor B. K. Ratha, Centre for Advance Study in Zoology, Banaras Hindu University, Varanasi whose kind inspiration, guidance and constant encouragement has enabled me to undertake this investigation.

I am also grateful to Professor (Mrs) V. Tandon, Head, Department of Zoology, North-Eastern Hill University, Shillong and Professor K. Chatterjee, Ex-Dean, School of Life Sciences for providing me the necessary laboratory facilities.

I thank my colleagues Dr. (Mrs) J. Chakravorty, Dr. (Miss) J. R. Das, Miss R.K. Rana and Mrs. B. Choudhury for their help during my research work. I also take the opportunity to thank my friends Miss R.D. Lyngdoh, Mrs. P. Saha, Miss M. Blah, Dr. (Miss) M. Roy, Miss M. Dutta and Miss P. Paul for their valuable assistance and co-operation.

I thank Mr. S. Roy Choudhury for preparing the graphs, Mr. B.K. Das for the photography, Mr. J. P. Rai and Mr. S Pohti for maintenance of fishes.

The award of fellowship from U.G.C., New Delhi is gratefully acknowledged.

Finally, I thank all my family members for their indispensable help, kind endurance and allotment of time needed throughout my research work.

Jacqueline DKhar

SHILLONG

Date: 21- December, 1994. MISS JACQUELINE DKHAR

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Dedicated to my

beloved father

INTRODUCTION

Nitrogen metabolism is one of the major pathways of energy supply in all animals. Dietary intake of proteins by animals provides amino acids in excess of the amount required for protein synthesis. Therefore, excess of amino acids which cannot be stored as proteins, as can be carbohydrates as glycogen and lipids as fat, are metabolized. They are deaminated releasing ammonia and the carbon residues which are either oxidized via the TCA cycle for energy production or used in gluconeogenesis or lipogenesis. Ammonia is highly toxic and cannot be stored in the body even in low concentrations (Smith, 1929; Jackson et al, 1986; Cooper and Plum, 1987; Campbell, 1991).

Ammonia toxicity to fish has been primarily attributed to the un-ionized form (NH_3) with the ionized form (NH_4^+) being relatively less toxic (EIFAC, 1970; Alabaster and Lloyd, 1982; Erickson, 1985; WHO, 1986; Hickey and Vikers, 1994). The proportion of un-ionized ammonia increases with increase in pH and temperature (Emerson et al, 1975). Acute ammonia toxicity includes decrease in oxygen carrying capacity of haemoglobin (Sousa and Meade, 1977), increased oxygen consumption, respiratory rate and heart beat (Smart, 1978; Chen and Nan, 1993), disturbances of ionic balance and acid-base balance (Maetz, 1973; Cameron and Heisler, 1983; Cameron, 1986; Paley et al, 1993; Wajsbrot et al, 1993) in fish. Acute toxicity of un-ionized ammonia to mysids and larval inland silversides was influenced by pH and salinity in a species specific manner (Miller et al,

1990). Sousa and Meade (1977) proposed that the mechanism of ammonia toxicity involved stimulation of glycolysis by the ammonium ion (NH_4^+) and the simultaneous suppression of the Krebs cycle due to the depletion of ∝-ketoglutarate which removes ammonia by amination to form first glutamate and then glutamine. These two concurrent actions would result in an increase of acidic metabolites from glycolysis and early Krebs cycle and would lower blood pH due to accumulation of pyruvate and lactate (Campbell, 1991). The resulting acidemia would shift the oxygen dissociation curve (Bohr effect) to reduce maximal oxygen saturation of haemoglobin and cause death by suffocation. The toxic action of ammonia might also involve an osmoregulatory disturbance in channel catfish (Tomasso et al, 1980) as it has been reported to increase the permeability of tissue to water (Dennis, 1966; Lloyd and Orr, 1969). The uncoupling of oxidative phosphorylation by NH_4^+ ion as suggested by Smart (1978) could be another adverse effect of ammonia to inhibit ATP production. Ammonia also affects the membrane potential and excitability of neurons (Cooper and Plum, 1987). Due to these wide ranging toxic effects , ammonia is either immediately excreted out or converted to some less toxic substances such as urea or uric acid for temporary storage in vivo.

In teleosts, ammonia usually is excreted out to the ambient water medium by diffusion through the gills (Smith, 1929; Forster and Goldstein, 1969; Watts and Watts, 1974; Kormanik and Cameron, 1981; Evans and Cameron, 1986;

Campbell, 1991; Wood,1993). In terrestrial animals, ammonia *in vivo* is converted either to urea or to some other compounds, which are excreted out mainly through urine utilizing lesser amount of water (Cohen, 1976; Hoar,1983; Campbell, 1991; Wood, 1993; Anderson, 1994a). Insoluble uric acid is found to be the excretory product of those animals where conservation of metabolic water is highly essential due to their arid environment (Hoar, 1983; Nener, 1988; Powers-Lee and Meister, 1988; Campbell, 1991; Wood, 1993).

Based on the type of primary nitrogenous excretory products, animals have been classified into three different groups :

(i) Ammoniotelic : Animals which excrete ammonia as the major excretory product as in most aquatic animals.

(ii) Ureotelic : Animals which excrete urea as the major excretory product as in mammals and amphibians.

(iii) Uricotelic : Animals which excrete uric acid as the major excretory product as in insects, birds and reptiles.

However, not all animals fall neatly into one category or another because many exhibit mixed patterns of nitrogen excretion, depending upon their physiological and environmental conditions. Amphibians, which can live in land as well as in water, excrete both ammonia and urea. They are ammoniotelic in water and ureotelic on land.

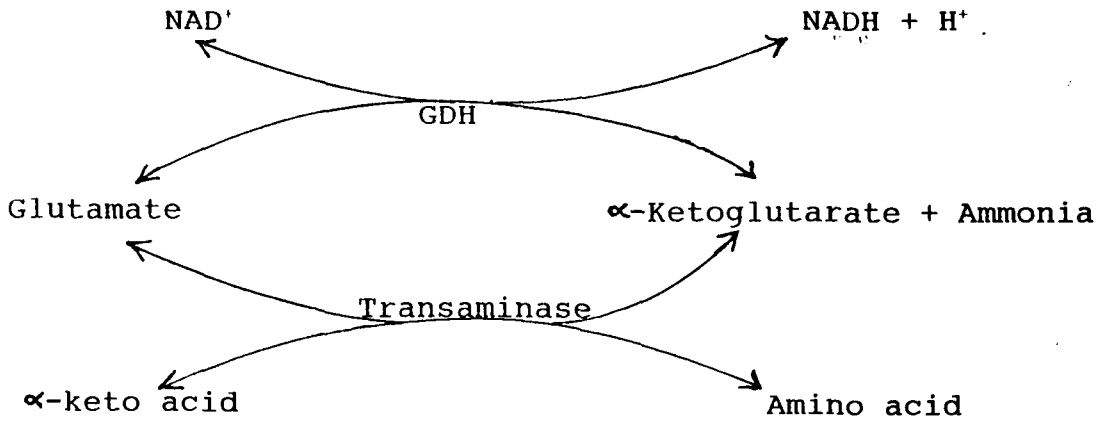
Tadpoles change from being ammoniotelic during early stages of development to ureotelic during later stages of development.

Ammonia has many advantages as a nitrogen excretory product. There is no expenditure of energy for the conversion of protein nitrogen to ammonia. Instead, some of the reactions involved in the formation of ammonia such as deamination of glutamate through glutamate dehydrogenase ultimately produce energy (Bessman and Pal, 1976). Due to its small size, high solubility in water and higher partition coefficient, ammonia is easily eliminated by diffusion (Forster and Goldstein, 1969). Evans and Cameron (1986) have demonstrated the ability of NH_4^+ to exchange with Na^+ absorption by the gills of freshwater fish. In freshwater fishes the exchange of NH_4^+ for Na^+ serves the dual purpose of elimination of nitrogenous waste product NH_4^+ and absorption of Na^+ from the external water medium.

Formation of ammonia : Ammonia can be formed by several pathways namely via deamination of amino acids, amides, purines, pyrimidines and hexosamines and through trans-deamination of amino acids (Cohen and Brown, 1960; Walton and Cowey, 1977, 1982; Randall and Wright, 1987).

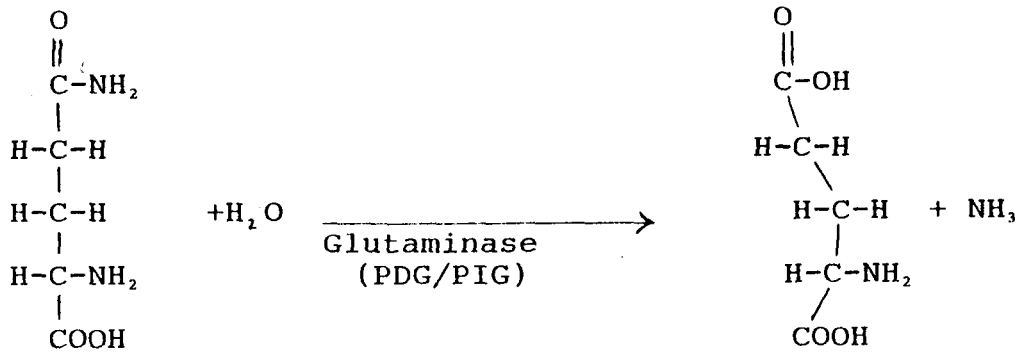
Transdeamination : The amino group from most of the amino acids with the exception of histidine, serine, cysteine, is transferred to another keto acid forming a new amino acid. The dissociated amino group tends to be channelized

directly or indirectly through the formation of glutamate. Glutamate undergoes oxidative deamination catalyzed by glutamate dehydrogenase (GDH) to form ammonia and ∝-keto-glutarate (Krebs et al, 1978). The overall reaction in the liberation of ammonia from amino acids via glutamate formation is known as transdeamination (Braunstein, 1939) which may be summarized in the following reaction



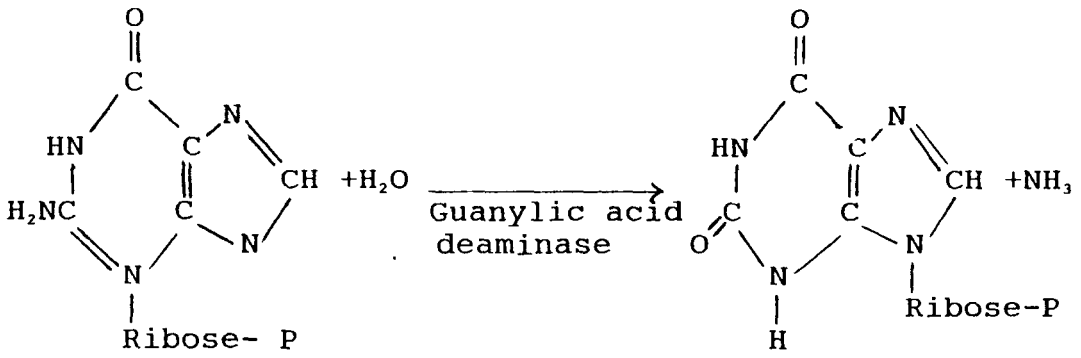
Transdeamination has been reported as the major pathway for ammoniogenesis in the liver of freshwater teleosts (Janssens, 1964;Campbell et al 1983; Campbell, 1991), and in the mudskippers, Boleopthalmus boddaerti and Periopthalmodon schlosseri (Chew and Ip, 1987).

Deamination : Van Slyke et al (1943) showed that glutamine, an amide, helps for temporary storage and transport of ammonia in animals. Glutamine is deaminated through hydrolytic removal of secondary amino group by the enzyme glutaminase which is found either as phosphate dependent (PDG) or phosphate independent (PIG) forms.

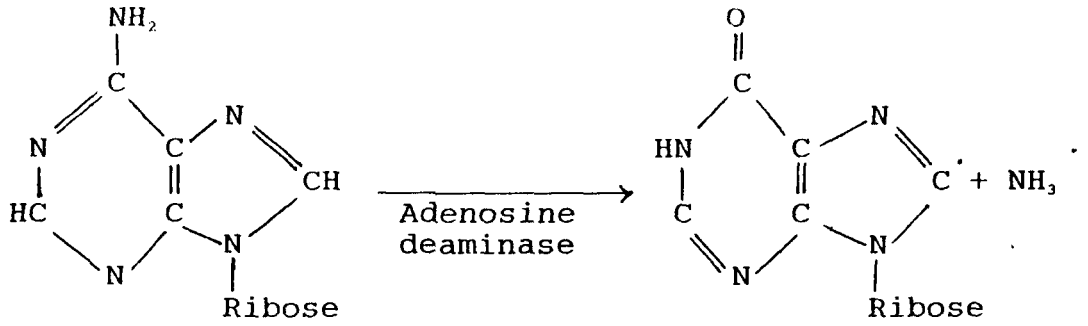


Glutamine Glutamate

Nucleodeamination : Nucleodeaminases catalyse the deamination of nucleosides and nucleotides to liberate ammonia (Cohen and Brown, 1960).

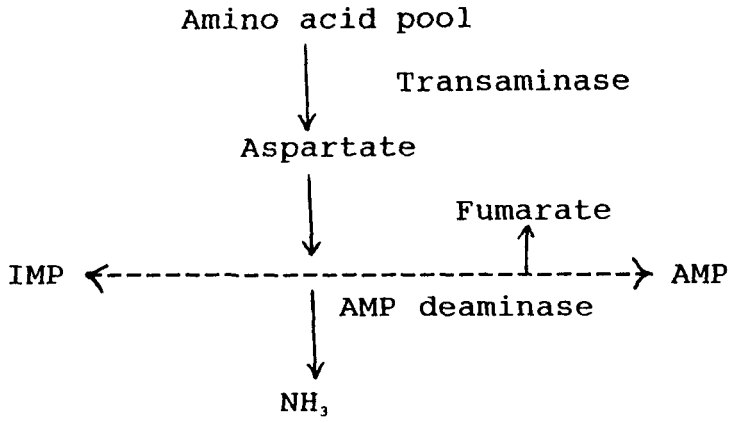


Guanylic acid Xanthylic acid



Adenosine Inosine

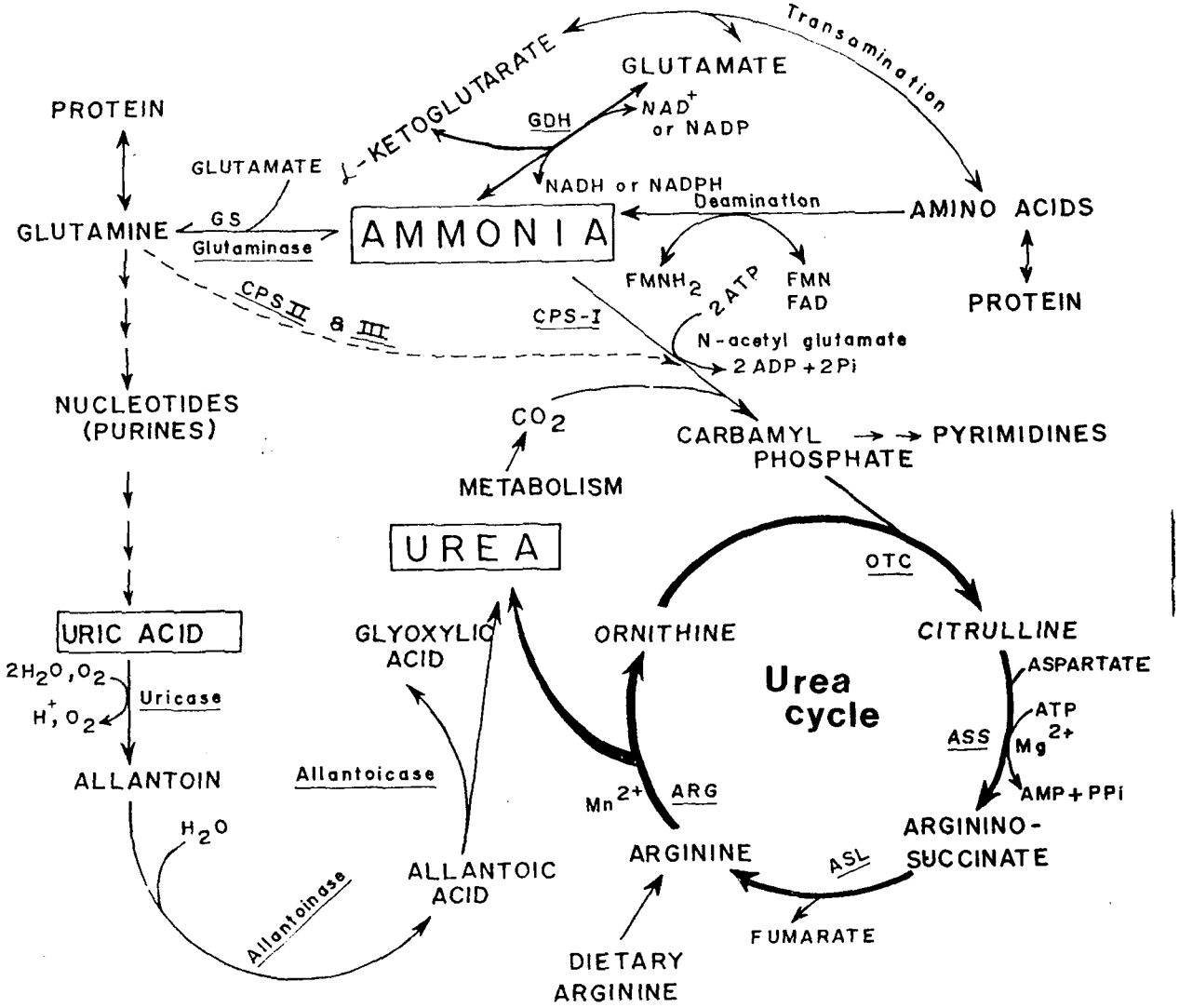
Hydrolysis of particularly AMP could be ultimately utilized for deamination of amino acids as follows :



The role of AMP deaminase has been shown to be more important in ammonia production in some fishes (Makarewicz and Zydowo, 1962; Makarewicz, 1963) and glutaminase in some others (Walton and Cowey, 1977).

Formation of urea : Although teleosts are primarily ammoniotelic, some amount of urea has been reported both in the excretory products (Holmes and Donaldson, 1969; Saha et al, 1988; Saha and Ratha, 1989) as well as in the tissues of several fishes (Smith, 1929; Burrows, 1964; Brett and Groves, 1979; Vellas, 1981; Ramaswamy and Reddy, 1983; Saha and Ratha, 1989) besides marine fishes (where urea production and retention serves the purpose of osmoregulation) (Alexander et al, 1968; Goldstein and Forster, 1971; Hoar, 1983; Campbell, 1991; Anderson,1994a). The formation of urea in fish has been suggested to be through either one or more of these pathways such as (i) Ornithine-urea (o-u) cycle (ii) uricolytic pathway and (iii) catabolism of dietary arginine.

o-u cycle : The o-u cycle involves a series of 5 enzymatic reactions (Krebs and Henseleit, 1932; Brown and Cohen, 1959). The five enzymes of the urea cycle are carbamyl phosphate synthetase (CPS), Ornithine transcarbamylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase (ARG)(Fig. 1). The first reaction of the o-u cycle involves the fixation of ammonia and CO_2 to carbamyl phosphate by the enzyme CPS. Carbamyl phosphate is then converted to citrulline in presence of ornithine by the enzyme OTC. Both the reactions in ureotelic vertebrates take place inside the mitochondria and citrulline produced is transported to the cytosol. It is converted ultimately to urea and ornithine by the other three cytosolic enzymes (ASS, ASL and ARG) of o-u cycle. The presence of a functional urea cycle in elasmobranchs and lungfishes (Brown and Cohen, 1959; Forster and Goldstein, 1966; Huggins et al, 1969; Schooler et al, 1966; Janssens and Cohen, 1966) and in marine teleosts (Huggins et al, 1969; Read, 1971; Mommsen and Walsh, 1989) have been reported. Brown and Cohen (1960) could not detect CPS and OTC in several species of freshwater teleosts studied. Huggins et al (1969) could detect all the enzymes of the o-u cycle enzymes in some freshwater teleosts but their activites were so low that no physiological significance could be attributed to them. They divided the urea producing animals into 3 categories on the basis of the role of urea synthesis. These are ureogenic, ureotelic and ureosmotic.



[CPS - Carbamylphosphate synthetase-I (ammonia and N-acetylglutamate dependent); - II (glutamine dependent and N-acetyl glutamate independent); - III (glutamine and N-acetyl glutamate dependent): OTC - Ornithine transcarbamylase; ASS - Argininosuccinate synthetase; ASL - Argininosuccinate lyase; ARG - Arginase; GS - Glutamine synthetase]

Fig.1. A brief diagramatic representation of nitrogen metabolic pathways in animals with special reference to ammonia and urea.

Ureogenic : The species having full complements of the o-u cycle enzymes indicating the potential for synthesizing urea, although for various reasons, its synthesis may be repressed in freshwater fishes.

Ureotelic : These animals are ureogenic and synthesize sufficient urea by the o-u cycle to account for the bulk of nitrogen excretion.

Ureosmotic : These animals produce urea for maintaining the osmotic equilibrium with the environment.

Uricolytic pathway : Another source of urea in teleosts could be purine degradation or uricolytic pathway which was first reported by Brunel (1937). Adenine and guanine produce uric acid as a catabolic product which further breakdown in a 3 step uricolytic pathway involving three enzymes - uricase, allantoinase and allantoicase to produce urea in most of the teleosts (Forster and Goldstein, 1969; Watts and Watts, 1974)(Fig.1). Cvancara (1969a) could find relatively high activity of uricase in nineteen species of freshwater teleosts and suggested that degradation of purines and nucleic acids might account for urea production at the levels of which it is found in the blood and excreted in teleosts. Saha and Ratha (1987) reported the presence of all the three uricolytic enzymes at least in the liver tissue of a freshwater air-breathing teleost, Heteropneustes fossilis and suggested that uricolysis could

be one of the pathway for the formation of urea in this fish in addition to the o-u cycle.

Dietary arginine : Arginase the last enzyme of the o-u cycle which converts arginine to urea and ornithine (Fig.1) has been reported to be present in various tissues of freshwater teleosts such as in liver (Hunter, 1929; Brown and Cohen, 1960; Huggins et al, 1969; Cvancara, 1969b; 1971; Wilson, 1973), kidney and heart (Hunter, 1929; Cvancara, 1969b), and to a lesser extent in spleen, gills, ovaries, testes and muscle of some teleosts (Cvancara, 1969b). Cvancara (1969b), therefore, has suggested that dietary arginine could be one of the major sources of urea in freshwater teleosts.

Active ureogenesis through o-u cycle has been confirmed in amphibians and terrestrial animals (Krebs and Henseleit, 1932; Cohen, 1976) and in marine fishes (Cohen, 1976; Pang et al, 1977; Hoar, 1983; Read, 1971; Mommsen and Walsh, 1989; Campbell, 1991; Wood,1993; Anderson,1994a). However, in freshwater teleosts the presence of a functional o-u cycle was not confirmed since some of the o-u cycle enzymes could not be detected in many of the teleosts studied (Manderscheid, 1933; Brown and Cohen, 1960; Wilson, 1973). Brown and Cohen (1960) could not detect CPS and OTC activity in several freshwater teleosts studied by them and therefore, suggested that the genes responsible for synthesizing some of these enzymes of o-u cycle, whose activities could not be detected, got deleted

and proposed the 'deletion' hypothesis. Huggins et al (1969) reported a full complement of o-u cycle enzymes in a variety of freshwater teleosts but with very low activity and suggested that the expression of the genes responsible for the synthesis of enzymes of the o-u cycle might have been altered as a result of an adaptational change in the freshwater teleosts when the excretion of ammonia was facilitated by diffusion. The presence of a regulatory physiological system for converting ammonia to urea via the o-u cycle has been well-documented in lungfishes (Janssens, 1964; Goldstein et al, 1967), mudskippers (Gregory, 1977; Gordon et al, 1969, 1978), and aquatic amphibians (Janssens and Cohen, 1968; Baldwin, 1970; Janssens, 1972; Balinsky, 1970) during their terrestrial life when the excretion of ammonia is not possible. Goldstein et al (1973) could also detect the activities of all o-u cycle enzymes in a well preserved sample of coelacanth liver which were comparable to those in elasmobranchs.

The lake Magadi (Kenya) tilapia (freshwater) Oreochromis alcalicus grahami, which lives in alkaline 'soda' lake having the water pH of 10 and osmolarity of 525 mOsm/kg, is reported to excrete large amounts of urea, rather than ammonia due to having a functional o-u cycle (Randall et al, 1989; Wood et al, 1989). This is the only known instance of complete ureotelism in a completely aquatic teleost fish. However, in Lahontan cutthroat trout, Oncorhynchus clarki henshawi, which also live in alkaline water of pH 9.4, the activities of o-u enzymes in

the liver were found to be low (Wilkie et al, 1993). High activities of all the o-u cycle enzymes in the liver of at least four species of freshwater air-breathing teleosts such as Heteropneustes fossilis, Clarias batrachus, Anabas testudineus and Amphipnous cuchia and in the kidney of three species (except A. testudineus) have been reported from our laboratory (Saha and Ratha, 1987; 1989). These fishes are primarily aquatic but breathe predominantly air by frequent surfacing. They usually inhabit stagnant and slow flowing shallow water bodies of ponds and lakes, and live in the mud during drought conditions and also frequently are being exposed to the air (Jhingran, 1983; Beavan, 1982). They are capable of tolerating temporary dehydration when kept outside water (Saha and Ratha, 1989). When they get exposed to outside water, an accumulation of toxic ammonia takes place in vivo since ammonia excretion into the surrounding environment is very difficult due to lack of water (Saha, 1986). At least in one of the above mentioned species, H. fossilis has been shown to tolerate a very high ambient ammonia (upto 75 mM NH_4Cl) which is unusual among freshwater teleosts and even for many amphibians (Saha, 1986; Saha and Ratha, 1990, 1991, 1994). The induction of o-u cycle enzymes in liver and kidney of H. fossilis and stimulation of urea production from accumulated ammonia, when these fishes were exposed to higher ambient ammonia (Saha and Ratha, 1986, 1991, 1994) and also when kept outside water (Saha, 1986, unpublished data), have been reported. In addition to the presence of

functional and regulatory o-u cycle, various other adaptations to nitrogen metabolism mainly to avoid ammonia toxicity have been reported in H. fossilis. The presence of higher physiological activity of glutamine synthetase (GS) and glutamate dehydrogenase (GDH) (reductive amination) in various tissues, and the induction of activities of these two enzymes under hyper-ammonia stress have been reported (Chakravorty, 1990; Chakravorty et al, 1989; Das, 1991; Das et al, 1991).

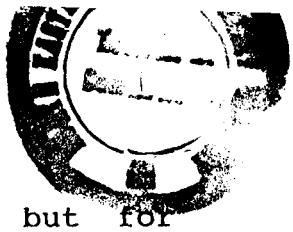
Adaptations to nitrogen metabolism, mainly a shift from ammoniotelism towards ureotelism, have been reported in various vertebrates such as amphibians during water shortage (Janssens and Cohen, 1968; McBean and Goldstein, 1970; Balinsky, 1970, 1981; Golstein, 1972), African lungfish during aestivation (Janssens, 1964; Goldstein et al, 1967), and mudskippers when exposed to air (Gordon et al, 1969, 1978) to avoid ammonia toxicity. Induction of o-u cycle and a shift towards ureotelism has been reported in a purely aquatic frog, Xenopus laevis when exposed to 10 mM NH_4Cl (Janssens, 1972), which is primarily ammoniotelic while living in water. Olson and Fromm (1971) found that goldfish, Carassius auratus when subjected to increased ambient ammonia level there was an increase in urea excretion rate. However, there is no report on the presence of a functional o-u cycle in goldfish. Three fold increase in urea production rate has been observed in ureotelic alkaline lake tilapia O. a. grahami when exposed to 0.5 mM ammonia at pH 10 (Wood et al, 1989) where the presence of

functional o-u cycle has been reported (Randall et al, 1989).

Induction of o-u cycle enzymes both in the liver and kidney tissues of H. fossilis was studied only when the fish was exposed to 50 mM NH_4Cl and also when exposed to the air in our laboratory. (Saha, 1986; Saha and Ratha, 1986, 1991, 1994).In both the cases the tissue ammonia concentration was significantly enhanced followed by the induction of the activity of the enzymes of o-u cycle (ureogenesis), suggesting that enhanced ammonia level in vivo was one of the factors to induce the activity of o-u cycle enzymes. However, the threshold concentration and maximum concentration of ammonia in vivo which was needed to cause initiation and maximum induction of all the o-u cycle enzymes could not be determined. This could be done by infusing different concentrations of NH_4Cl in perfused liver of H. fossilis and monitoring the level of activity of the o-u cycle enzymes.

Sub-cellular localization of o-u cycle enzymes and different isoenzymic forms of CPS :

Urea is synthesized in different groups of animals via o-u cycle, but for different purposes. In ureotelic species such as in mammals and amphibians, urea is synthesized from ammonia, a toxic metabolite, which is formed by the catabolism of amino acids and proteins, as a readily excretable form (Campbell, 1991). In ureosmotic marine elasmobranchs (sharks, skates and rays) urea is



synthesized via o-u cycle not for excretion but for retention for osmoregulation (Perlman and Goldstein, 1988). Nener (1988) have postulated that the o-u cycle is highly constrained in terms of enzyme composition and tissue localization among organism that produce urea for different purposes.

The synthesis of urea via the o-u cycle needs both mitochondrial and cytosolic enzymes. Some differences in the isoenzymic forms and the sub-cellular localization of some of the enzymes of o-u cycle have been reported, and correlated with their physiological functions in different groups of animals. In ureotelic species such as in mammals and amphibians, the first enzyme of the o-u cycle, carbamyl phosphate synthetase called CPS I is located in the mitochondrial matrix and utilizes ammonia as a nitrogen donating substrate for carbamyl phosphate synthesis, and requires the presence of N-acetyl glutamate (NAG) for catalytic activity (Ratner, 1973; Jackson et al, 1986). Another isozymic form of CPS (CPS II) found in ureotelic species is located in the cytosol and utilizes glutamine instead of ammonia as a nitrogen donating substrate for carbamyl phosphate synthesis. CPS II does not require NAG for its catalytic activity. Carbamyl phosphate formed by the cytosolic CPS II is utilized for pyrimidine synthesis and by the mitochondrial CPS I for urea synthesis (Ratner, 1973; Hager and Jones, 1967; Jones, 1980; Jackson et al, 1986; Campbell and Anderson, 1991). A third type of CPS, called CPS III was reported for the first time by Tramell

and Campbell (1970, 1971) in several species of invertebrates. CPS III requires NAG for catalytic activity like CPS I, but utilizes glutamine as the nitrogen donating substrate like CPS II and is located in the mitochondrial matrix (Campbell and Anderson, 1991; Anderson, 1994b). The role of CPS III has been reported to be in urea biosynthesis. The presence of CPS III has been reported in the liver of large mouth bass, Micropterus salmoides, a freshwater teleost (Anderson, 1976), alkaline lake Magadi tilapia, O. a. grahami (Randall et al, 1989), toad fish, O. beta (Mommsen and Walsh, 1989) and at much higher level in the liver of marine elasmobranch, Squalus acanthias (Anderson, 1980; 1981; Casey and Anderson , 1983). The second enzyme of the o-u cycle, ornithine transcarbamylase (OTC) has always been localized within the mitochondrial matrix in all ureotelic and ureosmotic vertebrates (Ratner, 1973; Gamble and Lehninger, 1973; Vorhaben and Campbell, 1977; Casey and Anderson, 1985; Campbell and Anderson,1991). The third, fourth and fifth enzyme of o-u cycle , arginino-succinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase (ARG) respectively, have been reported to be cytosolic in several ureotelic species (Ratner, 1973; Skrzypek-Osiecka et al, 1980; Jackson et al, 1986). In contrast to ureotelic species, ARG in uricotelic and ammoniotelic species is reported to be mitochondrial (Tsuyama et al, 1980; Taylor and Stewart, 1981; Carvajal et al, 1987, Dkhar et al, 1991). Casey and Anderson (1985) have reported the

mitochondrial localization of ARG in ureosmotic elasmobranch, S. acanthias. Mitochondrial localization of ARG has also been reported in the liver of gulf toadfish, O. beta (Mommsen and Walsh, 1989; Anderson and Walsh, 1994).

Mommsen and Walsh (1989) suggested that the urea cycle, which is a monophyletic trait in vertebrates, underwent two key changes during the course of vertebrate evolution : i) a switch over from CPS III to CPS I and ii) replacement of mitochondrial arginase by a cytosolic equivalent. The presence of a functional o-u cycle with comparatively higher physiological level of all the enzymes and mitochondrial ARG in H. fossilis are unique features among freshwater teleosts (Saha and Ratha, 1987,1989; Dkhar et al, 1991). The mitochondrial localization of glutamine synthetase (GS) in both liver and kidney of H. fossilis (Chakravorty et al, 1989) resembles those of elasmobrachs (Webb and Brown, 1976, 1980; Anderson, 1982, Smith et al, 1987) and uricotelic species (Vorhaben and Campbell, 1977; Campbell et al, 1983, 1984) where glutamine dependent CPS III isoenzyme have been reported to help in urea synthesis. However, the isoenzymic patterns of CPS has not yet been known in H. fossilis.

Annual variation of o-u cycle enzymes:

Induction of o-u cycle enzymes by hormones such as thyroxine, glucagon, glucocorticosteroid, corticosterone have been reported (Schultheiss, 1977; Balinsky et al,

1972; Husson et al, 1987; Kumar and Kalyankar , 1984; Marti et al, 1988; Lamers and Mooren, 1981). The extent of induction is dependent on the concentration of the hormones, time of exposure, temperature and the age of the animal. The levels of steroid hormones in vivo alter during various stages of reproductive cycle in fish in a year. The levels of these hormones are high during the pre-spawning and spawning periods and low during post-spawning phase (Sundararaj, 1959; Liley, 1969; Sundararaj and Goswami, 1969; Lamba et al, 1983). Bryla et al (1977) has reported that in perfused rat liver, glucagon increased citrulline production by about 1.6 times over the period from September to October, whereas from October to February, the rate of citrulline synthesis were reported to be less. Cohen et al (1982) have reported that isolated rat liver mitochondria showed extremely low rate of citrulline synthesis during the month of February. The activity of ARG from the liver of Clarias batrachus was found to be maximum in the month of July whereas from the month of September to October the activity was relatively low and did not change much during this period (Singh and Singh, 1988). GDH, which is one of the important enzymes in nitrogen metabolic pathway , has been reported to show annual variation in its activity in different tissues of H. fossilis (Das, 1991). GDH activity in reductive amination direction were found to be maximum in summer (May to June) and minimum in winter. However, in oxidative deamination direction it was found maximum in winter

(November to March) and minimum in summer (June to July). However, there is no report on the annual variation of the activity of the o-u cycle enzymes in ureogenic tissues of H. fossilis which is a seasonal breeder.

Molecular and kinetic properties of arginase :

Arginase is a ubiquitous enzyme and has been studied in various groups of organisms. This enzyme catalyzes the hydrolysis of arginine into urea and ornithine, and plays an important role in nitrogen metabolism (Lund and Wiggins, 1986; Morris, 1992). Ornithine is the precursor of polyamine synthesis in animals. Arginase occurs in almost all organisms including plants and bacteria (Ratner, 1973; Jackson et al, 1986; Nener, 1988) besides all ureogenic and non-ureogenic species and tissues (Baby et al, 1976; Huggins et al, 1969; Singh and Singh, 1988; Cvancara,1969b; Blachier et al, 1991; Dhanakoti et al, 1992; Jenkinson and Grigor. 1994). The universal distribution of arginase suggests that it appeared very early in the process of biochemical evolution. In insects, arginase helps in the conversion of arginine to proline via the formation of ornithine (Reddy and Campbell, 1969). Proline serves as the substrate for energy production in insects (Bursell, 1981).

Significant differences in arginase from ureotelic, uricotelic and ammoniotelic animals have been reported with respect to their molecular weight, substrate specificity, stability etc. (Reddy and Campbell, 1970; Hirsch-Kolb et

al, 1970; Rossi and Grazi, 1969). The Km value for arginine reported for several mammalian arginases lie in the range of 6 to 20 mM (Hirsch-Kolb et al, 1970). The pH optima for mammalian arginases lie in the range of 9.3 to 10.5 (Hirsch-Kolb et al, 1970). The pH optima for hepatic arginase from the teleost fish, Genypterus maculatus (Carvajal et al, 1987), Merluccius gayi (Carvajal et al, 1989) and from the freshwater air-breathing teleost, Clarias batrachus (Singh and Singh, 1990) was reported to be 9.5

Arginase requires Mn^{2+} for its catalytic activity and stability, although its oligomeric composition remains obscure (Hirsch-Kolb et al, 1971, Maggini et al, 1992; Turkoglu and Ozer, 1992). It has been reported that deficiency of hepatic Mn^{2+} in rat is associated with a decrease in arginase activity (Visek et al, 1992; Brock et al, 1994). Mg^{2+}, Co^{2+} and Ni^{2+} ions also serve as activators for arginase in addition to Mn^{2+}, whereas Zn^{2+} and Cd^{2+} ions act as inhibitors of arginase. Most mammalian arginases with the exception of beef liver arginase were inhibited by Ni^{2+} and Co^{2+} (Hirsch-Kolb et al, 1970). Rat hepatocyte plasma membrane bound arginase was inhibited by Cu^{2+}, Zn^{2+} and to a lesser extent by Co^{2+} (Fuentes et al, 1991). In the teleost fish, M. gayi, the metal ion requirement of arginase was accomplished by Mn^{2+}, and to a much lesser extent by Cd^{2+} and Co^{2+} (Carvajal et al, 1989). Mg^{2+}, Ca^{2+} and Co^{2+} activated the enzyme activity purified from the liver of guinea pig (Farooqui et al, 1978). Hepatic arginase of

freshwater air-breathing teleost, C. batrachus was activated by Co^{2+} and Ni^{2+} and inhibited by Cd^{2+} (Singh and Singh, 1988).

Bovine liver arginase was inhibited by many amino acids (Hunter and Downs, 1945). Ornithine, lysine and proline were competitive inhibitors of sheep liver arginase. On the other hand, activation of arginase with divalent cations altered the kinetics of inhibition of the enzyme by amino acids (Rao et al, 1973). Ornithine showed a mixed type of inhibition for the enzyme from rat kidney (Gasiorowska et al, 1970) and human liver (Bascur et al, 1966). Partial inhibition by proline, leucine, valine and isoleucine observed in the liver as well as in kidney arginase, indicated the existence of allosteric sites on both hepatic and renal enzymes (Carvajal and Cederbaum, 1986). Branched chain amino acids leucine, isoleucine, valine, ornithine, lysine and proline inhibited the enzyme from the liver of M. gayi (Carvajal et al, 1989). Ornithine and leucine acted as competitive inhibitors, whereas valine and isoleucine acted as non-competitive inhibitors for arginase purified from the liver of C. batrachus (Singh and Singh, 1990).Besides amino acids, polyamines also influence the activity of purified arginase (Subramanyam and Reddy, 1986).

Mammalian liver arginase is composed of 4 sub-units each having the molecular weight of 30,800 giving the enzyme an overall molecular weight of 120,000 (Hirsch-Kolb and Greenberg, 1968; Hirsch-Kolb et al, 1970;1971). However,

some data suggested that arginase from rat liver, though a mammal, was reported to be a trimer (Kanyo et al, 1992; Penninckx et al, 1974). The molecular weight of arginase reported from the gut of earthworm was 27,000 (Reddy and Campbell, 1968), from the land planarian was 2,40,000, from gull liver was 1,20,000 (Reddy and Campbell, 1970), from human erythrocyte was 1,05,000 (Ikemoto et al, 1989), from the liver of Squalus acanthias was 1,05,000 (Casey and Anderson, 1982), from Xenopus laevis liver was 76,000 (Peiser and Balinsky, 1982) and from the liver of C. batrachus was 87,000 (Singh and Singh, 1990). The existence of heterogeneity in size and charge of arginase sub-units from mouse liver have been reported (Spolarics and Bond, 1988). Different isozymic forms of arginase has been reported from the liver, kidney, sub-maxillary gland, intestine and pancreas on the basis of their tissue distribution, chromatographic behaviour, electrophoretic mobility and intereaction with antiserum (Herzfeld and Raper, 1976; Kaysen and Strecker, 1973; Venkatakrishnan and Reddy, 1983; Gasiorowska et al, 1970; Reddi et al, 1975; Porembska et al, 1971; Glass and Knox , 1973; Singh and Singh, 1988, 1990; Turkoglu and Ozer, 1991; Jenkinson and Grigor, 1994). The physiological level of arginase enzyme activity in the liver of ureogenic fish, H. fossilis has been reported to be quite high (Saha and Ratha, 1987)and about 60% of this activity is localized in the mitochondria and 40% in the cytoplasm. However, nothing is known about the molecular properties and isoenzymic pattern

of arginase in H. fossilis.

Objective :

It can be seen from the foregoing reports that the freshwater air-breathing teleost, Heteropneustes fossilis, is unique having ureogenic potential and adaptibility to shift from ammoniotelism to ureotelism under hyper-ammonia stress. It has mitochondrial glutamine synthetase and arginase activity in its liver and kidney. All these characters are unusual for a freshwater teleosts and show affinity with marine elasmobranchs, aquatic amphibians and reptiles. This requires a lot of additional studies on the effect of ammonia as an inducer for o-u cycle enzymes, possibility of glutamine besides ammonia being used as a substrate for ureogenesis, and the molecular and kinetic properties of the enzymes. Ubiquitous arginase was selected for purification and study of its properties in detail. To clarify these points,the following plan of work was made.

Plan of Work:

The work was planned as follows with the above objective in mind :

1. The liver of H. fossilis was perfused with haemoglobin-free media and different concentrations of NH_4Cl (0.01 to 1 mM) was infused into the liver for 60 min and the following observations were made.
   a) The level of ammonia in the perfused liver after infusing different concentrations of NH_4Cl for 60

min.

b) The amount of ammonia coming out into the effluent out of the total infused into the liver at every 2 min interval.

c) The activity of all the five o-u cycle enzymes such as CPS (ammonia dependent), OTC, ASS, ASL and ARG) in perfused liver.

d) The amount of urea-N coming out into the effluent at every 2 min interval of infusing the NH_4Cl.

2) In another set of experiment L-glutamine (1 mM and 2mM) instead of ammonia was infused into the perfused liver of H. fossilis for 60 min and the following observations were made:

a) The amount of urea-N coming out into the effluent at every 2 min interval of infusing L-glutamine.

b) The activity of CPS III (glutamine dependent) activity in the perfused liver after infusing L-glutamine for 60 min.

3) The occurence of three different isoenzymes of carbamyl phosphate synthetase (CPS I, II and III) were confirmed, and the sub-cellular localization of all the three isoenzymes of CPS and other four enzymes of o-u cycle such as OTC, ASS, ASL and ARG were studied both in the liver and kidney of H. fossilis.

4) The activity of all the o-u cycle enzymes both in the liver and kidney of H. fossilis were studied every month for one year to find out annual variation, if

any.

5) The enzyme arginase was purified from the liver of H. fossilis, and the fold of purification and percentage recovery were determined.

6) The molecular weight and various physico-chemical properties of purified arginase were determined.

7) The kinetic and the effect of different regulators such as metal ions and amino acids on the activity of purified arginase were studied.

MATERIALS AND METHODS

Animals :

Heteropneustes fossilis weighing 40-50 g were purchased from commercial sources. They were maintained in the laboratory at 25 ± 2 °C in plastic aquaria containing filtered tap water with 12 hr:12 hr light and dark period. Minced pork liver (5% of body weight) was supplied as food on every alternate day and water was changed regularly on the day after feeding. The fishes were used after their acclimatization to laboratory conditions for at least four weeks when death rate became zero and food consumption was normal. They were used for all estimations and for all experiments 24 hr after the last feeding. In all experiments the fishes were sacrificed at a fixed time of the day (12 noon).

Liver perfusion technique :

H. fossilis (40-50 g body wt.) acclimatized under laboratory conditions as mentioned above were used for perfusion of liver. Livers were perfused via the portal vein in a non-recirculating manner with the haemoglobin free media used by French *et al* (1981) with certain modifications. The media contained 119 mM NaCl, .5 mM $NaHCO_3$, 5.4 mM KCl, 0.35 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 0.81 mM $MgSO_4$ and 1.25 mM of $CaCl_2$ as a basic solution for perfusion. The osmolarity of the perfusing media was 265 mosmol per litre since the osmolarity of the plasma of *H. fossilis* was also 265 mosmol per litre determined by freezing point depression technique. Hence, an isotonic

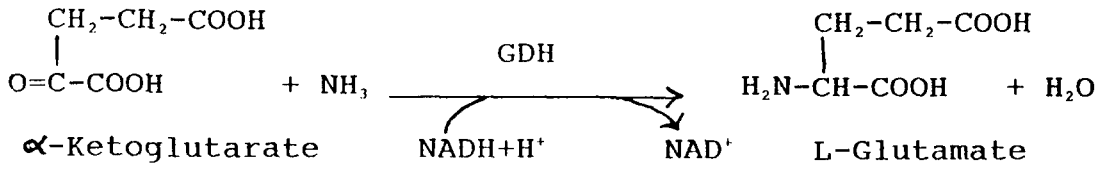
solution of same osmolarity was used for liver perfusion of this fish. The media also contained 5 mM glucose and 2 mM L-ornithine. Different concentrations of NH_4Cl (0.01 to 1 mM) were infused along with the perfusion media to study the effect of ammonia on o-u cycle enzymes activity, its accumulation rate and also its conversion to urea in the perfused liver. Ammonia was replaced by L-glutamine (1 and 2 mM) in some perfusions to study its conversion rate to urea. The media was gassed with oxygen before perfusing into the liver and temperature of the media was 30 °C. The media was infused into the liver at a flow rate of 5 to 6 ml per g of liver per min. The effluent which was coming out of the liver was collected through a pipe catheterised at the superior venacava for analysis of ammonia and urea-N.

Livers were perfused for 20 min with the standard media containing 5 mM D-glucose and 2 mM L-ornithine prior to infusion of NH_4Cl. Different concentrations of NH_4Cl and L-glutamine were infused for 60 min. Immediately after 60 min of infusion of NH_4Cl the perfused liver were deep frozen and stored at -20 ± 2 °C until used for measurement of o-u cycle enzymes activity and also the tissue ammonia level.

Estimation of ammonia and urea-N in the effluent : Concentration of ammonia and urea-N in the effluent were measured enzymatically based on the procedure of Kun and Kearney(1974). One ml of effluent was collected after every two min of perfusion. Samples were collected

just before infusing NH_4Cl or L-glutamine and during infusion of NH_4Cl or L-glutamine regularly after every two min of infusion in test tubes. Immediately after collecting the sample, 10 µl of 2 M PCA was added in each tube to precipitate out the protein present in the sample. The precipitate was separated by centrifugation and the supernatant was neutralised by adding 10 µl of 2 M NaOH in each tube before the measurement of ammonia and urea-N.

Ammonia : For measurement of ammonia in the effluent, all the ammonia was converted to glutamate by the enzyme glutamate dehydrogenase (GDH) in presence of ∝-keto-glutarate and NADH. The amount of NADH oxidized was equivalent to the amount of ammonia present in the effluent.

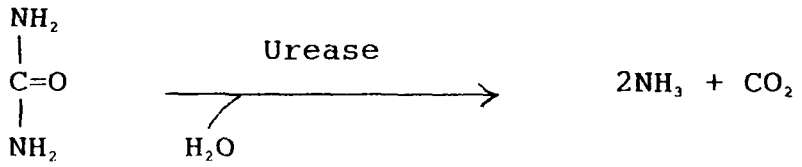


The assay mixture of 1 ml contained the following :

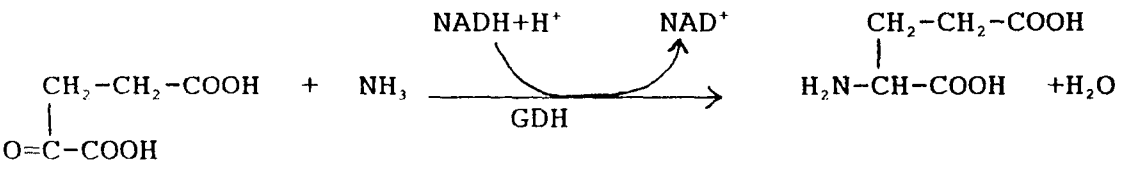
Tris-HCl buffer (pH 8.0)	66 µmoles
∝-Ketoglutarate	2.5 µmoles
EDTA	0.2 µmol
ADP	1.0 µmol
NADH	0.4 µmol
GDH	20 units
Effluent	0.2 ml.

The reaction mixture was incubated at 37 °C for 30 min. A control also was run simultaneously which contained everything in the reaction mixture as mentioned above except the effluent which was replaced by 0.2 ml of distilled water. O.D. was measured at 340 nm in a quartz microcuvette having 1 cm light path in a uv-visible spectrophotometer (Beckman, Model 26) both in the control and in the reaction mixture containing effluent. The differences in O.D. value obtained between these two was used to calculate the concentration of ammonia present in the effluent taking 6.22×10^3 as molar extinction coefficient for NADH.

<u>Urea-N</u> : For measurement of urea-N in the effluent, urea was first converted to ammonia by urease and then to glutamate in presence of ∝-Ketoglutarate and NADH by the enzyme GDH. The amount of NADH oxidized was equivalent to the amount of urea-N present in the effluent.



Urea



∝-Ketoglutarate L-Glutamate

The assay mixture of 1 ml contained the following :

Tris-HCl buffer (pH 8.0)	66 µmoles
α-Ketoglutarate	2.5 µmoles
EDTA	0.2 µmol
NADH	0.4 µmol
GDH	20 units
Urease	20 units
Effluent	0.2 ml

The reaction mixture was incubated at 37 °C for 30 min. A control also was run simultaneously which contained everything as mentioned above except the effluent which was replaced by 0.2 ml of distilled water. O.D. was measured at 340 nm in a quartz microcuvette having 1 cm light path in a uv-visible spectrophotometer (Beckman, Model 26) both in the control and in the reaction mixture containing effluent. The differences in O.D. value between these two was used to calculate the concentration of urea-N present in the effluent taking 6.22×10^3 as molar extinction coefficient for NADH.

Tissue processing :

Fishes were killed by decapitation and tissues such as liver and kidney were immediately removed, blotted dry and deep frozen at -20 °C until used for estimations. All the estimations were completed within 2 to 3 days after collecting the tissue. A 20% homogenate was prepared for both liver and kidney tissues in a Potter-Elvehjem type

motor driven glass homogenizer with a teflon pestle at 0 ± 2 °C. For annual variation studies, female fishes were killed on the first day of every month for one year, and liver and kidney tissues were collected.

To study the sub-cellular localization of all the o-u cycle enzymes and different isoenzymic forms of carbamyl phosphate synthetase (CPS I,II,III) along with the marker enzymes, each frozen tissue (liver and kidney) was thawed on ice and a 20% homogenate was prepared in 10 mM Tris-HCl buffer (pH 7.5) containing 0.1M KCl, 1 mM EDTA and 0.3 M mannitol. Different sub-cellular fractions such as nuclear, mitochondrial and cytoplasmic fractions were separated by differential centrifugation technique following the method as described in Chakravorty et al (1989) and Dkhar et al (1991). The pellet of centrifugation at 600 x g at 0 ± 2 °C for 15 min was resuspended in the homogenizing medium and recentrifuged once again at 600 x g at 0 ± 2 °C for 15 min. The pellet thus obtained was the nuclear fraction. The supernatant of the first and second centrifugations were pooled together and centrifuged at 14,000 x g at 0 ± 2 °C for 60 min. The pellet obtained was the mitochondrial fraction whereas the supernatant was the cytoplasmic fraction. The nuclear and mitochondrial pellets were resuspended in the homogenizing medium. Treatment of the tissue homgenates with Triton X-100 (0.5%) resulted in maximum release of o-u cycle enzyme activity within 20-30 min (Tables 8 and 9). Therefore, the different sub-cellular fractions were treated with 0.5%

Triton X-100 for 30 min before assaying of the enzyme activity.

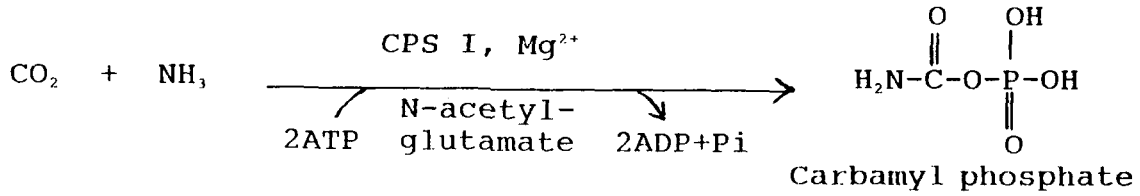
For other experiments, the frozen tissue was thawed on ice and a 20% homogenate of each was prepared in 0.1% cetyltrimethyl ammonium bromide (CTB). The homogenate was centrifuged at 600 x g at 0 ± 2 °C for 15 min and the supernatant was used for measurement of tissue ammonia level and also for assaying the enzymes such as carbamyl phosphate synthetase I,II and III, ornithine trans-carbamylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase (ARG) and also for measurement of proteins.

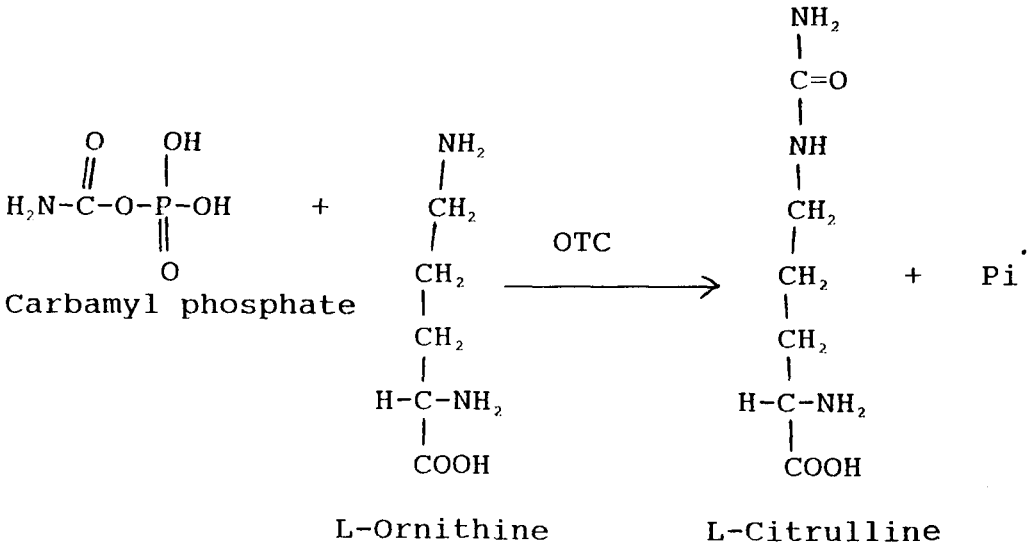
Estimation of ammonia in perfused liver: The perfused liver homogenates which were prepared for o-u cycle enzymes assay in 0.1% CTB, were also used for ammonia estimation. The supernatant obtained after centrifuging the homogenate at 600 x g for 10 min, was treated with 2M PCA at 1:0.5 ratio to precipitate the protein. The precipitated protein was separated out by centrifugation. The supernatant was neutralised with 2M NaOH before estimation of ammonia. Ammonia was estimated by the enzymatic method as mentioned above for estimation of ammonia in effluent.

Enzyme assay :

Carbamyl phosphate synthetase I (E.C.2.7.2.5)(CPS I): CPS I was assayed following the method of Saha and Ratha (1987) with certain modifications. In the reaction mixture

methionine sulfoxamine (25 mM) and UTP (1 mM), the inhibitors for glutamine synthetase (GS) and CPS II (glutamine dependent) respectively, were added so that CPS II and III activity do not interfere while measuring the CPS I activity. Carbamyl phosphate so formed by CPS I during the period of incubation was converted to citrulline in presence of excess of OTC and L-ornithine. The resultant citrulline was estimated for expressing the activity of CPS I.





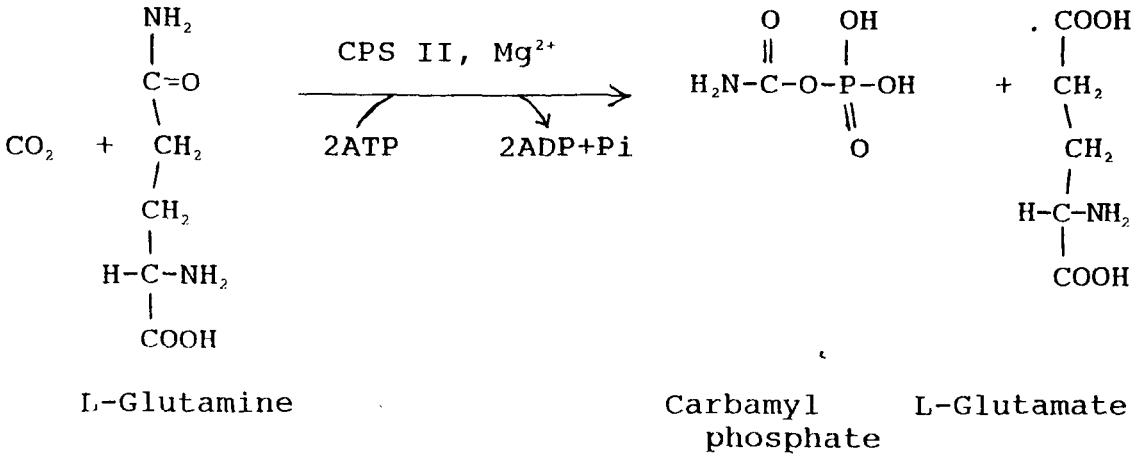
The assay mixture in a final volume of 1.0 ml contained :

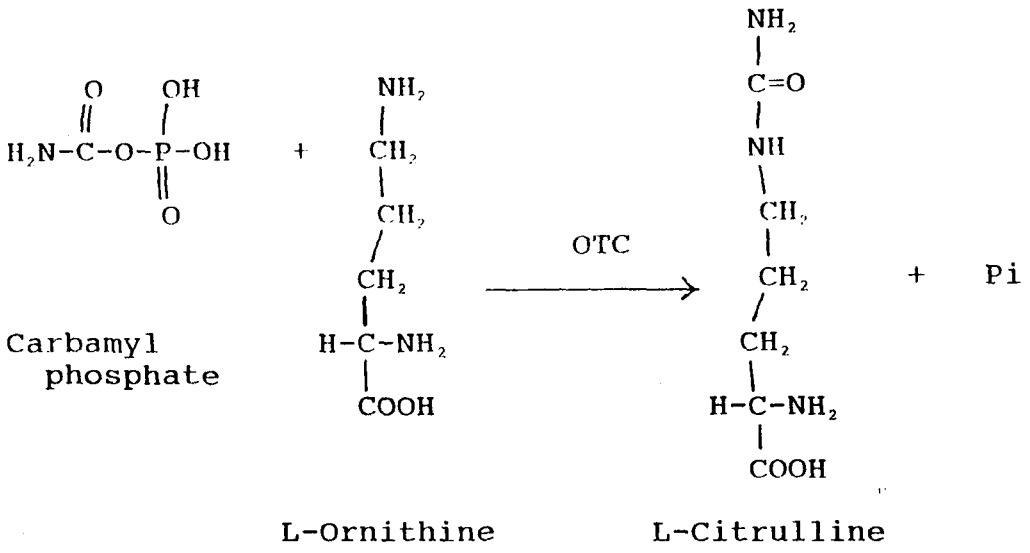
Potassium phosphate buffer (pH 7.5)	50 µmoles
Ammonium chloride	50 µmoles
Sodiumbicarbonate	50 µmoles
ATP	5 µmoles
L-ornithine	5 µmoles
N-acetyl glutamate	5 µmoles
$MgSO_4$	10 µmoles
Methionine sulfoxamine	25 µmoles
UTP	1 µmol
OTC	10 units
Suitably diluted tissue extract	0.3 ml

The assay mixture without tissue extract, methionine sulfoxamine and UTP, and tissue extract with methionine sulfoxamine and UTP were preincubated separately for 5 min at 30 °C. The reaction was initiated by mixing these two mixtures. After 30 min the reaction was stopped by adding 0.5 ml of 10% perchloric acid (PCA). A tissue blank was prepared simultaneously by adding PCA to the reaction mixture prior to the addition of tissue extract. The precipitated protein was separated out by centrifugation. The supernatant was used for citrulline estimation following the method of Moore and Kauffman (1970). 1.0 ml of suitably diluted supernatant was treated with 2.5 ml of acid mixture (300 ml H_3PO_4, 100 ml H_2SO_4, 0.237 g $MnSO_4$ in 398 ml distilled water and 1.8 ml of 0.1M $FeCl_3$) followed by 0.25 ml of 3% (w/v) diacetyl monoxime. The mixture was

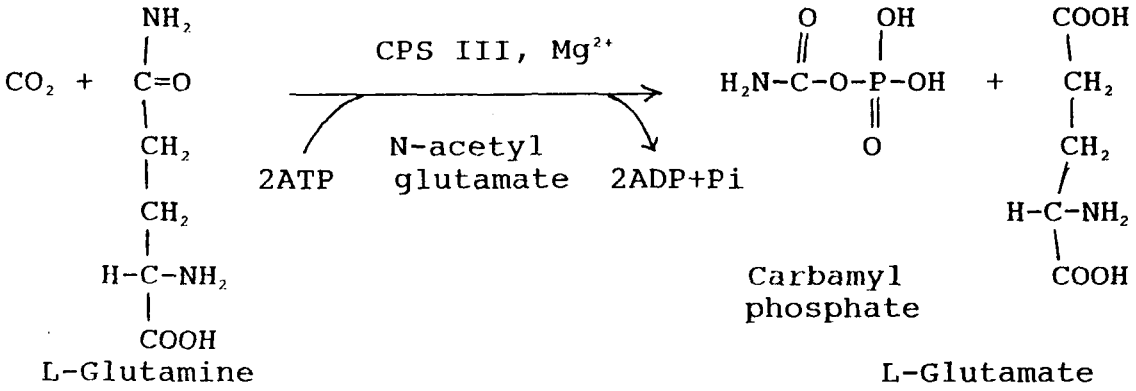
kept for boiling for 30 min, cooled and O.D was read at 490 nm in a spectrophotometer (Beckman, Model 26) against the tissue blank. The amount of citrulline present was calculated from the standard graph prepared using different concentrations of citrulline (0.04-0.1 μmol) which was linear. One unit of CPS I activity was expressed as that amount of enzyme which catalyzed the formation of 1 μmol of citrulline per hr at 30 °C.

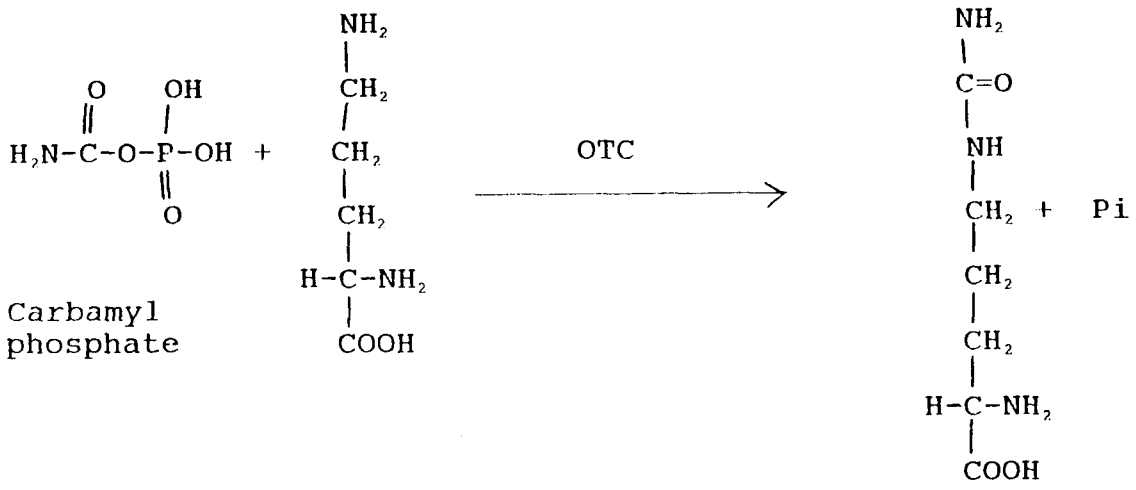
Carbamyl phosphate synthetase II (CPS II) : CPS II was assayed exactly in the same way as for CPS I with the following modifications. Glutamine (20 mM) was taken as nitrogen donor instead of ammonium chloride. N-acetylglutamate (co-factor), methionine sulfoxamine (inhibitor for GS) and UTP (inhibitor for CPS II) were not added in the reaction mixture. The resultant carbamyl phosphate formed during the period of incubation was converted to citrulline in presence of excess of OTC and L-ornithine. The citrulline so formed was estimated for expressing the activity of CPS II.



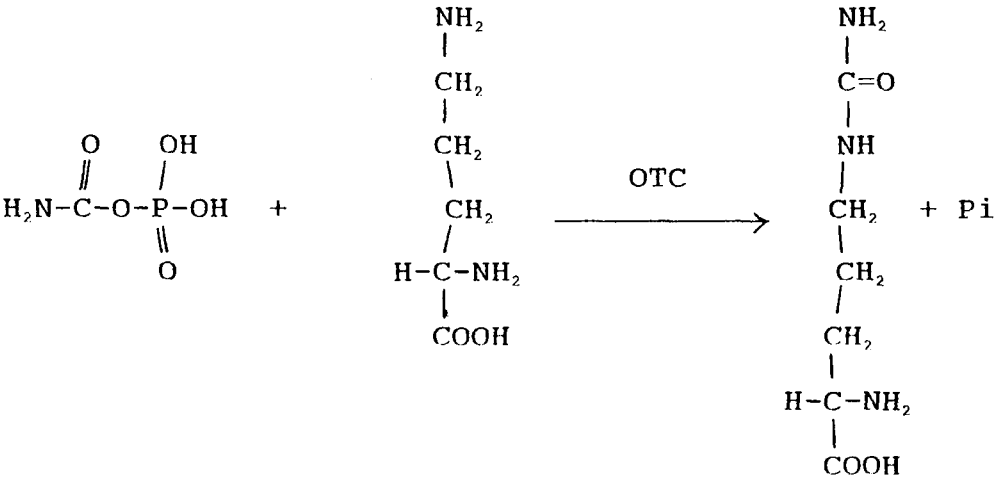


Carbamyl phosphate synthetase III (CPS III) : CPS III was assayed exactly in the same way as for CPS I with the following modifications. Glutamine (20 mM) was taken in the reaction mixture as nitrogen donor instead of ammonium chloride. Methionine sulfoxamine (inhibitor for GS) was not added in the reaction mixture. The carbamyl phosphate so formed by CPS III during the period of incubation was converted to citrulline in presence of excess of OTC and L-Ornithine. Resultant citrulline was estimated following the method as mentioned for CPS I, for expressing the activity of CPS III.





Ornithine transcarbamylase (E.C.2.1.3.3) (OTC) : OTC was assayed following the method described by Brown and Cohen (1959) by estimating the product (citrulline) formed.



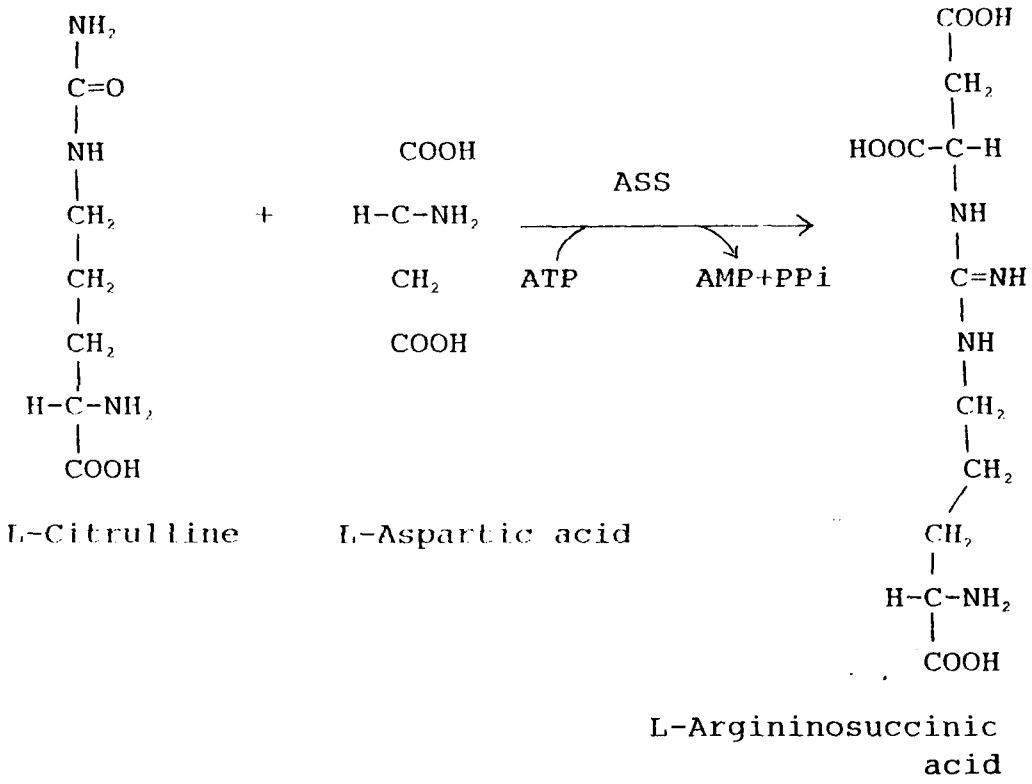
Carbamyl phosphate L-Ornithine L-Citrulline .

The assay mixture in a final volume of 2.0 ml contained :

Glycylglycine buffer (pH 8.3)	90 µmoles
L-Ornithine	20 µmoles
Dilithium carbamyl phosphate	20 µmoles
Suitably diluted tissue extract	0.3 ml

The assay mixture without tissue extract was pre-incubated for 5 min at 30 °C. The reaction was initiated by addition of tissue extract. After 20 min of incubation the reaction was stopped by the addition of 0.5 ml of 10% PCA to the reaction mixture. A tissue blank was prepared by adding PCA to the reaction mixture prior to the addition of tissue extract. The precipitated protein was separated out by centrifugation. Citrulline so formed during the period of incubation was estimated in the supernatant following the method of Moore and Kauffman (1970) as described above for CPS I assay. One unit of OTC activity was expressed as that amount of enzyme which catalyzed for the formation of 1 μmol of citrulline per hr at 30 °C.

Argininosuccinate synthetase (E.C.6.3.4.5) (ASS) : ASS activity was assayed following the method of Ratner (1955) with the following modification as described by Saha and Ratha (1987). In the assay mixture, 20 units of urease (Sigma Type IV) were taken to convert all the urea present or formed to ammonia to avoid interference with citrulline estimation. The amount of citrulline utilized per unit time was used to express ASS activity.



L-Citrulline L-Aspartic acid

L-Argininosuccinic acid

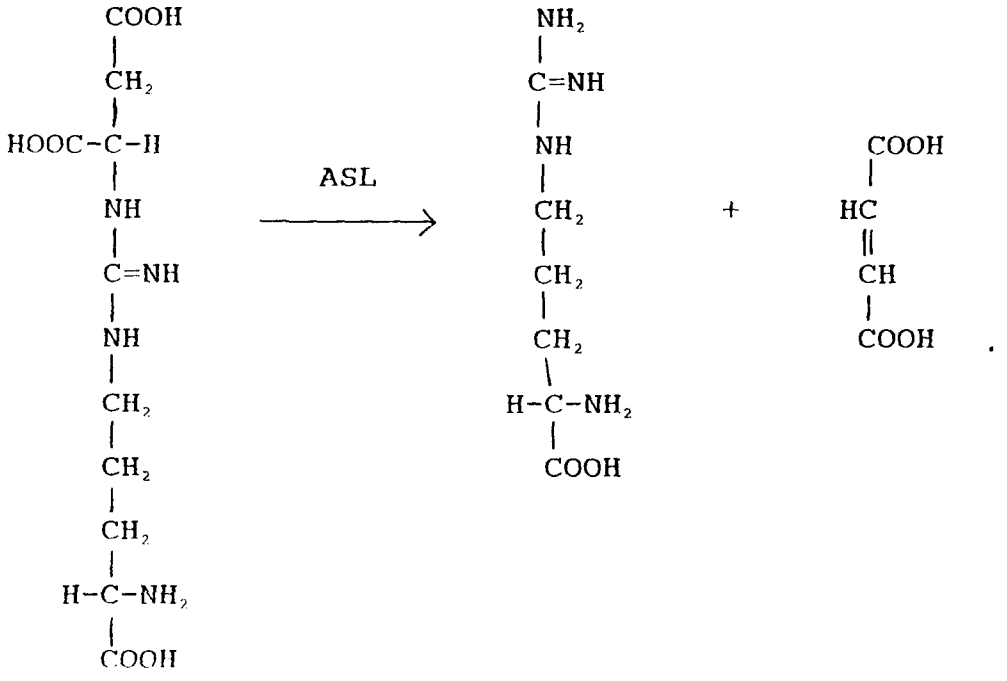
The assay mixture (pH 7.0) in a final volume of 1.0 ml contained :

Potassium phosphate buffer (pH 7.0)	50 µmoles
L-Citrulline	3 µmoles
L-Aspartic acid	5 µmoles
$MgSO_4$	8.75 µmoles
ATP	5 µmoles
Urease	20 units
Suitably diluted tissue extract	0.2 ml.

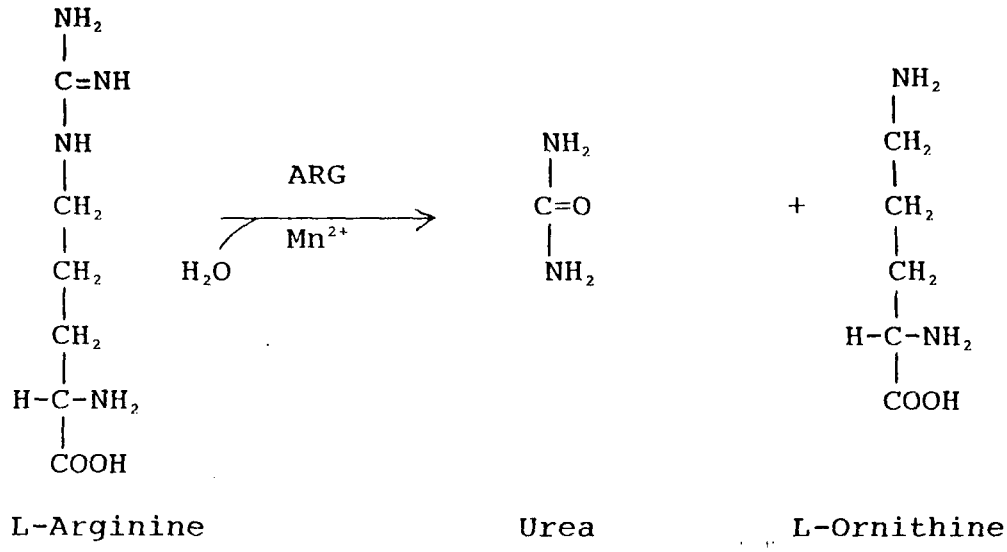
The reaction mixture without citrulline was pre-incubated for 5 min at 30 °C. The reaction was initiated with the addition of citrulline and incubated for 30 min at 30 °C. The reaction was stopped by the addition of 0.5 ml of 10% PCA. In the tissue blank PCA was added in the reaction mixture just before the addition of citrulline.

The precipitated protein was separated out by centrifugation. The amount of citrulline utilized during the incubation period was estimated in the supernatant following the method of Moore and Kauffman (1970) as described above in CPS I assay. One unit of ASS activity was expressed as that amount of enzyme which catalyzed the utilization of 1 μmol of citrulline per hr at 30 °C.

Argininosuccinate lyase (E.C.4.3.2.1) (ASL) : ASL activity was assayed following the method of Brown and Cohen (1959) with certain modifications suggested by Saha and Ratha (1987). The concentration of L-argininosuccinic acid was increased from 2 μmoles to 4 μmoles and 20 units of arginase (from Sigma) was added in each reaction mixture. Arginine formed by ASL activity in the tissue was converted to urea in presence of excess arginase and the amount of urea formed was estimated to express the ASL activity.



L-Argininosuccinic acid L-Arginine Fumaric acid



L-Arginine Urea L-Ornithine

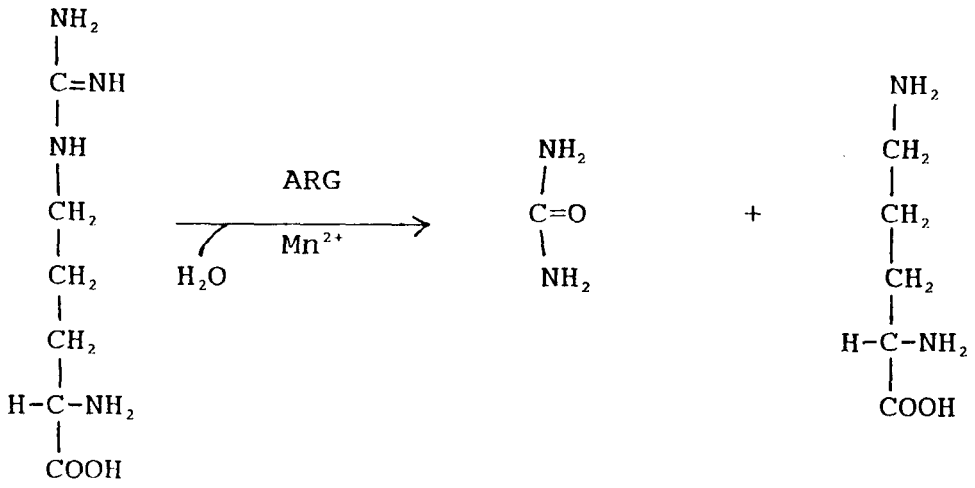
The assay mixture in a final volume of 1.0 ml contained :

Potassium phosphate buffer (pH 7.3)	50 µmoles
L-Argininosuccinic acid	4 µmoles
Arginase	20 units
Suitably diluted tissue extract	0.2 ml.

The reaction mixture without tissue extract was pre-incubated for 5 min at 30 °C. The reaction was initiated by adding tissue extract and the reaction was stopped after 30 min by the addition of 0.5 ml 10% PCA. At tissue blank was prepared with each assay by adding PCA to the reaction mixture prior to the addition of tissue extract. The precipitated protein was separated out by centrifugation. Urea was estimated following the method of Moore and Kauffman (1970). The method was same as described for citrulline estimation except that the O.D. was taken at 478 nm. The concentration of urea was calculated from a linear

standard graph prepared with different concentrations (0.01 to 0.05 μmol) of urea. One unit of ASL activity was expressed as that amount of enzyme which catalyzed the formation of 1 μmol of urea per hr at 30 °C.

Arginase (E.C.3.5.3.1) (ARG) : ARG activity was assayed following the method of Brown and Cohen (1959) with the following modification as suggested by Saha and Ratha (1987). The concentration of L-arginine was increased to 50 μmoles per assay. The urea formed during the incubation was estimated to express ARG activity.



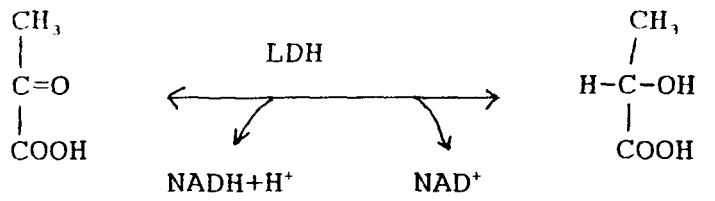
L-Arginine Urea L-Ornithine

The assay mixture in a final volume of 2.0 ml contained :

Sodium glycinate buffer (pH 9.5)	50 μmoles
L-Arginine	50 μmoles
$MnCl_2$	0.5 μmol
Suitably diluted tissue extract	0.1 ml.

The reaction mixture without L-arginine was pre-incubated for 10 min at 30 °C. The reaction was initiated by the addition of L-arginine and incubated for 15 min at 30 °C. The reaction was stopped by adding 1.0 ml of 10% PCA.A tissue blank was prepared with each assay by adding PCA to the reaction mixture prior to addition of L-arginine. The precipitated protein was separated out by centrifugation and the supernatant was used for urea estimation. The amount of urea formed during the incubation period was estimated following the method of Moore and Kauffman (1970) as described above in ASL assay. One unit of arginase activity was expressed as that amount of enzyme which catalyzed the formation of 1 μmol of urea per hr at 30 °C.

Lactate dehydrogenase (E.C.1.1.1.27) (LDH) : LDH activity was assayed following the method of Vorhaben and Campbell (1972) :



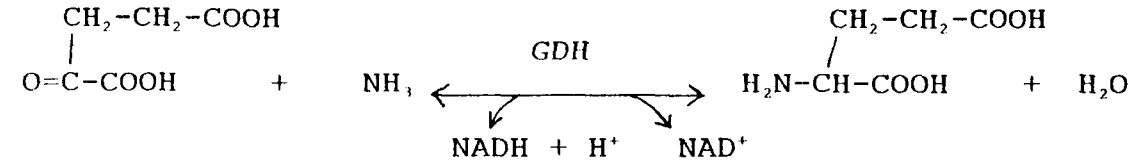
Pyruvate Lactate

The reaction mixture of 3.0 ml contained the following :

Potassium phosphate buffer (pH 7.0)	120 μmoles
Sodium pyruvate	1 μmol
NADH	0.5 μmol
Suitably dilted tissue extract	50 μl.

The assay mixture was incubated in a rectangular guartz cuvette having 1 cm light path directly in a uv-visible spectrophotometer (Beckman, Model 26). The reaction mixture without the tissue extract was pre-incubated for 5 min at 30 °C. The reaction was started by adding the tissue extract. The decrease in O.D. was recorded at 340 nm at 30 sec intervals and the period of linear decrease was used for calculation of LDH activity. The amount of NADH utilized per hr was calculated taking 6.2 $x10^3$ as molar extinction co-efficient for NADH. One unit of LDH activity was expressed as that amount of enzyme which catalyzed the oxidation of 1 µmol of NADH to NAD^+ per hr at 30 °C.

Glutamate dehydrogenase (E.C.1.4.1.3)(GDH) : GDH was assayed following the method of Olson and Anfinsen (1952) with some modifications described by Das et al (1991).



∝-Ketoglutarate Ammonia L-Glutamate

The reaction mixture of 3.0 ml contained :

Potassium phosphate buffer (pH 8.5)	200 µmoles
L-Glutamate	50 µmoles
NAD^+	6 µmoles
ADP	6 µmoles
Suitable diluted tissue extract	50 µl.

The reaction mixture without NAD^+ was pre-incubated in a rectangular quartz cuvette at 30 °C for 5 min in a uv-visible spectrophotometer (Beckman, Model 26). The O.D. was adjusted to zero and the reaction was initiated by adding NAD^+ in the reaction mixture directly into the cuvette. The increase in O.D. at 340 nm was recorded at 30 sec intervals and the period of linear increase was used for calculation. The rate of reduction of NAD^+ to NADH was calculated taking 6.22×10^3 as molar extinction co-efficient for NADH. One unit of GDH activity was expressed as that amount which reduced 1 µmol of NAD^+ to NADH per hr at 30 °C.

Protein : Protein was determined following the method of Lowry et al (1951) using bovine serum albumin as the standard.

Purification of hepatic arginase :

Step 1 - Crude extract : Fishes were killed irrespective of sex and weight by decapitation and the liver was immediately removed and kept deep frozen at -20 ± 2 °C. The frozen tissue was thawed on ice and a 20% homogenate was prepared in 10 mM Tris-HCl buffer (pH 7.5) containing 5 mM $MnCl_2$, 0.1 M KCl using a Potter-Elvehjem type motor driven glass homogenizer fitted with a teflon pestle. The homogenate was diluted to 10% with Triton X-100 to a final concentration of 0.5% and kept for 30 min with constant stirring to release the mitochondrial portion of arginase. The homogenate was then centrifuged at 14,000 xg for 30

min. The supernatant obtained is the crude extract and was used in the subsequent purification steps. All the steps involved in the purification of arginase was carried out at 0 ± 2 °C.

Step 2 - Ammonium sulphate fractionation : Ammonium sulphate crystals were added to the crude extract gradually with constant stirring to 40% saturation (243 g/l). The mixture was centrifuged at 15,000 xg for 30 min. The pellet was discarded and the supernatant was adjusted to 60% saturation (132 g/l) of ammonium sulphate with constant stirring. The mixture was centrifuged at 15,000 xg for 30 min. The pellet obtained was resuspended in 10 mM Tris-HCl buffer (pH 7.5) containing 5 mM $MnCl_2$ and 1 mM 2-mercapto-ethanol. This solution was dialysed for 18 hr against the same buffer with at least three to four changes of buffer.

Step 3 - Heat treatment : The dialysed solution was heated at 55 °C for 10 min with constant stirring. The precipitated protein was removed by centrifugation at 10,000 xg for 20 min. The supernatant was used in further steps of purification.

Step 4 - Ion-exchange chromatography on DEAE-Sephacel : After heat treatment the solution was loaded on to a DEAE-Sephacel column (20 x 1.6 cm) previously equilibrated with 10 mM Tris-HCl buffer (pH 7.5). The unbound protein was eluted out first while washing the column with the same

buffer. Arginase was eluted with a linear gradient of KCl prepared in Tris-HCl buffer at a flow rate of 16 ml per hr. Fractions of 5 ml were collected. Arginase was eluted approximately at 0.1 and 0.15 M KCl. 10 µl of 2.5 M $MnCl_2$ was added in each collecting tube just to protect the arginase from denaturation. The active fractions were pooled and dialysed against 10 mM Tris-HCl buffer (pH 7.5) to remove KCl.

Step 5 - Affinity chromatography on Arginine Sepharose-4B: The dialysed sample obtained from step 4 was loaded on to Arginine Sepharose-4B column (10 x 1.0 cm) previously equilibrated with 10 mM Tris-HCl buffer (pH 7.5). The column was washed with the same buffer at a flow rate of 8 ml per hr. All the unbound proteins were eluted first. Arginase was then eluted with a linear gradient of KCl. Fractions of 3 ml were collected. Arginase was eluted approximately at 0.07 M KCl. The active fractions were pooled and dialysed against 50 mM Tris-HCl buffer (pH 7.5) to remove KCl.

Step 6 - Gel filtration on Sephadex G-150 : The dialysed sample was applied on to Sephadex G-150 column (40 x 2.5 cm) previously equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 0.1 M KCl. Arginase was eluted with the same buffer at a flow rate of 25 ml per hr. Fractions of 2 ml were collected. In each fraction 10 µl of 1 M $MnCl_2$ was added. The active fractions were pooled and used for

kinetic and molecular studies.

Polyacrylamide gel electrophoresis (PAGE) : Polyacrylamide gel electrophoresis of the different purification fractions was carried out according to Hames (1981) using 7% polyacrylamide gels. The ratio of acrylamide to N, N-methylenebisacrylamide was 30:0.8. Anodic electrophoresis was carried out in an electrophoretic chamber using Tris-glycine buffer (pH 8.3) at a constant current of 2 mA per tube. A pre run of the gel was done before loading the sample. At the end of electrophoresis the gels were removed from the glass tubes using water jet.

The gels were fixed in 50% TCA for 30 min prior to staining. Protein staining was done as described by Hames (1981) using 0.1% Coomassie brilliant blue prepared freshly by mixing glacial acetic acid, methanol and water in the ratio of 1:6:8 by volume. The excess stain was removed by repeated washing with a mixture of glacial acetic acid, methanol and water prepared in the ratio of 3:2:35 by volume. The gels were then preserved in distilled water.

Molecular weight determination : Molecular weight of the purified ARG was determined by gel filtration on Sephadex G-150 column as described by Andrews (1964). The column (40x2.5 cm) was equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 0.1 M KCl and elution was carried out with the same buffer. A constant flow rate was maintained at 25 ml/hr using a peristaltic pump and 2 ml fractions were

collected. The calibrating proteins were dissolved in the same buffer in a concentration of 4 mg/ml. Two to three ml of the protein solution was introduced on to the top of the gel bed under the eluting buffer. Blue Dextran 2000 (Pharmacia) was used to determine the void volume. Molecular weight of purified arginase was determined from the plot of elution volume (V_e) versus log molecular weight.

Temperature optima and thermal stability : ARG activity was assayed at different incubation temperatures such as 10, 20, 25, 30, 35, 40, 45, 50, 55 and 60 °C to determine the temperature optima.

Thermal stability of the purified ARG was studied by incubating the enzyme at different temperatures such as 10, 20, 30, 35, 40, 50, 55 and 60 °C. Aliquots for each were taken at different time intervals such as 5, 10, 20, 30, 40, 50 and 60 min and enzyme activity was assayed.

In another set of experiment the purified enzyme was pre-incubated at a fixed temperature (55 °C) in presence of either the substrate (L-arginine, 100 mM) or the co-factor ($MnCl_2$,5 mM) or both together. Aliquots for each were taken at different time intervals such as 10, 20, 30, 40, 50 and 60 min and enzyme activity was assayed to find out the protective role of either substrate, or co-factor, or in combination of substrate and co-factor against thermal denaturation. Purified arginase alone was pre-incubated in Tris-HCl buffer, pH 7.5 (10 mM) for different time intervals at 55 °C which served as control.

pH optima : ARG activity was assayed at different pH values ranging from 6.0 to 12.0. Tris-HCl buffer was used for lower pH values and sodium glycinate buffer was used for higher pH values.

Kinetic studies : The Km for L-arginine and Vmax of the purified arginase were determined both by Michaelis-Menten and Lineweaver-Burk plots. The arginase activity was measured between 1 mM to 100 mM L-arginine concentration.

Inhibition studies : The effect of some inhibitors (amino acids) was studied using fixed concentration of the inhibitor (25 mM). The percentage activity was calculated taking the activity in absence of the inhibitor as 100%. The nature of inhibition of some of the amino acids (L-ornithine, L-leucine, L-isoleucine and L-valine) was determined by Lineweaver-Burk plot and the Ki of the above inhibitorswas determined by Dixon plot.

Effect of metal ions : The purified enzyme was pre-incubated at 30 °C for 10 min with various metal ions at a concentration of 5, 10 and 20 mM and the enzyme activity was assayed. Values of arginase activity were expressed as % of activity taking 100% of activity in the absence of any metal ion.

Chemicals : All the enzymes, substrates, co-enzymes and inhibitors used for various enzyme assays and also for estimations , dialysis tubing, cetyltrimethyl ammonium bromide (CTB)and Arginine Sepharose-4B were obtained from

Sigma Chemical Company, St. Louis, Missouri, USA. DEAE-Sephacel, Sephadex G-150 and molecular weight kit used for column chromatography were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. All the other chemicals used were of analytical grade and were obtained from indigenous sources. Deionized double glass distilled water was used in all preparations.

Expression of enzyme activities: Total activity of the enzyme was expressed as units per g wet wt. of tissue and the specific activity was expressed as units per mg protein.

Statistical analysis and presentation of data : The data was calculated from at least three to five observations for each point and presented as mean ± standard error of the mean (S.E.M). The level of significance between two sets of data were calculated by student's 't' test (Croxton et al, 1982) and 'p' value above 0.05 were taken as non-significant (N.S.). Besides the presentation of data in tabulated form, graphs and histograms were also prepared to highlight the results.

RESULTS

Alterations of ammonia level in perfused liver of H. fossilis when infused with different concentrations of NH_4Cl

The increase of ammonia level was negligible upto 0.29 µmol/g of liver/min of NH_4Cl addition in perfused liver (Table 1). Significant accumulation of ammonia was seen only from 0.58 µmol/g liver/min of NH_4Cl addition into the perfused liver. The ammonia level in perfused liver raised from 12.75 to 33.81 µmoles/g wet wt. of liver with the rate of addition of 5.55 µmoles/g liver/min of NH_4Cl. The percentage increase of ammonia level was 5, 10, 24, 42, 93, 152 and 165% with the addition of 0.058, 0.12, 0.29, 0.58, 1.18, 2.8 and 5.55 µmoles/g liver/min of NH_4Cl into the perfused liver for 60 min.

Uptake and release of ammonia by the perfused liver:

There was about 75 to 85% uptake of ammonia out of the total added into the perfused liver upto 0.58 µmol/g liver/min of NH_4Cl addition (Table 2; Fig. 3). Similarly, the rate of release of ammonia into the effluent was also very low and was between 15 to 25% out of the total ammonia added upto the addition of 0.58 µmol/g liver/min of NH_4Cl (Table 2, Fig.2). At higher rate of addition, the percentage uptake of ammonia gradually decreased and percentage release into the effluent gradually increased with increasing rate of addition (Table 2, Figs.2 and 3). The rate of uptake of ammonia is a saturable process although the Vmax of uptake did not reach even at 5.55 µmoles/g liver/min of NH_4Cl addition (Fig.3). The Vmax

would have reached at still higher rate of addition of NH_4Cl.

Formation of urea-N in perfused liver :

The rate of release of urea-N by the perfused liver while infusing NH_4Cl at different rates have been shown in Table 2 and Fig.4. At lower rates of ammonia addition, i.e. upto 0.58 µmol/g liver/min, about 80 to 90% of the total ammonia taken up by the liver were converted to urea-N. The rate of synthesis of urea-N by the perfused liver at different rates of ammonia addition was again a saturable process (Fig. 4). The Vmax of urea-N excretion was obtained with the addition of 1·18 µmoles/g liver/min of NH_4Cl.

Induction pattern of o-u cycle enzymes in perfused liver:

The induction pattern of different o-u cycle enzymes (both total and specific) activity in the perfused liver while infusing NH_4Cl at different rates have been shown in Tables 3 and 4, and in Figs. 5-8. The induction of total activity of o-u cycle enzymes except OTC and ASL was not significant with the addition ammonia upto 0.29 µmol/g liver/min. Significant and maximum induction of total activity of CPS (ammonia dependent) and OTC was obtained with the addition of 0.58 µmol/g liver/min, and for ASS and ASL the maximum induction was obtained with the addition of 2.8 µmoles/g liver/min of NH_4Cl (Table 3, Fig.5). No significant change of total activity of ARG was noticed in any of the concentration of ammonium chloride added (Table 3, Fig.5).

In percentage wise, the induction of total activity of CPS was found to be maximum (204%) followed by ASS (153%), OTC (119%) and ASL (117%) respectively in decreasing order (Table 3, Fig.6).

No significant induction of specific activity was found for any of the o-u cycle enzymes activity upto the addition of 0.12 μmol/g liver/min of NH_4Cl. Significant induction of specific activity of all the enzymes were seen from 0.29 μmol/g liver/min of NH_4Cl having the maximum induction at 1.18 μmoles/g liver/min of NH_4Cl addition (Table 4, Fig.7). Like total activity, the specific activity of ARG also did not show any alterations in any of the concentrations of NH_4Cl added. The percentage induction of specific activity of CPS was found to be maximum (234%) followed by ASS (168%), ASL (140%) and OTC (132%) respectively in decreasing order (Table 4, Fig. 8).

Isoenzymes of CPS (I,II and III) in the liver and kidney of H. fossilis:-

The activity of CPS increased more than 3 times both in the liver and kidney tissues of H. fossilis when N-acetylglutamate (NAG) was taken in the reaction mixture along with ammonia with comparison to ammonia alone (Table 5). Addition of methionine sulfoxamine (25 mM),an inhibitor for GS, and UTP (1 mM), an inhibitor for CPS II, along with ammonia and NAG did not change in the CPS activity. This confirmed the presence of CPS I (ammonia and NAG-dependent) activity in both the tissues of H. fossilis.

There was significant activity of CPS when glutamine alone was taken in the reaction mixture indicating the presence of CPS II isoenzymes. However, when UTP was taken along with glutamine in the reaction mixture without the addition of NAG, only about 10% of the CPS activity could be detected in both the tissues. This confirmed further the presence of CPS II (glutamine dependent and NAG independent) activity in both the tissues. Addition of NAG along with glutamine in the reaction mixture caused further increase of CPS activity suggesting also the presence of CPS III activity. This activity was inhibited to the extent of CPS II activity when the CPS II inhibitor (UTP, 1 mM) was added in the reaction mixture. This confirmed the presence of CPS III activity also in the liver and kidney tissue of H. fossilis. Thus the presence of all the three isoenzymes of CPS (I, II and III) could be confirmed in the two ureogenic tissues such as liver and kidney of H. fossilis.

Table 6 shows the activities of the three isoenzymes of CPS (I, II and III) in liver and kidney of H. fossilis, studied by using specific inhibitors in the reaction mixture as mentioned in the materials and methods and also in Table 6. Both total and specific activity of CPS I was found to be maximum in both the tissues followed by CPS III and CPS II respectively.

Induction of CPS I and CPS III, and rate of synthesis of urea from ammonia and glutamine in perfused liver of H. fossilis: The induction pattern of CPS I (ammonia

dependent) after infusing NH_4Cl (1 and 2 mM) and CPS III (glutamine dependent) after infusing glutamine (1 and 2 mM) for 60 min were studied in perfused liver of H. fossilis (Table 7). The rate of synthesis of urea from either ammonia or glutamine, while infusing in different concentrations of these two, were also measured in the effluent (Table 7). There was a significant ($p < 0.001$) induction (145%) of CPS I activity even at 1 mM NH_4Cl infusion (equivalent to ammonia addition of 5.5±0.25 µmoles/g liver/min). There was apparently no further increase in CPS I activity while infusing 2 mM of NH_4Cl (equivalent to ammonia addition of 11.5±1.0 µmoles/g liver/min) into the perfused liver. The rate of synthesis of urea was 10.7±1.5 and 11±2.1 µmoles/g liver/hr at 1 and 2 mM of NH_4Cl respectively.

There was only about 20% increase in CPS III (non-significant) activity (3.9 units/ g wet wt.) as compared to control (3.2 units/g wet wt.) while infusing 1 mM of glutamine (equivalent to glutamine addition of 5.55 ±0.5 µmoles/g liver/min). A significant increase (47%) in CPS III (4.7±0.61 units/g wet wt.) was observed while infusing 2 mM glutamine (equivalent to 11.5±1.7 µmoles/g liver/min) in perfused liver. Accordingly, the rate of synthesis of urea (5.8±0.41 µmoles/g liver/hr) from glutamine at 2 mM concentration was 35% higher than at 1 mM concentration of glutamine infused (4.3±0.32 µmoles/g liver/min).

Sub-cellular localization of different isoenzymes of CPS (I, II and III) and other o-u cycle enzymes : The effect

of 0.5% Triton X-100 treatment on the activity of CPS (I, II and III) and other o-u cycle enzymes of liver and kidney homogenates of H. fossilis for different periods has been presented in Tables 8 and 9 respectively. Treatment of tissue homogenates with Triton X-100 (0.5%) increased activity of CPS I and III, and mitochondrial enzymes of o-u cycle such as OTC and ARG by 70-120% in liver (Table 8) and 80-160% in kidney (Table 9) within 30 min. However, the activity of CPS II, and soluble enzymes of o-u cycle such as ASS and ASL did not show any significant alteration even after 60 min of treatment.

The localization of the three isoenzymes of CPS and other o-u cycle enzymes in different sub-cellular fractions of the liver (Table 10) and kidney (Table 11) of H. fossilis was studied along with marker enzymes. The activities of CPS I (73%), CPS III (72%), OTC (66%) and ARG (58%) were found mostly in the mitochondrial fraction of liver tissue along with the 67% of activity of mitochondrial marker enzyme GDH (Table 10). Whereas, the activities of CPS II (65%), ASS (74%) and ASL (65%) were found mostly in the soluble cytosolic fraction of liver tissue along with the 79% of activity of cytosolic marker enzyme LDH (Table 10).In kidney tissue, most of the activities of CPS I (71%), CPS III (74%), OTC (72%) and ARG (67%) were found in the mitochondrial fraction with 73% of activity of mitochondrial marker enzyme GDH (Table 11). Whereas, the activities of CPS II (69%), ASS (71%) were mostly found in the soluble cytosolic fraction of kidney tissue with 73% of activity of cytosolic marker enzyme LDH

(Table 11).

Annual variation of o-u cycle enzymes:

The annual variation of the activity (both total and specific) of the o-u cycle enzymes were studied for one year in the liver and kidney tissues of H. fossilis maintained in the laboratory under control conditions (Tables 12-15; Figs. 9-12). The five o-u cycle enzymes studied showed different pattern of annual variation in their activity in the two tissues. In general, the enzymes showed higher activity during summer (June-July) and lower activity during winter (November to March) months.

All through the year arginase activity was very high followed by OTC, ASS, ASL and CPS. The activity of CPS was very low in both the tissues studied. The activity of all the enzymes started increasing at the end of winter around March/April, reaching a peak in June (Summer) and decreasing thereafter. The highest activity of all the enzymes was observed in June (summer). However, the activity of OTC and CPS in kidney did not show any appreciable annual variation. The specific activity of OTC and CPS in kidney showed a further peak in June., The specific activity of ARG continued in a higher level from June till October.

Purification of hepatic arginase (Table 16; Fig. 13):

The protocol of purification of hepatic arginase has been presented in Table 16. The fold of purification and percentage recovery of the enzyme activity obtained in the last step of purification were 306 and 36% respectively. Heat treatment at 55°C resulted in an increase in specific activity and percentage recovery. The enzyme obtained after heat treatment was loaded to DEAE-Sephacel column and while washing the column with Tris-HCl buffer (pH 7.5) a small peak of enzyme activity was obtained in the void volume, which accounted for about 17% of the total enzyme loaded (Fig. 13). 83% of the arginase activity which got bound to the ion exchanger, was eluted from the column by passing a linear gradient of KCl (0-0.3 M). The enzyme activity was eluted at approximately 0.1 -0.15 M of KCl coinciding with a major protein peak. Affinity chromatography of the above eluate on Arginine Sepharose-4B resulted in a single enzyme peak (Fig.13) at approximately 0.07 M of KCl. The eluate from the affinity column was finally passed through a Sephadex G-150 column. The enzyme activity eluted as a single peak and the two 5 ml fractions of eluate having highest specific activity were pooled and used as purified hepatic arginase source for further studies.

Polyacrylamide gel electrophoresis (PAGE) :

The enzyme samples from different stages of purification were separated by polyacrylamide gel electrophoresis (Fig.14). The gels were stained with

Coomassie Brilliant Blue. The purified enzyme showed a single band on PAGE.

Physico-chemical properties of purified arginase:

Molecular weight determination : The apparent molecular weight of the purified arginase was 81,000 as determined by gel filtration chromatography on Sephadex G-150 (Fig. 15). The void volume was determined by Blue Dextran followed by calibration of the column with marker proteins such as bovine serum albumin (67,000); ovalbumin (43,000), cytochrome c (12,400), bovine arginase (115,000) and lactate dehydrogenase (140,000). Molecular weight was determined from the plot of log molecular weight versus elution volume (Ve) (Fig. 15).

Temperature optima (Table 17; Fig. 16) : The activity of arginase assayed at different temperatures showed a gradual increase from 10 to 30°C followed by a sharp increase upto 50°C. However, the enzyme activity decreased sharply after 50 °C with no activity detected at 70°C and above incubation temperate. The optimal temperature for arginase activity was found to be 50°C.

Thermal stability (Fig. 17; Table 18) : Thermal stability of purified arginase was studied by pre-incubating the enzyme alone at different temperature and then assaying the activity at different time intervals upto 60min in a standard reaction mixture at 30°C as mentioned under

materials and methods. At lower temperature (10 and 20°C) the enzyme was very stable having 95% of the activity retained upto 60 min of incubation. At 30 and 35°C, about 65% of activity was retained and at 40 and 50°C only 30 to 35% of arginase activity was found after 60 min of incubation. However, at 55 and 60°C the enzyme activity was lost very fast. There was only about 10% of enzyme activity at 55°C after 60 min of incubation and at 60°C the activity was completely lost after 30 min of incubation.

Factors influencing thermal stability (Table 19; Fig. 18): Purified hepatic arginase activity showed fast denaturation when pre-incubated at 55°C for 60 min. Therefore, the enzyme was incubated with the substrate (arginine, 100 mM) and co-factor ($MnCl_2$, 5 mM) either individually or in combination to study their effects on thermal stability (Table 19; Fig. 18). In the absence of co-factor or substrate, arginase activity decreased sharply retaining only about 5% of the activity after 60 min of pre-incubation. $MnCl_2$ (5 mM) and arginine (100 mM) when added separately to the buffer containing the enzyme, did protect the enzyme from the thermal denaturation, and 40% of the enzyme activity was retained after 60 min of pre-incubation. However, $MnCl_2$ (5 mM) and L-arginine (100 mM) when incubated together with the purified enzyme gave a better protection to the enzyme against the thermal denaturation. The loss of enzyme activity was slow for about 40 min loosing about 10-13% of the activity. Even after 60 min of incubation about 60% of activity could still be detected loosing only about 40% of

activity.

pH optima (Table 20; Fig. 19) : The purified arginase was assayed at a pH range of 6 to 12 to find out its pH optima. The enzyme activity increased gradually with increasing pH from pH 6 to 9.5 after which the arginase activity decreased more sharply to a very low level by pH 11. The pH optima of hepatic arginase of *H. fossilis* was very narrow with a sharp peak at pH 9.5.

Effect of amino acids (Table 21) : Most of the amino acids tested except L-alanine, L-cysteine, L-glutamate, L-serine and L-tyrosine inhibited arginase activity at 25 mM concentration. However, the level of inhibition varied from 68% to 11%. L-ornithine. L-isoleucine. L-valine and L-leucine inhibited arginase activity by more than 60%; L-aspartate, L-histidine, L-threonine. L-lysine and L-phenylalanine between 40-50%; and L-proline, L-methionine and L-glycine between 20-30%.

Effect of metal ions (Table 22) : Effect of several divalent and monovalent metal ions on the activity of purified arginase was studied . Out of all the metal.ions studied divalent ions Mn^{2+}. Ca^{2+}, Mg^{2+}, Co^{2+}, Cu^{2+} and Cd^{2+}, and monovalent ions Na^{+} and K^{+} showed induction, and divalent ions Hg^{2+}, Ag^{2+} and Zn^{2+} showed inhibition of arginase activity. Mn^{2+} was the most suitable divalent metal ion showing induction of purified hepatic arginase activity by 113% at 5 mM concentration. However, at 10 and 20 mM

concentrations of Mn^{2+}, the induction level decreased to 98 and 58% respectively. In general, 5 mM concentration was optimum for most ions showing induction. However, the inhibiting ions showed increased effect at higher (10 and 20 mM) concentrations. Fe^{2+} showed unique feature with a small induction (+13%) at 5 mM concentration, no effect at 10 mM and a small inhibition (-8%) at 20 mM concentration.

Kinetics of purified arginase:

Km and Vmax (Figs. 20,21) : The apparent Michaelis-constant (Km) of the purified arginase obtained was 10 mM and 12.5 mM , and Vmax 8.4 and 10 units/ml by Michaelis-Menten (M.M.) and Lineweaver-Burk (L.B.) plot respectively. There was a decrease in activity when the substrate concentration exceeded 0.1 M, which decreased further at a still higher concentration.

Nature of inhibition of arginase by amino acids and Ki values : The nature of inhibition of the four amino acids Orn, Val, Leu and Ile has been presented in Table 23 as determined by L.B. plot (Figs.22-25). Orn and Leu were found to be competitive , whereas, Ile and Val acted as non-competitive inhibitors for arginase. The Ki values for the four amino acids were determined by Dixon's plot (Figs.26-29). The Ki values obtained for Orn, Val, Leu and Ile were found to be 4.25, 5.3, 9.3 and 7.25 mM respectively.

TABLES

Table 1 : Levels of ammonia (μmoles/g wet wt.) in perfused liver after infusing different concentrations of NH_4Cl for 60 min. Values are expressed as mean ± S.E.M.(n=3-4)

NH_4Cl conc. (mM)	Ammonia added (μmoles/g liver/min)		Ammonia in liver (μmoles/g wet wt.)
0	0		12.75±1.65
0.01	0.058±0.015		13.43±1.87(5)
		p	N.S.
0.02	0.12±0.03		13.96±1.10(10)
		p	N.S.
0.05	0.29±0.04		15.85±1.01(24)
		p	N.S.
0.10	0.58±0.05		18.10±1.06(42)
		p	<0.05
0.20	1.18±0.12		24.55±1.17(93)
		p	<0.05
0.50	2.80±0.20		32.15±2.22(152)
		p	<0.02
1.00	5.55±0.25		33.81±2.57(165)
		p	<0.02

% increase of ammonia level with relation to control are given in parentheses.

N.S. = Non-significant

Table 2 : Addition of ammonia into the perfused liver, uptake of ammonia by the perfused liver, release of ammonia and urea-N (μmoles/g liver/min) into the effluent by the perfused liver of H.fossilis. Values are expressed as mean ± S.E.M. (n=3-4).

Concentration of NH_4Cl(mM)	Addition of ammonia	Uptake of ammonia	Ammonia release	Urea-N release
0.01	0.058+0.015	0.048+0.015(83)*	0.01+0.002(17)	0.04+0.01 (83)**
0.02	0.12+0.03	0.09+0.03 (75)	0.03+0.004(25)	0.08+0.015(89)
0.05	0.29+0.04	0.22+0.05 (76)	0.07+0.02 (24)	0.17+0.02 (77)
0.10	0.58+0.05	0.43+0.07 (74)	0.15+0.05 (26)	0.35+0.02 (81)
0.20	1.18+0.12	0.73+0.10 (62)	0.45+0.07 (38)	0.40+0.02 (55)
0.50	2.80+0.20	1.10+0.11 (39)	1.70+0.25 (61)	0.41+0.025(37)
1.00	5.55+0.25	1.35+0.11 (24)	4.20+0.30 (76)	0.39+0.025(29)

* % of total ammonia added
** % of total ammonia uptake

Table 3 : Alterations of total activity (units/g wet wt.) of different o-u cycle enzymes in perfused liver of H. fossilis when infused with different concetrations of NH_4Cl. values are expressed as mean ± S.E.M. (n = 3-4)

Enzymes	Concentrations of NH_4Cl (mM)							
	0	0.01	0.02	0.05	0.1	0.2	0.5	1.0
CPS (ammonia dependent)	2.9±0.13	2.9±0.12 (0)	3.0±0.26 (3±0.6)	3.7±0.39 (28±10)	8.9±0.8 (204±20)	8.8±0.87 (202±15)	8.7±0.87 (197±17)	8.8±0.80 (203±23)
P		N.S.	N.S.	N.S.	<0.001	<0.001	<0.001	<0.001
OTC	153±15	165±15 (10±3)	171±14 (15±4)	247±20 (61±6)	332±20 (119±11)	328±32 (113±13)	317±22 (109±18)	306±21 (105±14)
P		N.S.	N.S.	<0.05	<0.01	<0.02	<0.01	<0.01
ASS	33.2±2.1	39.8±1.7 (20±4)	35.8±3.1 (8±3)	46.4±7.7 (40±5)	76.4±3.6 (130±23)	82.3±5.2 (148±17)	84.0±6.0 (153±22)	82.4±10.3 (148±21)
P		N.S.	N.S.	N.S.	<0.01	<0.01	<0.01	<0.02
ASL	18.5±2.3	22.0±1.7 (19±5)	21.7±2.4 (17±5)	30.4±2.1 (65±6)	35.0±2.5 (90±12)	38.8±3.0 (110±13)	40.0±1.6 (117±10)	38.7±1.2 (110±9)
P		N.S.	N.S.	<0.05	<0.02	<0.02	<0.01	<0.01
ARG	1756±132	1773±166 (1±4)	1861±127 (6±6)	1721±98 (-2±6)	1847±139 (5±4)	1872±100 (7±6)	1879±193 (7±9)	1835±168 (5±7)
P		N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

% change of activity as compared to control are given in parentheses.
1 mM NH_4Cl infusion was equivalent to 5.55 µmoles/g liver/min of ammonia addition.
N.S.= non-significant; CPS= carbamyl phosphate synthetase; OTC= ornithine transcarbamylase; ASS= argininosuccinate synthetase; ASL= argininosuccinate lyase; ARG= arginase

Table 4 : Alterations of specific activity (units/mg protein) of different o-u cycle enzymes in the perfused liver of H. fossilis infused with different concentrations of NH_4Cl. Values are expressed as mean ± S.E.M. (n=3-4)

Enzymes	Concentrations of NH_4Cl (mM)							
	0	0.01	0.02	0.05	0.1	0.2	0.5	1.0
CPS (ammonia dependent)	0.055±0.003	0.059±0.006 (6±1.5)	0.067±0.004 (21±8)	0.084±0.01 (50±9)	0.178±0.02 (221±17)	0.184±0.02 (234±5)	0.175±0.02 (216±26)	0.182±0.02 (232±13)
P		N.S.	N.S.	< 0.05	<0.001	< 0.001	<0.001	< 0.001
OTC	2.99±0.33	3.38±0.40 (15±3)	3.97±0.28 (37±8)	5.50±0.27 (87±14)	6.70±0.65 (125±9)	6.93±0.88 (132±14)	6.37±0.40 (116±14)	6.36±0.59 (122±13)
P		N.S.	N.S.	<0.01	<0.02	< 0.05	< 0.01	<0.02
ASS	0.56±0.05	0.71±0.05 (11±4)	0.78±0.04 (23±6)	1.02±0.12 (65±9)	1.51±0.06 (136±17)	1.71±0.14 (168±16)	1.69±0.13 (168±17)	1.72±0.014 (172±16)
P		N.S.	N.S.	< 0.05	<0.01	<0.01	< 0.01	<0.01
ASL	0.36±0.03	0.45±0.05 (11±4)	0.48±0.04 (36±9)	0.80±0.07 (122±14)	0.70±0.12 (101±17)	0.82±0.08 (140±14)	0.80±0.03 (130±13)	0.80±0.03 (130±17)
P		N.S.	N.S.	<0.02	<0.02	<0.02	<0.02	<0.02
ARG	33.9±1.6	35.8±2.4 (6±6)	41.6±3.9 (23±12)	38.5±1.0 (14±6)	37.0±2.4 (10±4)	39.4±2.7 (17±9)	37.8±4.1 (12±7)	38.2±4.4 (12±9)
p		N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

% change as compared to control are given in parentheses.
1 mM NH_4Cl infusion was equivalent to 5.55 μmoles/g liver/min of ammonia addition.
N.S.= non-significant; CPS= carbamyl phosphate synthetase; OTC= ornithine transcarbamylase; ASS= argininosuccinate synthetase; ASL= argininosuccinate lyase; ARG= arginase

Table 5: Activity (units/g wet wt.) of carbamyl phosphate synthetase in the liver and kidney of H. fossilis. Values are expressed as mean ± S.E.M.

Components in reaction mixture	CPS activity	
	Liver	Kidney
Ammonia	1.21±0.12 (3)	0.94±0.11 (3)
Ammonia + N-acetylglutamate (CPS I)	3.83±0.13 (3)	3.00±0.21 (3)
Ammonia + N-acetylglutamate + methionine sulfoxamine + UTP(CPS I)	3.54 (1)	2.60 (1)
Glutamine (CPS II)	2.77±0.19 (3)	1.65±0.05 (3)
Glutamine + UTP	0.25 (1)	0.18 (1)
Glutamine + N-acetylglutamate (CPS II & III)	6.02±0.29 (3)	3.29±0.16 (3)
Glutamine + N-acetylgutamate + UTP (CPS III)	3.60±0.70 (3)	1.90±0.30 (3)

The homogenate prepared in CTB (0.1%), was used for CPS assay. The standard assay mixture as mentioned in materials and methods were used for measuring CPS activity except where indicated N-acetylglutamate (5mM) was absent, and either NH_4Cl (50 mM) or glutamine (20 mM) was used as nitrogen donor. In some of the reaction mixture either UTP (1mM), an inhibitor for CPS II or methionine sulfoxamine (25 mM), an inhibitor for glutamine synthetase, or both were added.
Number of observations in each case are given in parentheses

Table 6: Total (units/g wet.wt.) and specific (units/mg protein) activities of different isoenzymes of CPS (I,II,III) in the liver and kidney of H. fossilis. Values are expressed as mean ± S.E.M. (n=3)

Enzymes	LIVER		KIDNEY	
	Total	Specific	Total	Specific
CPS I	4.2±0.35	0.16±0.04	2.8±0.26	0.20±0.03
CPS II	2.4±0.40	0.09±0.04	1.4±0.20	0.10±0.04
CPS III	3.6±0.70	0.15±0.07	1.9±0.30	0.12±0.03

The homogenate prepared in CTB (0.1%), was used for CPS assay. The standard assay mixture, as mentioned in materials and methods, was used for the assay of CPS. However, for CPS I, the reaction mixture contained NH_4Cl (50 mM) as nitrogen donor,the co-factor N-acetylglutamate (NAG) (5 mM), and also methionine sulfoxamine (25 mM) and UTP (1 mM) as inhibitors for GS and CPS II (ammonia dependent) respectively. For CPS II activity, the reaction mixture contained glutamine (20 mM) as nitrogen donor. For CPS III activity, the reaction mixture contained glutamine (20 mM) as nitrogen donor,the co-factor NAG and also UTP (1 mM) as inhibitor for CPS II.

Table 7 : Induction of CPS I (ammonia and NAG-dependent) and CPS III (Glutamine and NAG-dependent) and the rate of urea synthesis from ammonia and glutamine in perfused liver of *H. fossilis*. Values are expressed as mean ± S.E.M.(n=3)

Enzymes		Activity (units/g wet wt.)	Urea synthesis (μmol/g liver/hr)
CPS I	Control	2.9±0.13	BLD
	With ammonia (1 mM)	7.1±0.66* (+144.8)	10.7±1.50
	With ammonia (2 mM)	7.2±0.71* (+148.3)	11.0±2.10
CPS III	Control	3.2±0.30	BLD
	With glutamine (1 mM)	3.9±0.45 (+21.9)	4.3±0.32
	With glutamine (2 mM)	4.7±0.61** (+46.8)	5.8±0.41*

* Significant at <0.001 level
** Significant at <0.05 level

Livers were perfused with standard isotonic media containing 5 mM glucose and 2 mM ornithine as mentioned in materials and methods for 20 min before infusion of NH_4Cl (1 and 2 mM) and glutamine (1 and 2 mM). The urea in the effluent between 50 to 60 min of infusion was measured at 2 min intervals and were expressed as the rate of urea synthesis from either ammonia or glutamine. CPS I and III activity were also measured in the liver after perfusing for 80 min with the same media but without NH_4Cl and glutamine and were taken as control. Figures in the parentheses indicate the % change.

Table 8 : Effect of Triton X-100 (0.5%) on the activity (units/g wet wt.) of o-u cycle enzymes and different isoenzymes of CPS(I,II,III) in the liver of H. fossilis. Values are expressed as mean ± S.E.M (n=3-5)

Time (min)	CPS I	CPS II	CPS III	OTC	ASS	ASL	ARG
0	1.8±0.12	2.50±0.22	2.0±0.2	98±4.9	25±1.3	23±0.9	2454±101
10	2.5±0.23 (39)	2.50±0.21 (0)	2.7±0.3 (35)	116±6.3 (18)	27±1.8 (8)	24±1.3 (4)	2987±88 (22)
20	4.0±0.23 (122)	2.70±0.2 (8)	3.9±0.33 (95)	141±6.7 (44)	27±1.3 (8)	24±0.9 (4)	3783±115 (54)
30	3.9±0.57 (117)	2.60±0.24 (4)	4.0±0.34 (100)	169±6.3 (72)	26±2.2 (4)	26±2.2 (13)	4178±120 (70)
40	3.9±0.12 (117)	2.55±0.21 (2)	3.8±0.31 (90)	152±7.2 (55)	26±1.8 (4)	24±1.8 (4)	4039±134 (65)
50	3.8±0.47 (111)	2.5±0.2 (0)	3.7±0.3 (85)	151±5.4 (54)	27±2.7 (8)	24±1.8 (4)	4011±119 (63)
60	3.7±0.23 (106)	2.5±0.18 (0)	3.7±0.3 (85)	141±5.8 (44)	27±1.3 (8)	24±2.2 (4)	3982±97 (62)

% increase of activity with respect to zero time value are given in parentheses

Table 9 : Effect of Triton X-100 (0.5%) on the activity (units/g wet wt.) of o-u cycle enzymes and different isoenzymes of CPS (I,II,III) in the kidney of *H. fossilis*. Values are expressed as mean ± S.E.M (n=3-5)

Time (min)	CPS I	CPS II	CPS III	OTC	ASS	ASL	ARG
0	1.00±0.12	1.45±0.3	0.8±0.25	33±2.2	20±1.3	23±1.8	646±49
10	1.25±0.12 (25)	1.50±0.4 (3)	1.2±0.27 (50)	41±1.8 (24)	20±0.9 (0)	23±0.9 (0)	693±43 (7)
20	2.65±0.17 (165)	1.60±0.4 (10)	1.7±0.32 (113)	47±3.1 (42)	21±0.9 (5)	24±1.3 (4)	986±38 (53)
30	2.60±0.12 (160)	1.55±0.31 (7)	2.0±0.30 (150)	59±3.6 (79)	22±1.3 (10)	23±0.9 (0)	1289±54 (100)
40	2.50±0.22 (150)	1.50±0.42 (3)	1.8±0.32 (125)	59±2.2 (79)	20±1.3 (0)	24±1.3 (4)	1217±60 (88)
50	2.45±0.23 (145)	1.50±0.33 (3)	1.8±0.31 (125)	59±1.8 (79)	20±0.9 (0)	22±1.8 (-4)	1199±45 (86)
60	2.45±0.37 (145)	1.45±0.30 (0)	1.7±0.28 (113)	58±3.1 (76)	20±0.9 (0)	22±0.9 (-4)	1188±41 (84)

% increase of activity with respect to zero time value are given in parentheses

Table 10: Sub-cellular distribution of o-u cycle enzymes and different isoenzymes of CPS (I,II,III) in the liver of H. fossilis. Values are expressed as mean ± S.E.M .(n=3-5)

Enzymes	Homogenate	Nuclear	Mitochondrial	Soluble	Recovered activity
CPS I	4.2±0.40	0.40±0.02 (9)	3.07±0.34 (73)	0.84±0.08 (20)	4.31 (103)
CPS II	2.4±0.4	0.22±0.1 (9)	0.48±0.2 (20)	1.54±0.5 (64)	2.24 (93)
CPS III	3.6±0.7	0.36±0.1 (10)	2.59±0.4 (72)	0.65±0.2 (18)	3.6 (100)
Total CPS	10.2	0.98 (10)	6.14 (60)	3.03 (30)	10.5 (100)
OTC	205.0±11.2	25.0±1.4 (12)	135.o±5.8 (66)	51.0±2.7 (25)	211 (103)
ASS	27.0±1.3	3.0±0.13 (11)	7.0±0.09 (26)	20.0±0.31 (74)	30 (111)
ASL	26.0±1.3	2.0±0.02 (8)	6.0±0.04 (23)	17.0±0.20 (65)	25 (96)
ARG	4970±230	189±6.3 (4)	2862±69 (58)	1885±27 (38)	4936 (99)
GDH	437±13.5	32±0.9 (7)	293±36 (67)	115±4.0 (26)	440 (101)
LDH	4291±216	302±14 (7)	830±36 (19)	3396±112 (79)	4528 (106)
Protein mg/g tissue	121.0±9.0	16.0±0.9 (13)	42±1.8 (35)	65.0±3.1 (54)	123 (102)

% recovery of activity of different enzymes are given in parentheses

Table 11: Sub-cellular distribution of different o-u cycle enzymes and different isoenzymes of CPS (I,II,III) in the kidney of H. fossilis. Values are expressed as Mean ± S.E.M.(n=3-5)

Enzymes	Homogenate	Nuclear	Mitochondrial	Soluble	Recovered activity
CPS I	2.8±0.26	0.22±0.02 (8)	2.0±0.12 (71)	0.67±0.07 (24)	2.91 (104)
CPS II	1.4±0.2	0.20±0.05 (14)	0.28±0.1 (20)	0.97±0.1 (69)	1.45 (104)
CPS III	1.9±0.3	0.19±0.04 (10)	1.4±0.2 (74)	0.38±0.1 (20)	1.97 (104)
Total CPS	6.1	0.61 (10)	3.68 (60)	2.02 (33)	6.31 (103)
OTC	53±2.7	3±0.2 (6)	38±1.8 (72)	14±0.9 (26)	55 (104)
ASS	21±2.2	2±0.22 (10)	5±0.45 (24)	15±1.8 (71)	22 (105)
ASL	24±0.9	2±0.09 (8)	6±0.3 (25)	19±0.9 (79)	27 (113)
ARG	1169±47.5	171±4.9 (15)	789±23.8 (67)	216±8.5 (18)	1176 (101)
GDH	250±10.3	17±0.45 (7)	182±6.7 (73)	64±2.7 (26)	263 (105)
LDH	2689±100	112±3.6 (4)	649±12.6 (24)	1955±54.7 (73)	2716 (101)
Protein (mg/g tissue)	118±7.2	15±0.9 (13)	37±2.2 (31)	65±3.1 (55)	117 (99)

% recovery of activity of different enzymes are given in parentheses

Table 12 : Annual variation of the total activity (units/g wet wt.) of o-u cycle enzymes in the liver of *H. fossilis*. Values are expressed as mean ± S.E.M.(n=3-5)

Enzymes	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
CPS (ammonia dependent)	1.85± 0.12	1.95± 0.16	1.85± 0.15	2.05± 0.2	2.01± 0.22	4.9± 0.23	3.11± 0.24	2.40± 0.17	2.55± 0.14	1.98± 0.11	1.69± 0.13	1.36± 0.22
OTC	120± 8.1	150± 8.0	161± 8.0	175± 16.0	205± 16.0	232± 12.1	241± 16.0	191± 12.0	155± 12.1	145± 23.9	142± 16.0	105± 16.1
ASS	24.2± 1.5	25.5± 1.6	27.6± 1.2	31.2± 1.9	34.1± 2.0	38.4± 1.6	29.1± 1.2	31.4± 2.4	28.1± 2.4	29.2± 2.2	27.0± 2.2	25.3± 2.1
ASL	20.5± 1.2	22.3± 1.2	24.6± 0.8	27.5± 1.2	30.9± 1.6	35.6± 1.6	32.2± 1.2	26.9± 1.2	29.4± 1.6	21.4± 2.4	19.5± 2.0	17.0± 1.6
ARG	2550± 200	2116± 240	2496± 280	2914± 320	3421± 160	5632± 360	4941± 280	4184± 240	3014± 280	2998± 200	2143± 160	1999± 200

CPS= carbamyl phosphate synthetase; OTC= ornithine transcarbamylase; ASS= argininosuccinate synthetase; ASL= argininosuccinate lyase; ARG= arginase

Table 13: Annual variation of the specific activity (units/mg protein) of o-u cycle enzymes in the liver of H. fossilis. Values are expressed as mean ± S.E.M.(n=3-5)

Enzymes	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
CPS(ammonia dependent	0.014± 0.002	0.017± 0.002	0.016± 0.002	0.018± 0.002	0.021± 0.002	0.026± 0.002	0.024± 0.002	0.019± 0.002	0.015± 0.002	0.018± 0.002	0.016± 0.002	0.014± 0.002
OTC	0.96± 0.08	1.33± 0.08	1.46± 0.08	1.53± 0.05	1.70± 0.08	1.69± 0.10	1.54± 0.08	1.41± 0.05	1.40± 0.05	1.56± 0.05	1.48± 0.05	0.92± 0.08
ASS	0.19± 0.013	0.22± 0.015	0.23± 0.018	0.22± 0.015	0.20± 0.013	0.24± 0.018	0.21± 0.015	0.21± 0.015	0.20± 0.015	0.19± 0.018	0.17± 0.018	0.18± 0.02
ASL	0.17± 0.008	0.16± 0.012	0.18± 0.013	0.17± 0.013	0.22± 0.01	0.23± 0.016	0.19± 0.015	0.16± 0.013	0.17± 0.015	0.20± 0.017	0.20± 0.017	0.19± 0.016
ARG	20.4± 2.7	21.6± 3.5	30.4± 3.0	30.7± 2.5	33.5± 2.7	41.3± 2.7	38.1± 3.5	37.3± 2.8	36.4± 3.1	36.0± 2.5	29.9± 3.1	24.2± 2.7

CPS= carbamyl phosphate synthetase; OTC= ornithine transcarbamylase; ASS= argininosuccinate synthetase; ASL= argininosuccinate lyase; ARG= arginase

Table 14 : Annual variation of the total activity (units/g wet wt) of o-u cycle enzymes in the kidney of H. fossilis. Values are expressed as mean ± S.E.M.

Enzymes	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
CPS(ammonia dependent)	0.85± 0.24	1.45± 0.27	1.79± 0.28	2.14± 0.24	2.13± 0.27	2.85± 0.23	2.11± 0.28	1.86± 0.32	1.55± 0.27	1.05± 0.30	1.07± 0.36	0.79± 0.31
OTC	18.0± 4.3	28.9± 4.4	34.2± 4.5	38.4± 4.4	34.5± 4.7	47.9± 5.2	30.7± 5.2	34.5± 4.0	25.4± 5.2	26.9± 5.3	19.5± 5.9	16.2± 4.0
ASS	11.5± 1.57	11.4± 2.01	14.4± 1.99	15.5± 1.19	14.0± 1.23	18.7± 0.83	13.5± 0.79	13.2± 1.20	12.9± 1.61	13.7± 2.8	12.4± 2.4	11.2± 1.6
ASL	12.4± 1.99	14.5± 2.03	16.4± 1.22	22.4± 2.00	21.4± 2.37	22.4± 3.19	17.8± 3.22	14.3± 3.20	16.3± 2.78	12.3± 2.40	10.3± 1.58	10.2± 2.11
ARG	505 ±60	584 ±64	554 ±52	675 ±36	654 ±35	849 ±52	645 ±48	655 ±52	594 ±47	559 ±40	510 ±36	489 ±43

CPS= carbamyl phosphate synthetase; OTC= ornithine transcarbamylase; ASS= arginosuccinate synthetase; ASL= argininosuccinate lyase; ARG= arginase

Table 15: Annual variation of the specific activity (units/g wet wt.) of o-u cycle enzymes in the kidney of *H. fossilis*. Values are expressed as mean ± S.E.M.(n=3-5)

Enzymes	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
CPS(ammonia dependent	0.010± 0.002	0.012± 0.002	0.010± 0.002	0.014± 0.003	0.015± 0.002	0.024± 0.002	0.019± 0.002	0.013± 0.002	0.014± 0.003	0.01± 0.003	0.01± 0.002	0.011± 0.003
OTC	0.24± 0.005	0.23± 0.008	0.24± 0.008	0.23± 0.005	0.22± 0.005	0.29± 0.005	0.22± 0.008	0.21± 0.008	0.24± 0.008	0.26± 0.005	0.26± 0.008	0.25± 0.005
ASS	0.116± 0.01	0.119± 0.01	0.122± 0.008	0.128± 0.008	0.127± 0.01	0.132± 0.013	0.124± 0.008	0.116± 0.008	0.118± 0.01	0.121± 0.01	0.119± 0.01	0.115± 0.013
ASL	0.120± 0.01	0.124± 0.008	0.126± 0.008	0.130± 0.01	0.136± 0.01	0.14± 0.01	0.137± 0.137	0.132± 0.008	0.134± 0.008	0.131± 0.01	0.13± 0.008	0.128± 0.009
ARG	7.3± 0.005	8.1± 0.007	8.6± 0.005	9.1± 0.006	9.8± 0.007	10.0± 0.008	8.4± 0.005	7.6± 0.007	7.3± 0.006	7.9± 0.007	7.4± 0.006	7.2± 0.006

CPS= carbamyl phosphate synthetase; OTC= ornithine transcarbamylase; ASS= argininosuccinate synthetase; ASL= argininosuccinate lyase; ARG= arginase

Table 16 : Purification protocol of hepatic arginase from H. fossilis

Purfication Steps	Volume (ml)	Protein (mg/ml)	Arginase activity (units/ml)	Total protein (mg)	Total units (units)	Specific activity (units/mg protein)	Purification fold	% Recovery
Homogenate (Triton X-100 treated)	60	15.77	4.26	946.2	255.6	0.27	-	100
$(NH_4)SO_4$ fractionation (40-60%)	30	9.62	7.12	288.6	213.6	0.74	2.7	84
Heat treatment	28	5.33	10.33	149.2	289.2	1.94	7.2	113
DEAE-Sephacel	20	1.27	9.88	25.4	197.6	7.8	29.0	77
Arginine Sepharose-4B	15	0.22	9.98	3.3	149.7	45.4	168.1	59
Sephadex G-150	10	0.11	9.10	1.10	91.0	82.7	306.3	36

Table 17: Effect of temperature on the activity (units/ml) of arginase purified from the liver of H. fossilis.

Temp °C	Arginse activity
5	0.49
10	1.05
15	1.25
20	1.50
25	2.05
30	2.25
35	3.40
40	4.85
45	5.70
50	7.46
55	5.95
60	3.99
70	-

Table 18 : Effect of pre-incubation time and temperature on the activity (%) of arginase purified from the liver of H. fossilis.

Temp. of pre-incubation(°C)	Time of pre-incubation (min)						
	5	10	20	30	40	50	60
10	99.5	98.0	97.5	96.5	96.5	92.5	91.5
20	99.5	96.5	95.0	94.0	93.0	90.0	89.0
30	97.5	95.0	94.0	90.0	82.5	74.0	65.0
35	95.5	95.0	92.0	82.5	78.5	67.0	62.5
40	91.0	90.0	75.0	70.0	57.5	46.0	34.0
50	91.0	85.0	70.0	62.0	50.0	37.5	26.0
55	90.0	73.5	62.0	48.0	30.0	12.5	8.0
60	75.0	51.0	21.0	1.0	-	-	-

Table 19 : Effect of cofactor ($MnCl_2$, 5mM), substrate (L-arginine, 100 mM) separately and together on arginase activity purified from the liver of H. fossilis.

Time (min)	Enzyme + buffer	Enzyme + $MnCl_2$ (5 mM)	Enzyme + L-Arginine	Enzyme + $MnCl_2$(5 mM)+ L-Arginine (100 mM)
0	6.84 (100)	7.4 (100)	7.47 (100)	8.75 (100)
10	5.49 (80)	5.94 (80)	6.87 (92)	8.53 (98)
20	4.0 (56)	5.74 (78)	5.90 (79)	8.31 (95)
30	3.28 (48)	5.36 (73)	5.54 (74)	7.91 (90)
40	2.07 (30)	5.18 (70)	5.38 (72)	7.65 (87)
50	0.77 (11)	3.70 (50)	4.18 (56)	6.34 (73)
60	0.37 (5)	2.22 (30)	3.06 (41)	5.11 (58)

% change of activity are given in parentheses.
The zero time activity in each case was taken as 100% of activity.

Table 20: Activity (units/ml) of arginase purified from the liver of H. fossilis at different pH.

pH	Arginase activity (units/ml)
6.0	0.8
6.5	1.05
7.0	2.25
7.5	2.40
8.0	3.15
8.5	3.65
9.0	5.40
9.5	6.50
10.0	2.75
10.5	1.45
11.0	0.25
12.0	0

Table 21: Effect of different amino acids on the activity (units/ml) of arginase purified from the liver of *H. fossilis*.

Amino acids (25mM)	Arginase activity (units/ml)	% Change
None	7.2	-
L-Alanine	7.2	0
L-Aspartate	3.7	-48.6
L-Histidine	3.7	-48.6
L-Methionine	5.7	-20.6
L-Proline	5.3	-26.4
L-Tryptophan	6.4	-11.1
L-Cysteine	7.0	-2.7
L-Glutamate	7.0	-2.7
L-Leucine	2.9	-60.0
L-Ornithine	2.3	-68.1
L-Serine	7.3	+1.3
L-Tyrosine	7.1	-1.3
L-Glycine	5.7	-20.8
L-Isoleucine	2.7	-62.5
L-Phenylalanine	4.3	-40.3
L-Threonine	3.7	-48.6
L-Valine	2.8	-61.1
L-Lysine	3.8	-47.2

Table 23: Nature of inhibition and Ki values for different inhibitors for the enzyme arginase purified from the liver of H. fossilis.

Inhibitors	Nature of inhibition	Ki (mM)
L-Ornithine	Competitive	4.25
L-Leucine	Competitive	9.30
L-Valine	Non-competitive	5.30
L-Isoleucine	Non-competitive	7.25

FIGURES

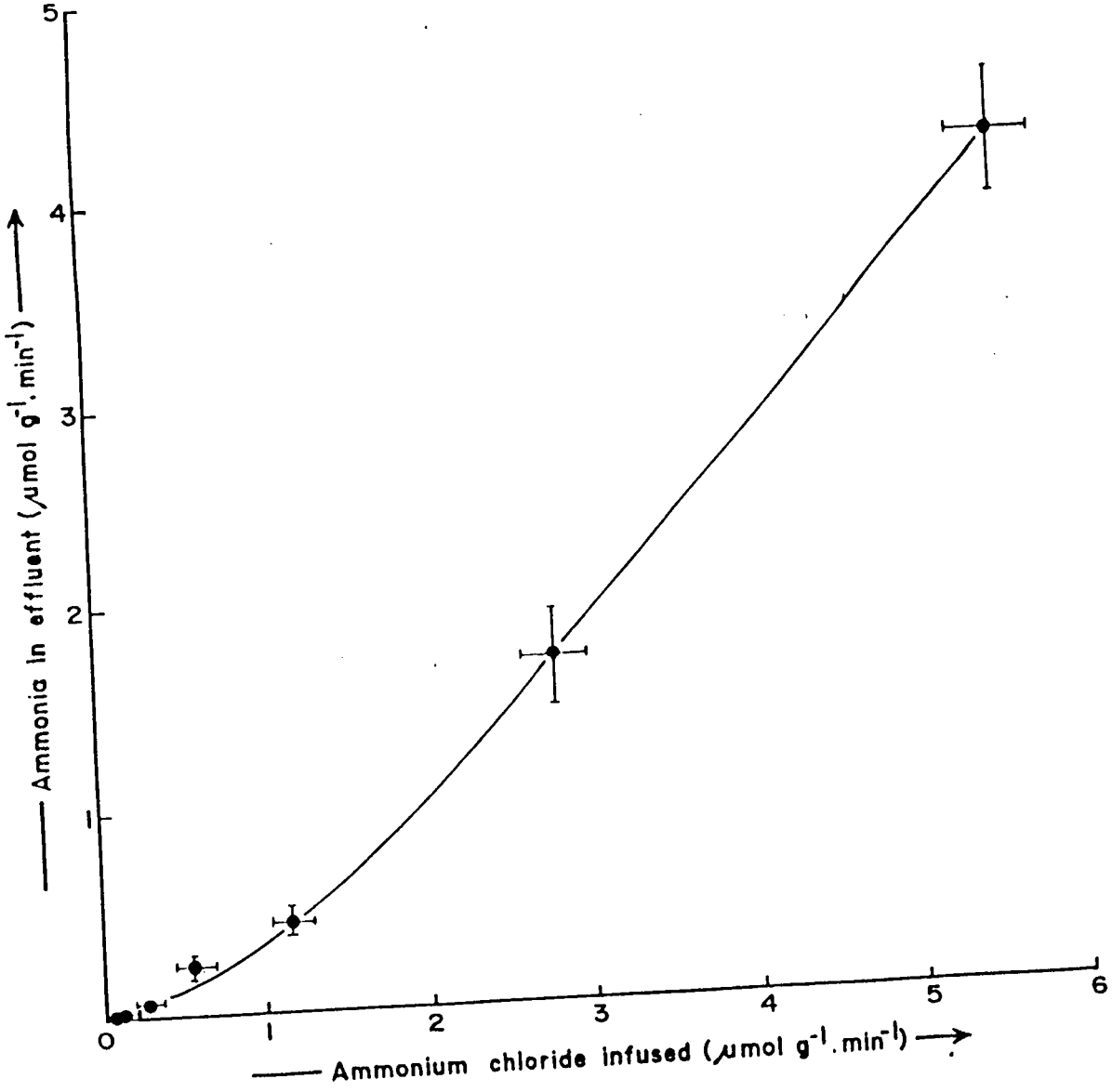


Fig. 2 Ammonia excretion rate (µmol g^{-1} **liver. min^{-1}) into the effluent by the perfused liver of H. fossilis while infusing ammonium chloride at different rates.**

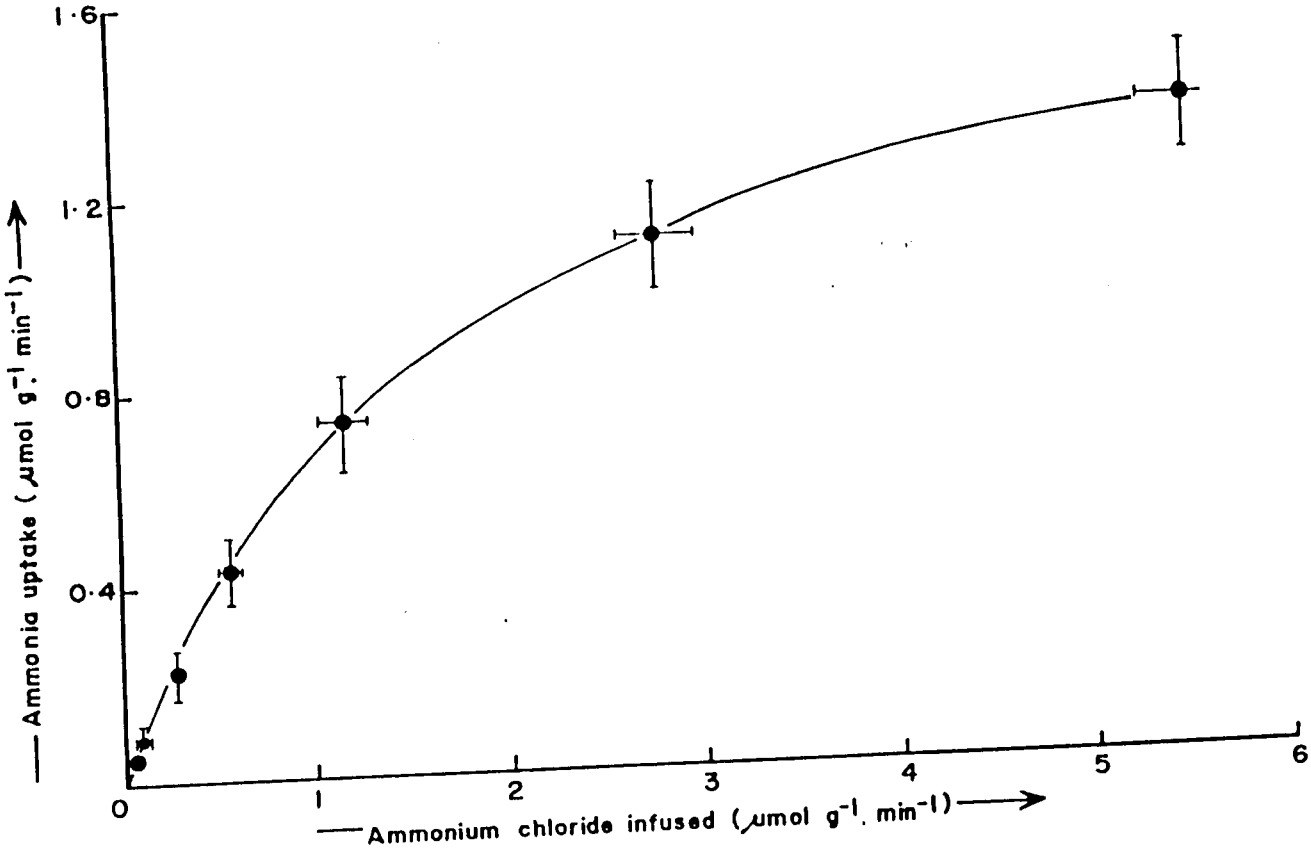


Fig. 3 The rate of ammonia uptake (µmol g^{-1} liver .min^{-1}) by the perfused liver of H. fossilis while infusing ammonium chloride at different rates.

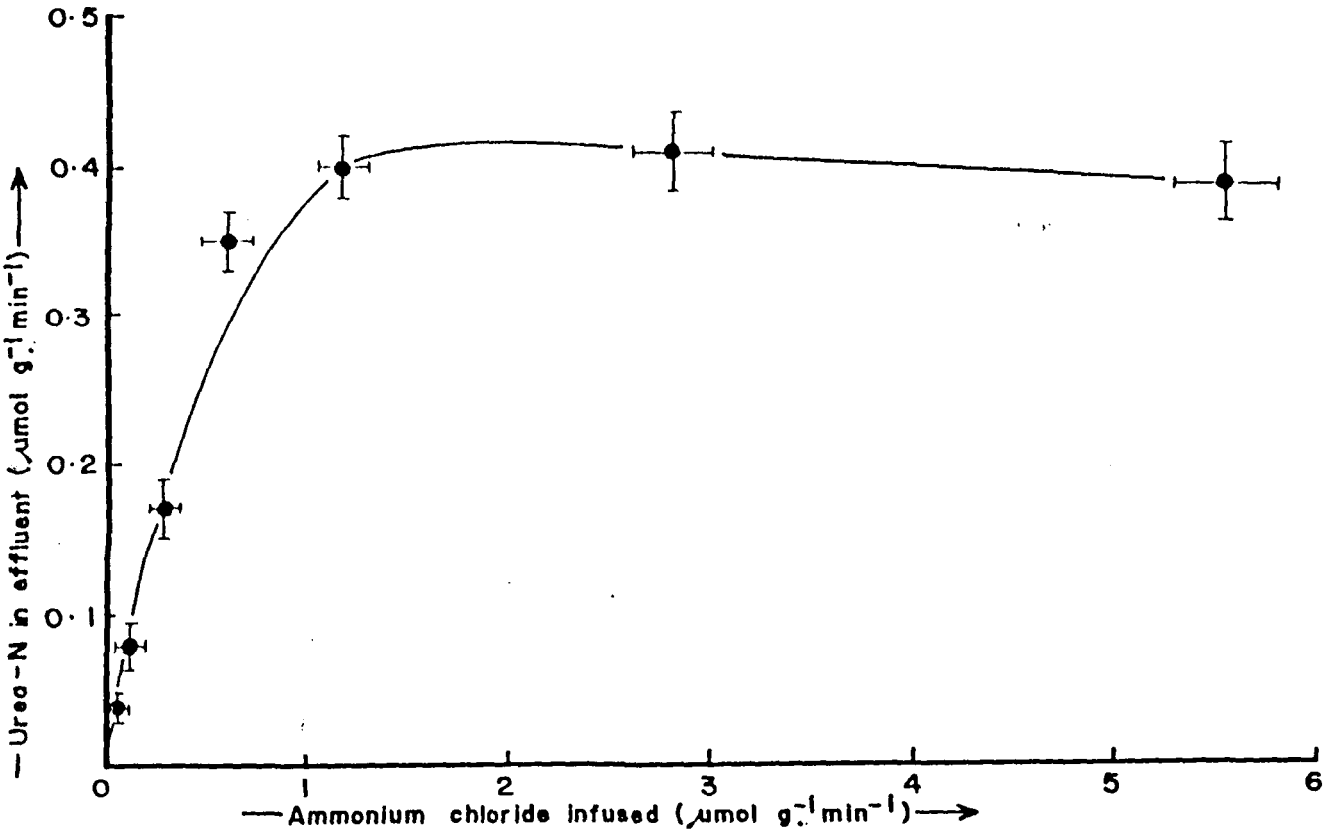


Fig. 4 The rate of urea-N release (μmol g^{-1} liver. min^{-1}) into the effluent by the perfused liver of H. fossilis while infusing ammonium chloride at different rates.

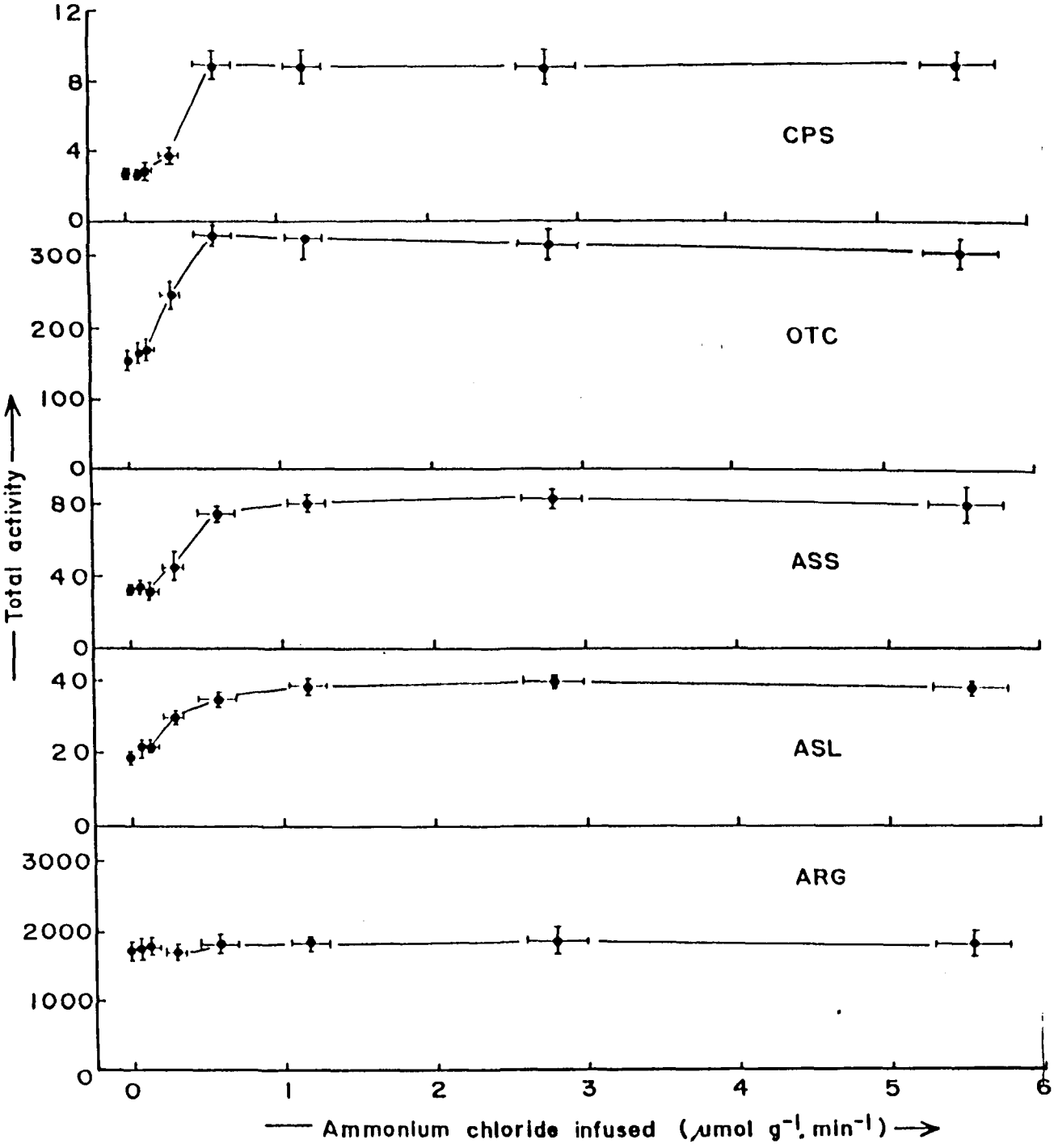


Fig. 5 **Alterations of total activity (units g^{-1}. wet wt.) of o-u cycle enzymes in the perfused liver of H. fossilis while infusing ammonium chloride at different rates.**

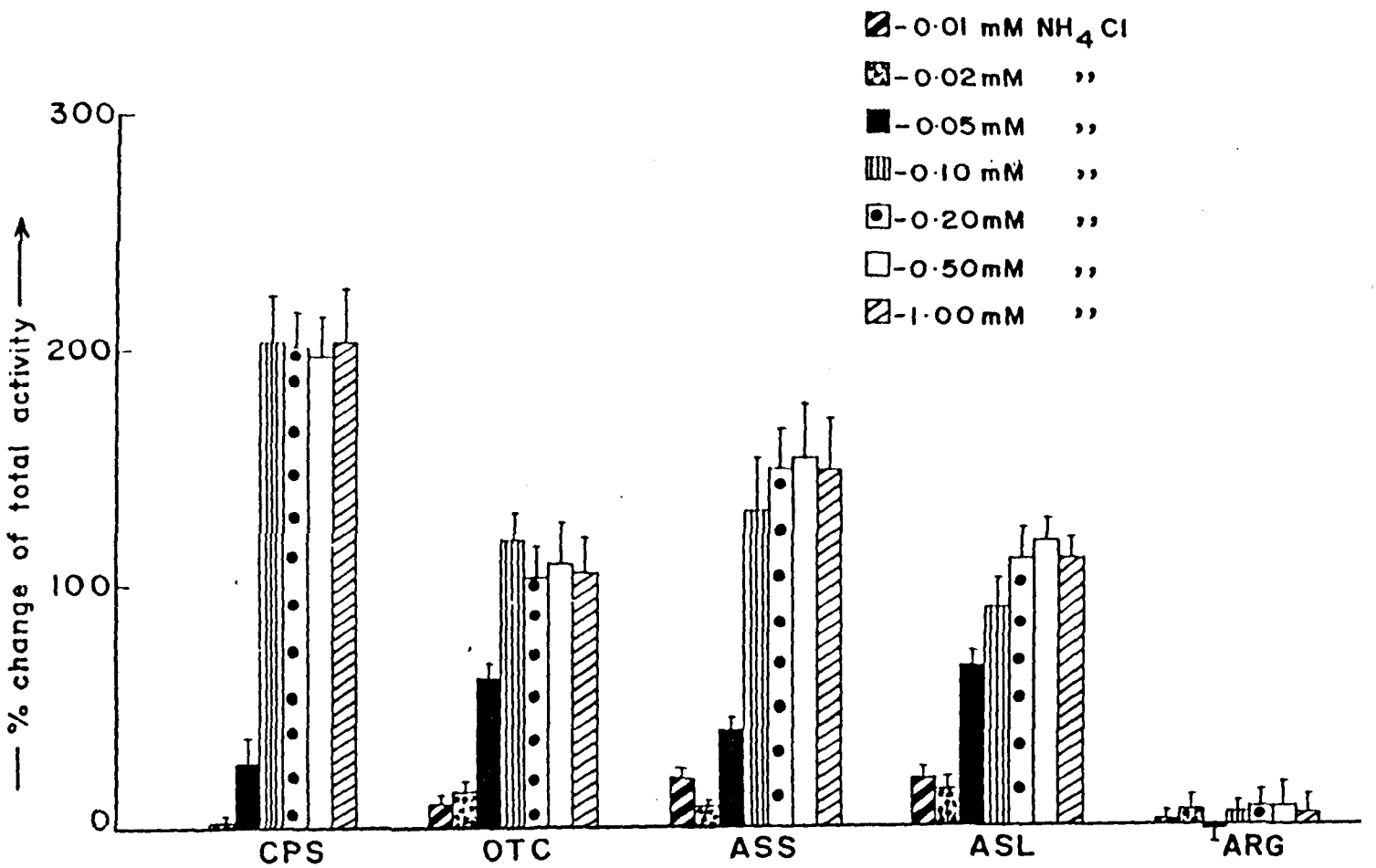


Fig. 6 % change of total activity of o-u cycle enzymes in the perfused liver of H. fossilis while infusing ammonium chloride at different rates.

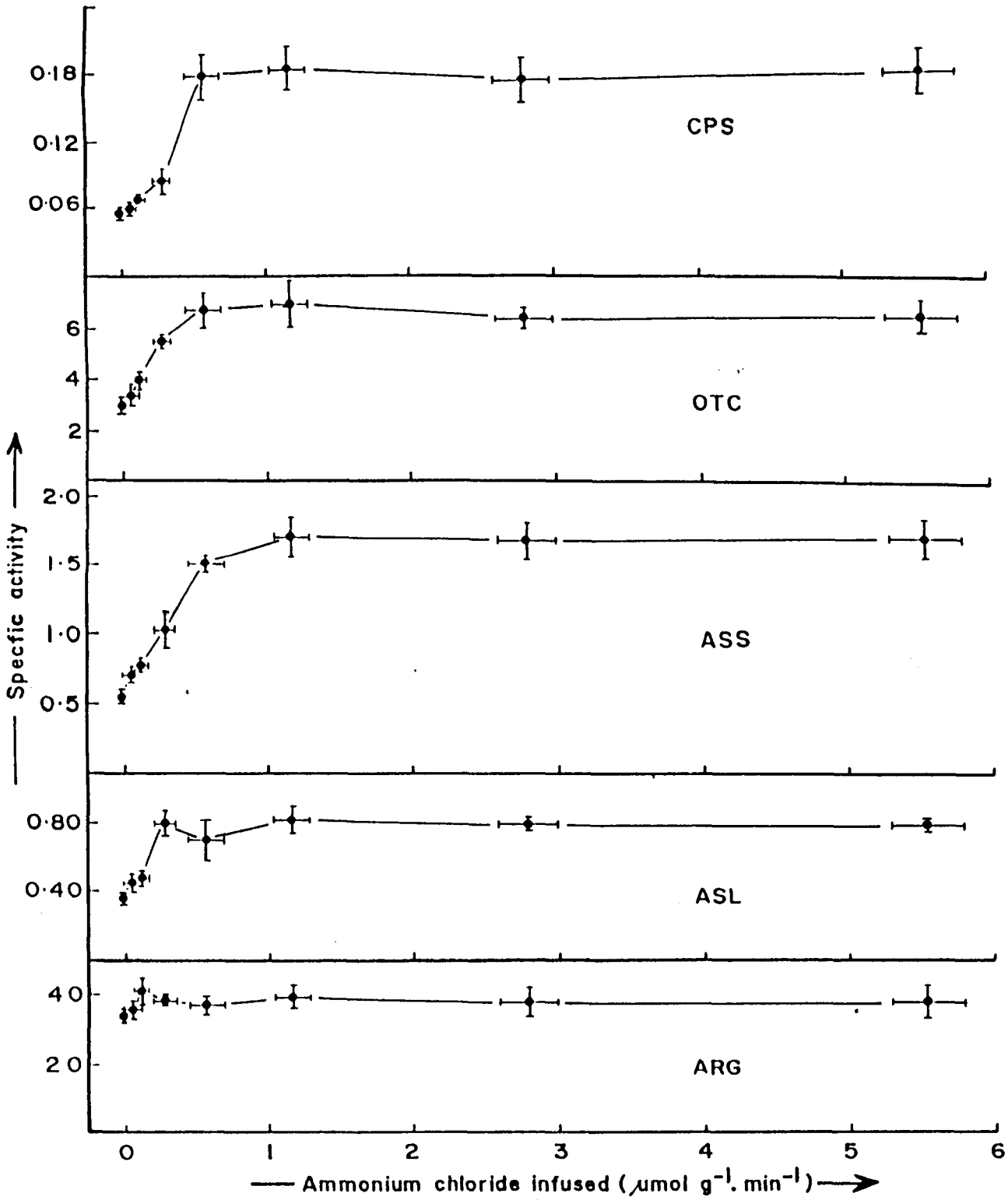


Fig. 7 Alterations of specific activity (units mg^{-1}. protein) of o-u cycle enzymes in the perfused liver of H. fossilis while infusing ammonium chloride at different rates.

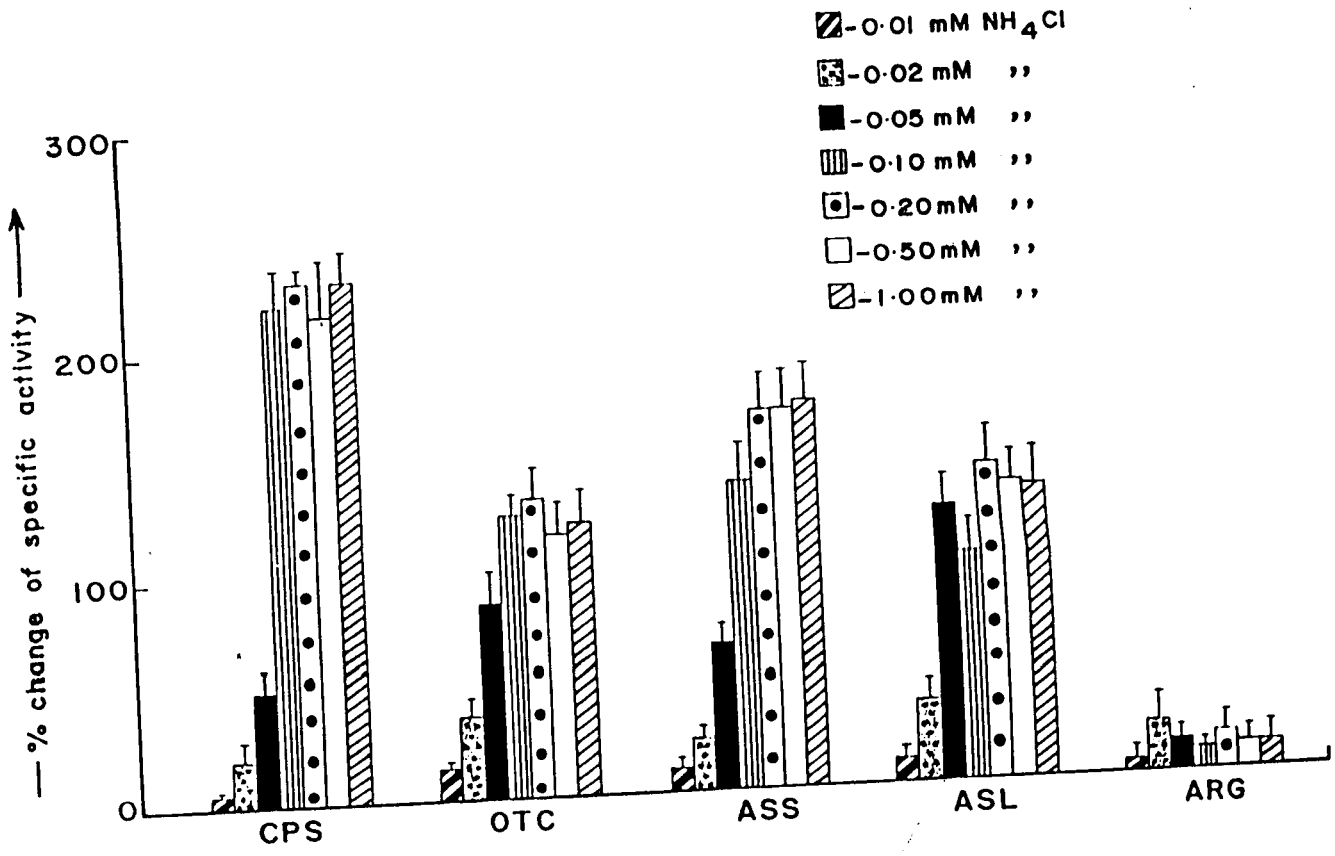


Fig. 8 % change of specific activity of o-u cycle enzymes in the perfused liver of H. fossilis while infusing ammonium chloride at different rates.

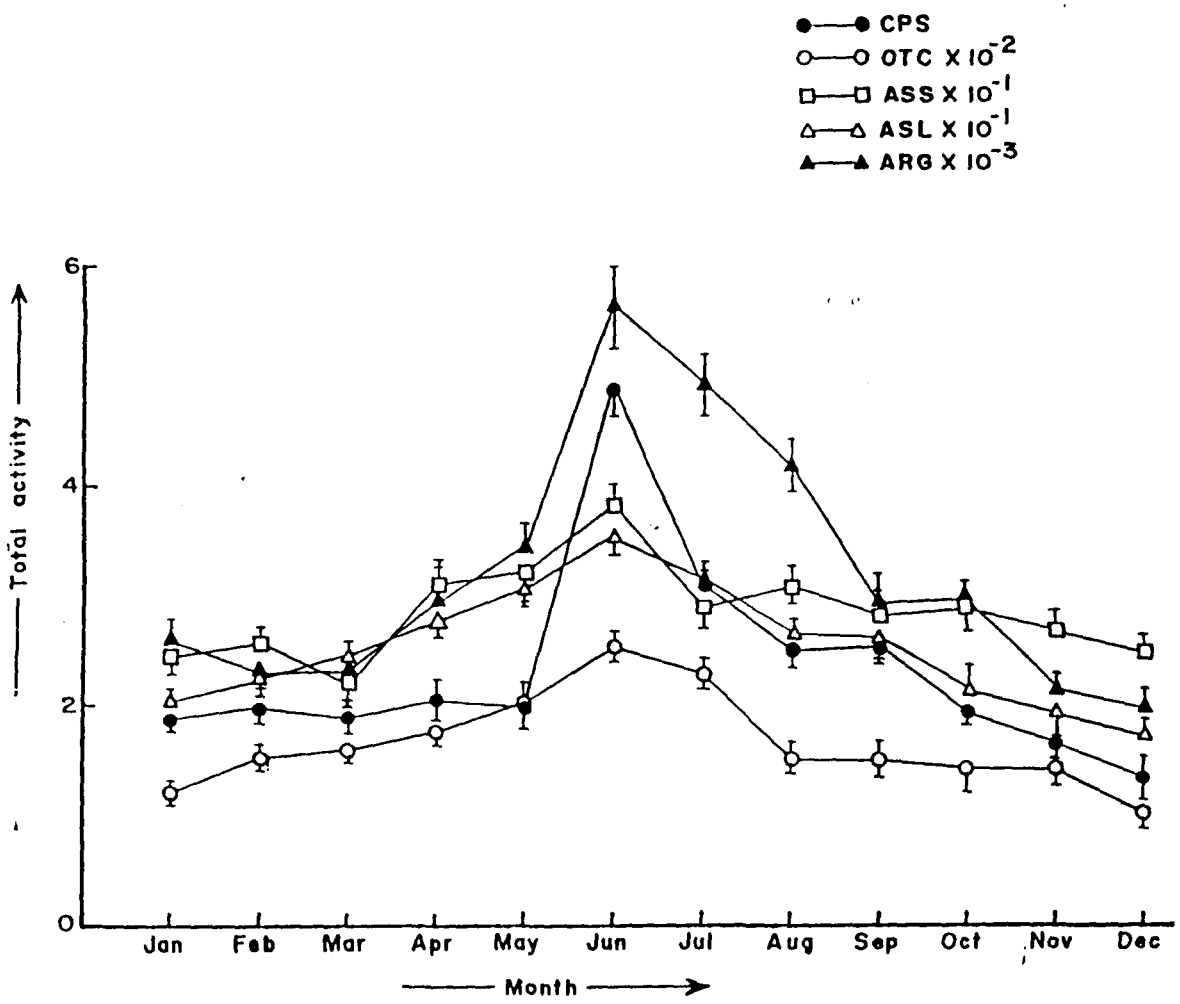


Fig. 9 Annual variation of total activity (units/g wet wt.) of the o-u cycle enzymes in the liver of H. fossilis during the year 1991

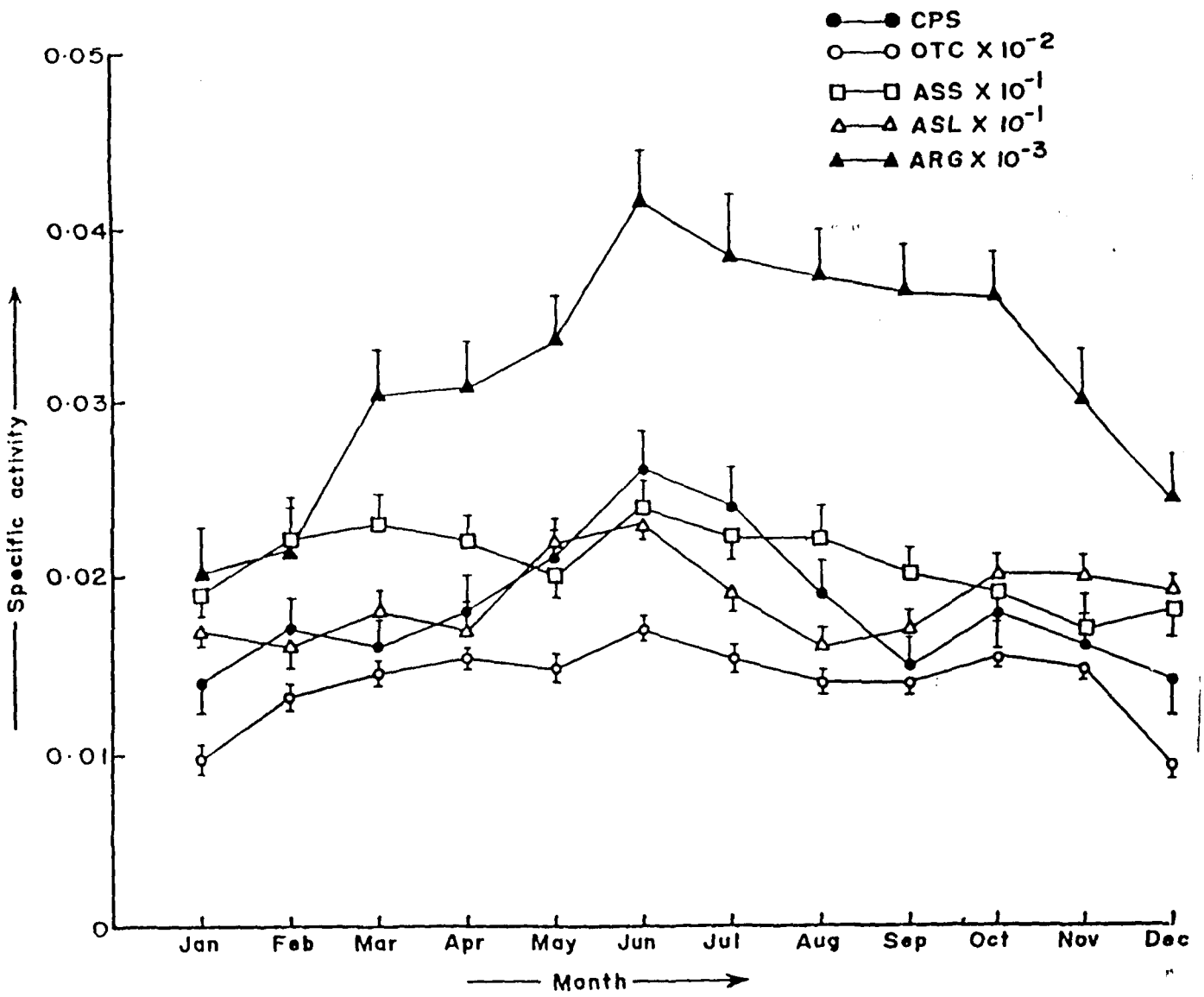


Fig. 10 Annual variation of specific activity (units/mg protein) of o-u cycle enzymes in the liver of H. fossilis during the year 1991

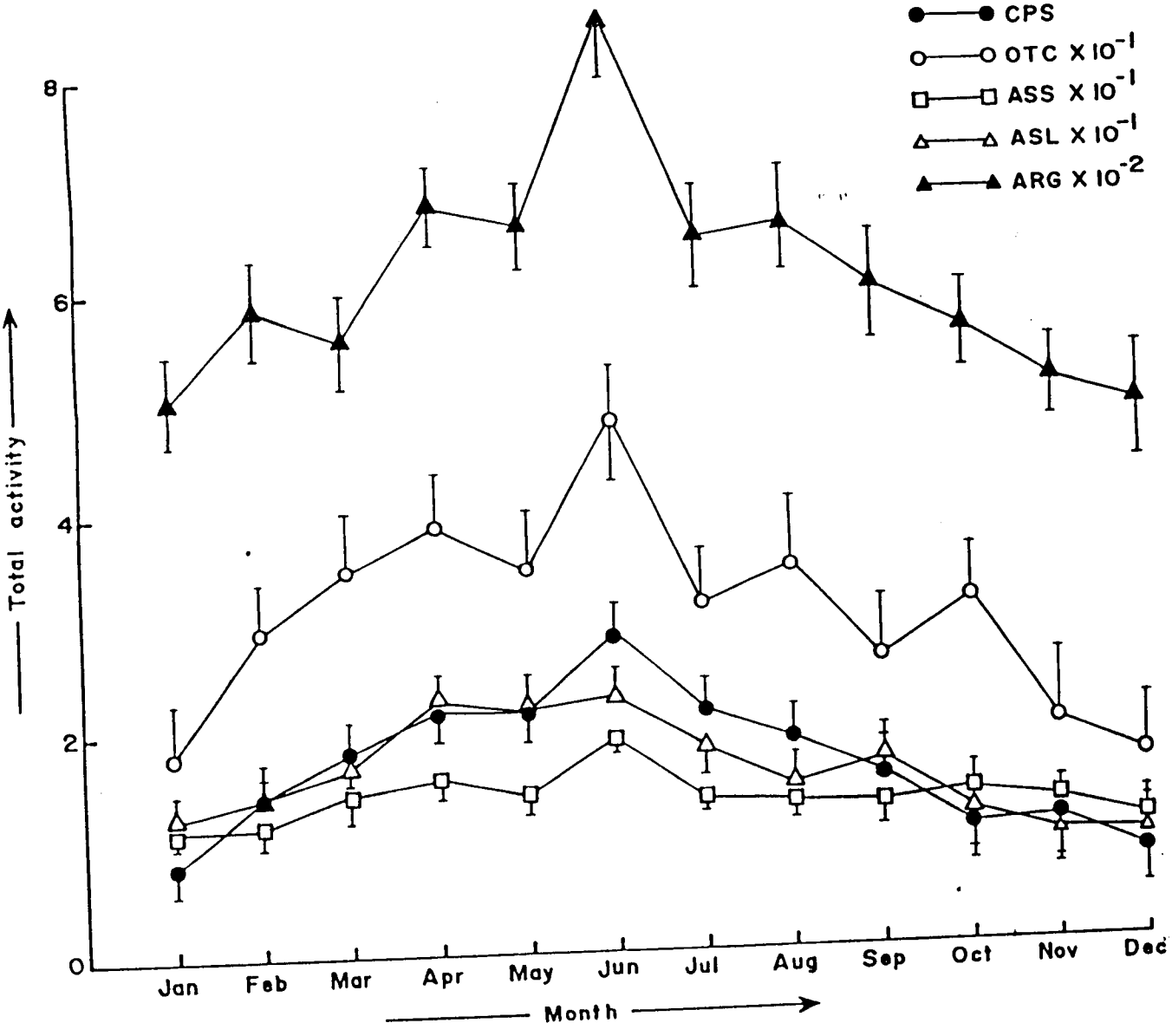


Fig. 11 Annual variation of total activity (units/g wet wt.) of o-u cycle enzymes in the kidney of H. fossilis during the year 1991

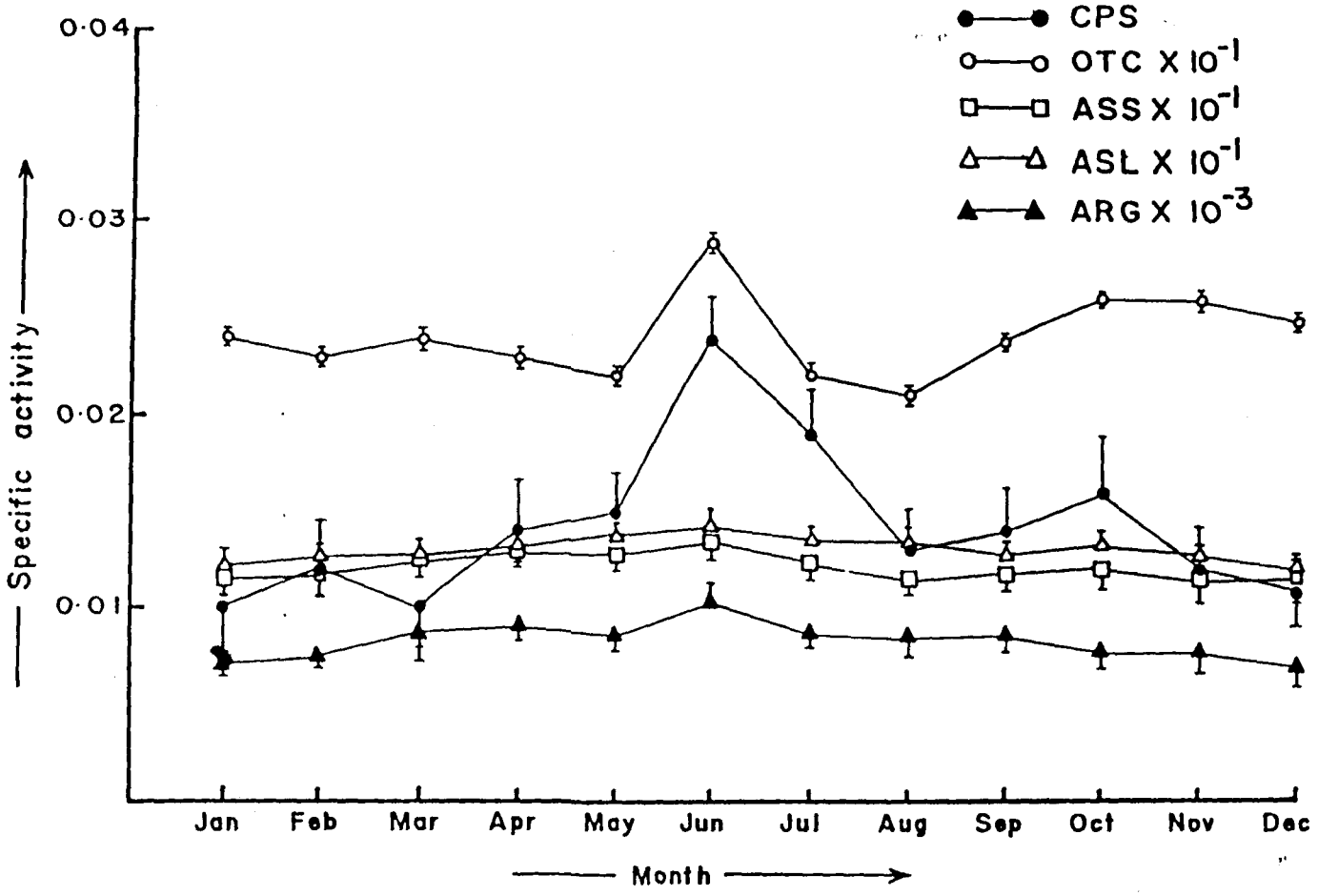


Fig. 12 *Annual* variation of specific activity (units/mg protein) of o-u cycle enzymes in the kidney of H. fossilis during the year 1991

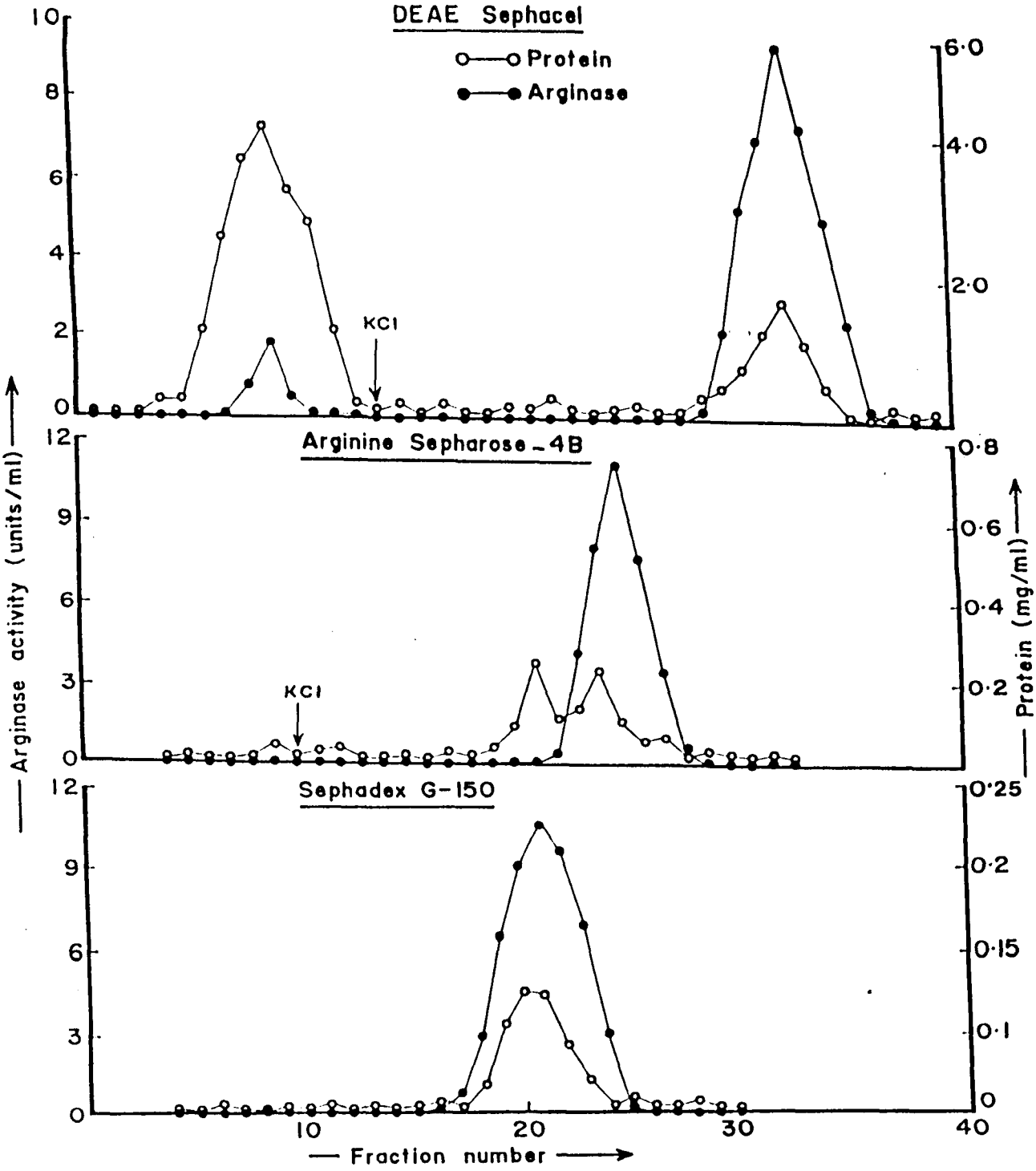


Fig. 13 Elution pattern of arginase from DEAE-Sephacel, Arginine Sepharose 4B and Sephadex G-150 columns during purification from the liver of H. fossilis.

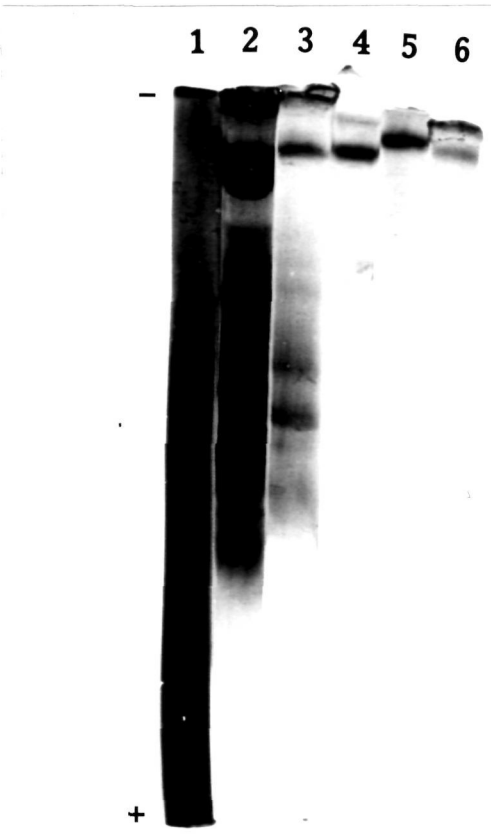


Fig. 14 Protein staining (Coomasie Blue) of hepatic arginase from H. fossilis on polyacrylamide gel electrophoresis at different stages of purification.

1. Homogenate
2. $(NH_4)_2SO_4$ fractionation
3. Heat treatment
4. DEAE - Sephacel
5. Arginine Sepharose 4B
6. Sephadex G-150

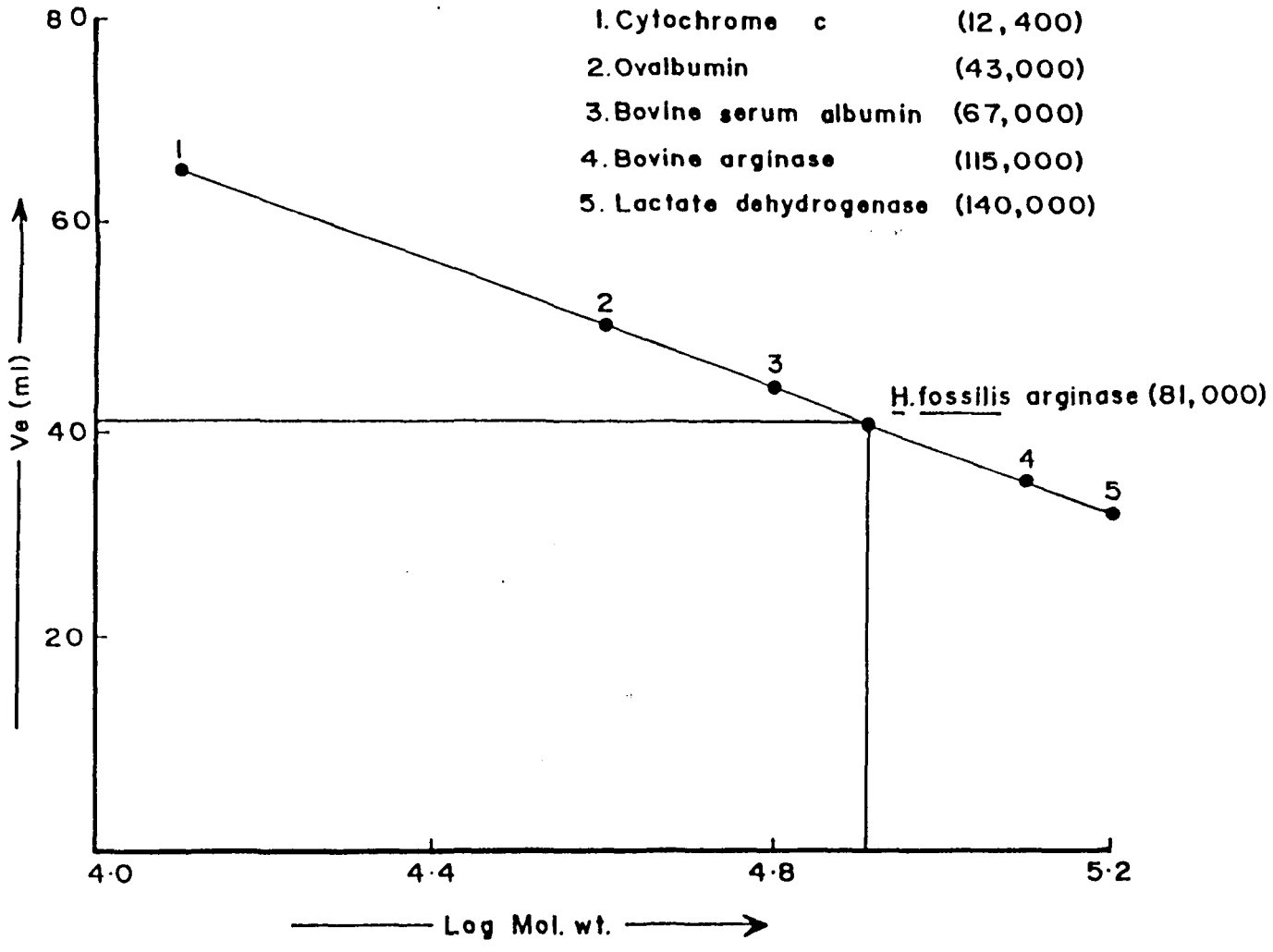


Fig. 15 Determination of molecular weight of arginase purified from the liver of H. fossilis by gel filtration on Sephadex G-150 column.

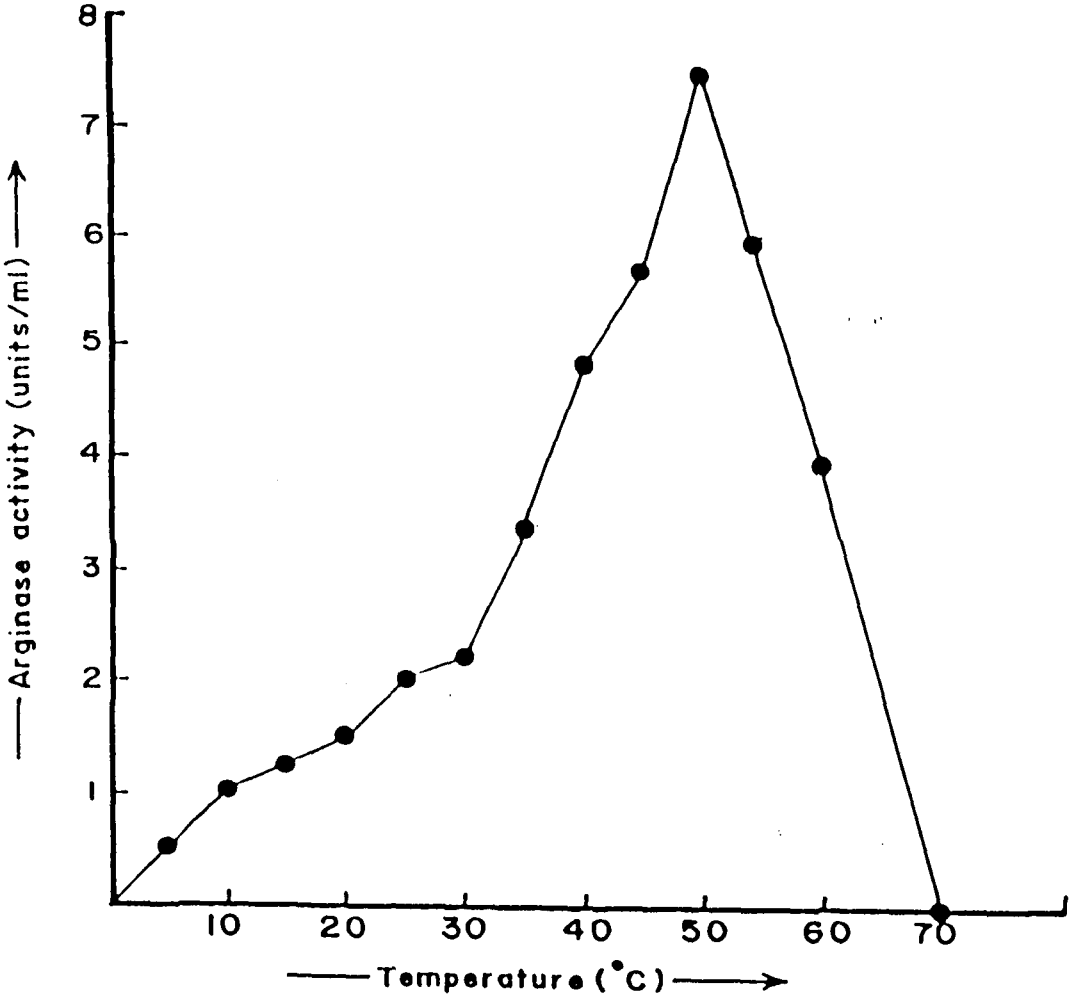


Fig. 16 Effect of incubation temperature on arginase activity purified from the liver of H. fossilis.

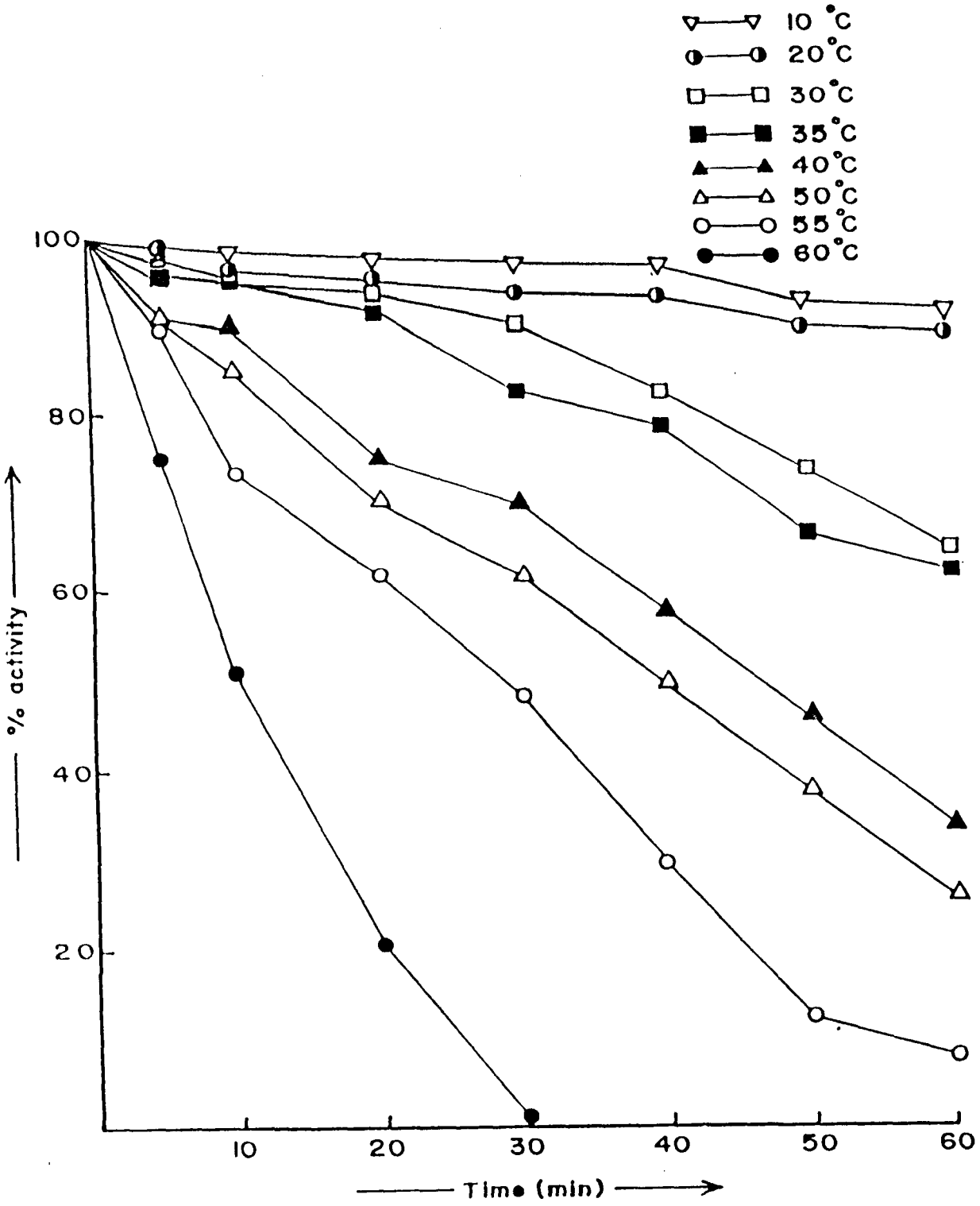


Fig. 17 Effect of pre-incubation time and temperature on the % change of activity of arginase purified from the liver of H. fossilis.

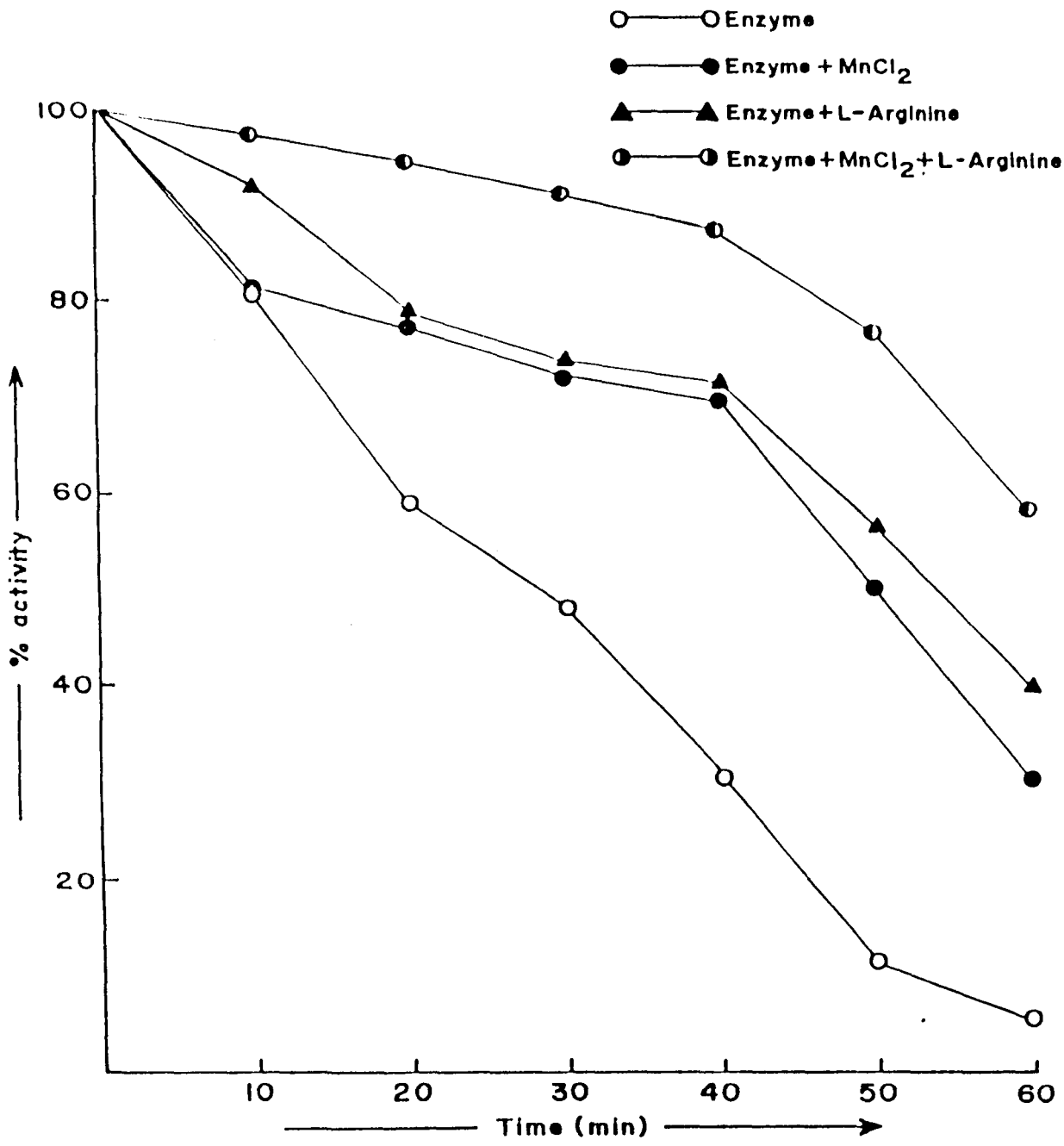


Fig. 18 Temporal change in the activity of arginase purified from the liver of H. fossilis pre-incubated at 55 °C with the substrate (arginine, 100 mM) and co-factor(Mn_2Cl, 5mM) separately and together.

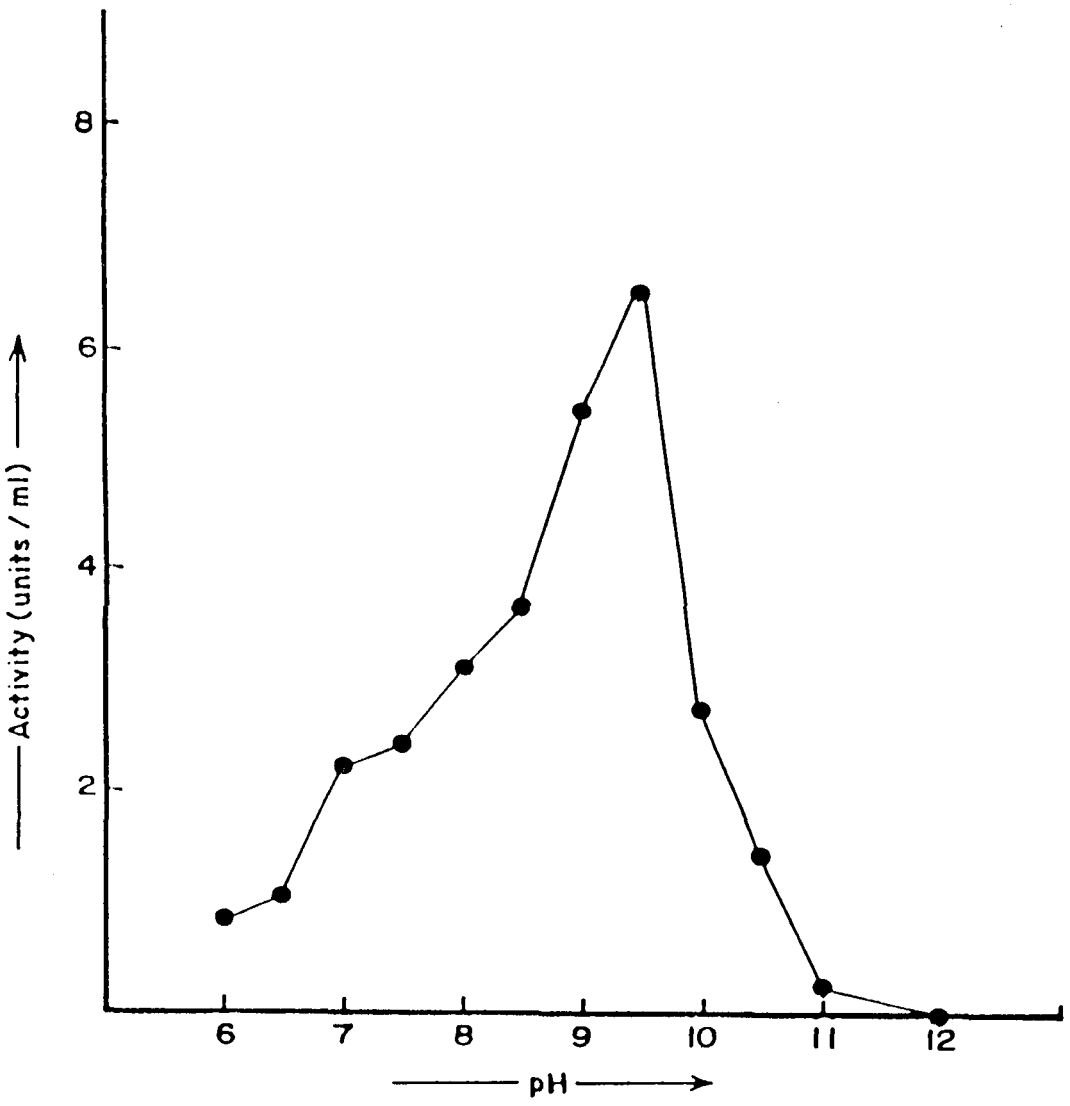


Fig. 19 Effect of pH on arginase activity purified from the liver of H. fossilis.

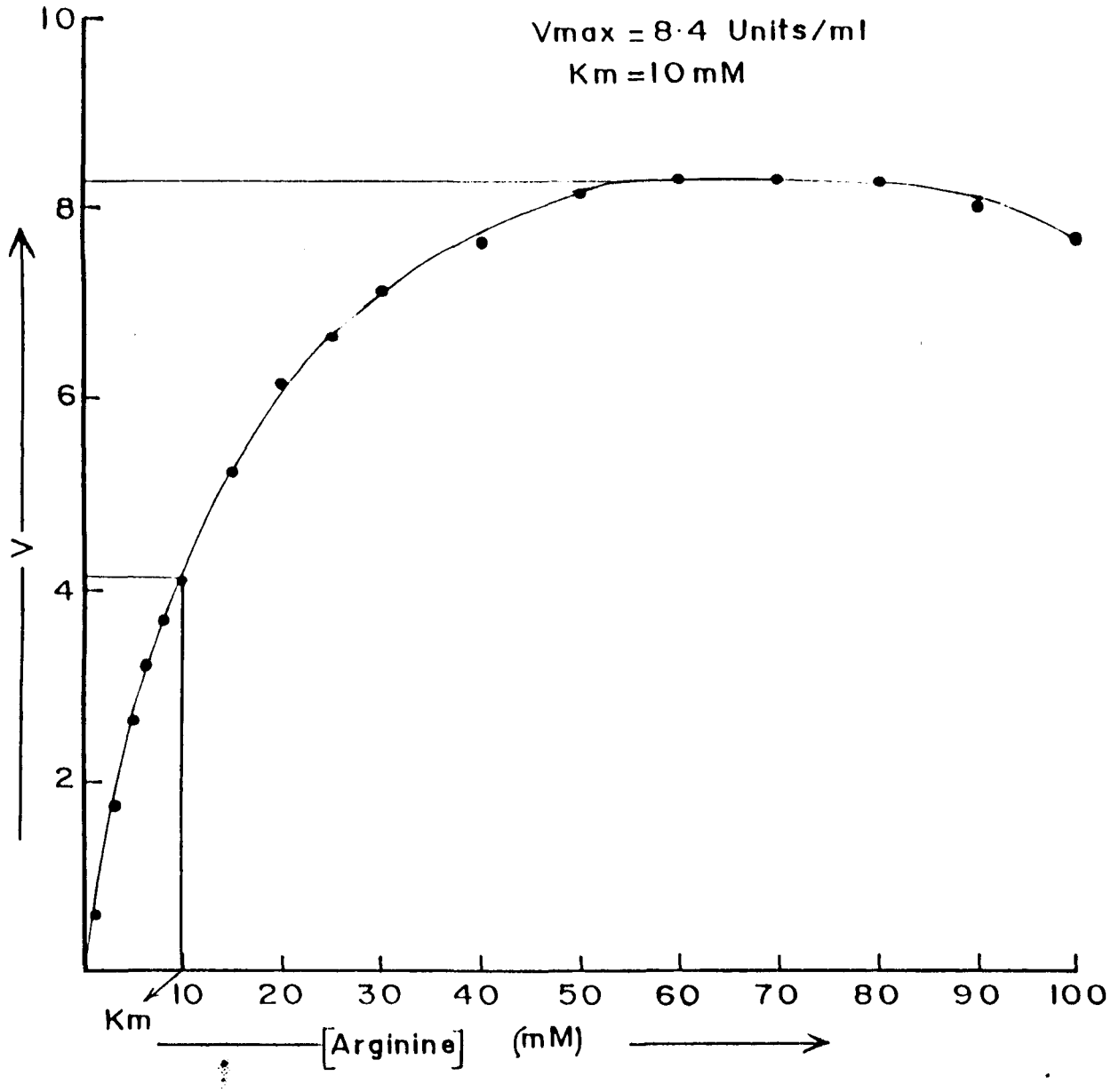


Fig. 20 Michaelis-Menten plot for determination of Km and Vmax of arginase purified from the liver of H. fossilis.

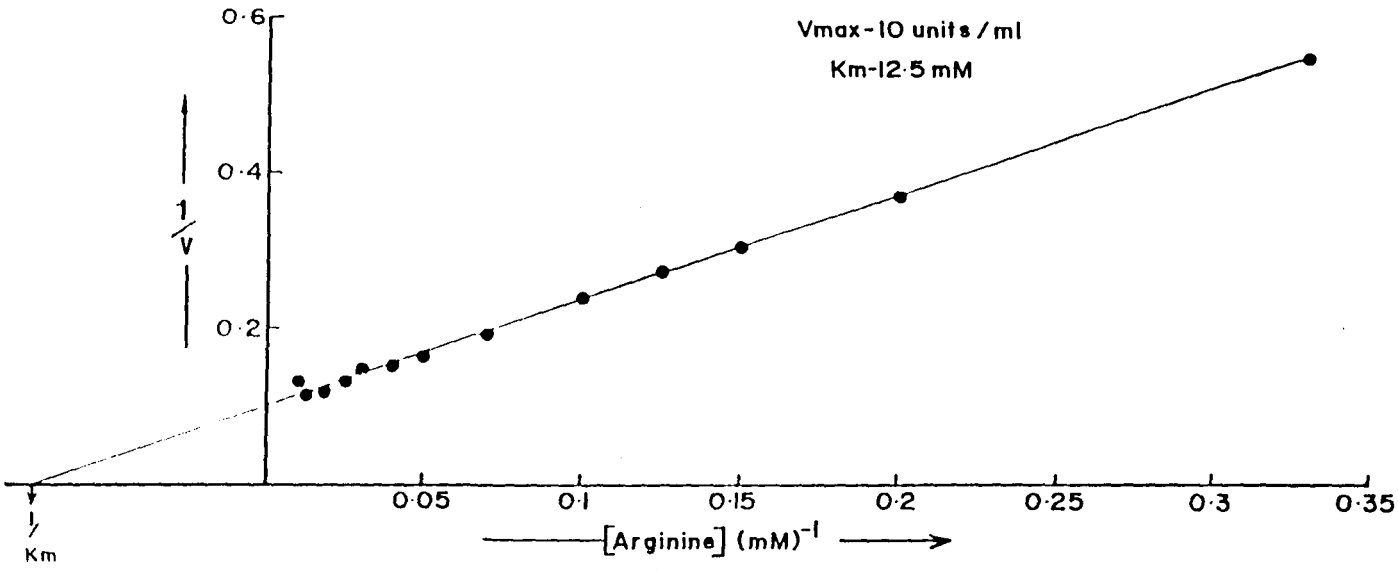


Fig. 21 Lineweaver-Burg plot for determination of Km and Vmax of arginase purified from the liver of H. fossilis.

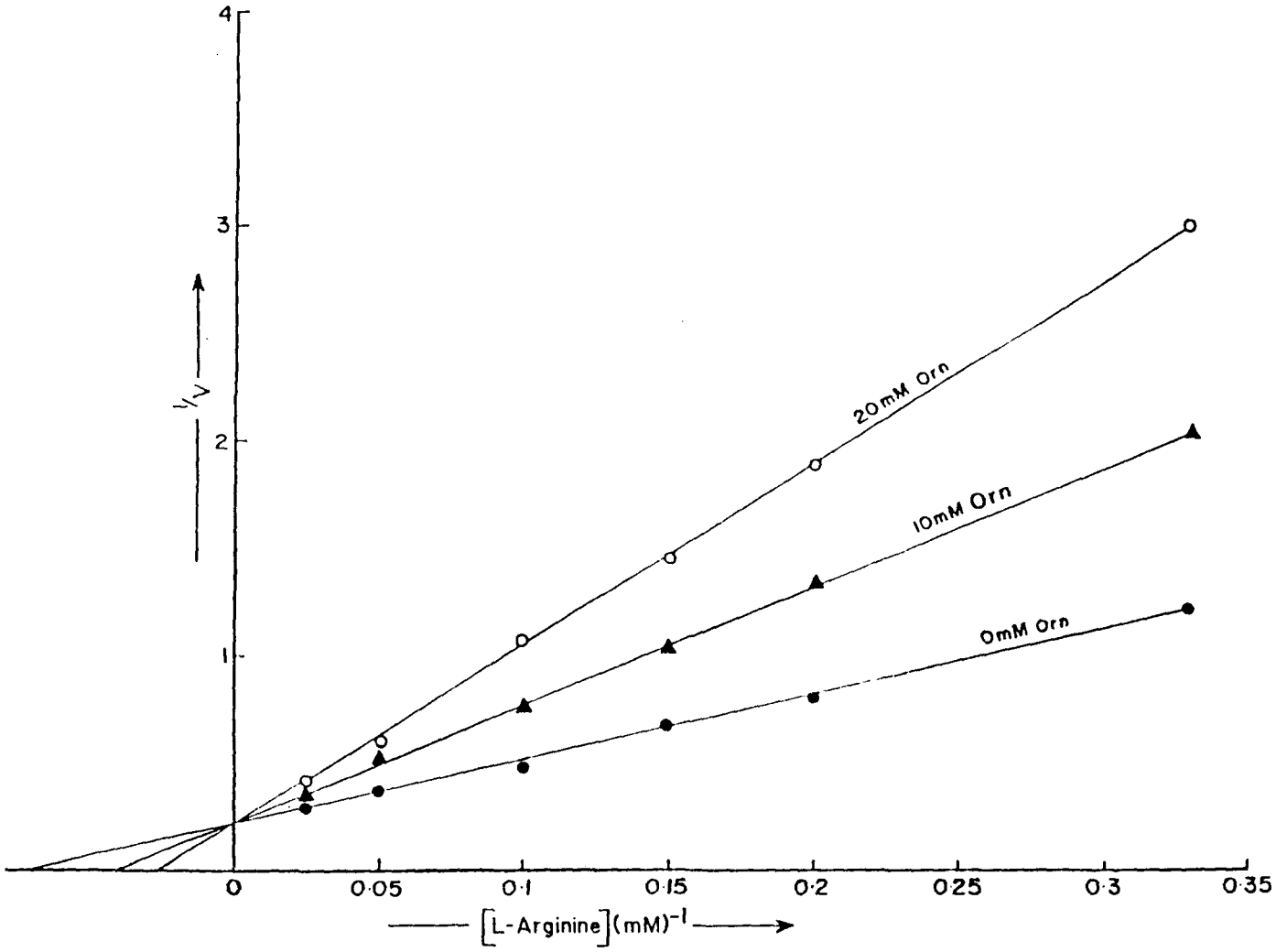


Fig. 22 Lineweaver-Burk plot for determination of the nature of inhibition of L-ornithine on the activity of arginase purified from the liver of H. fossilis.

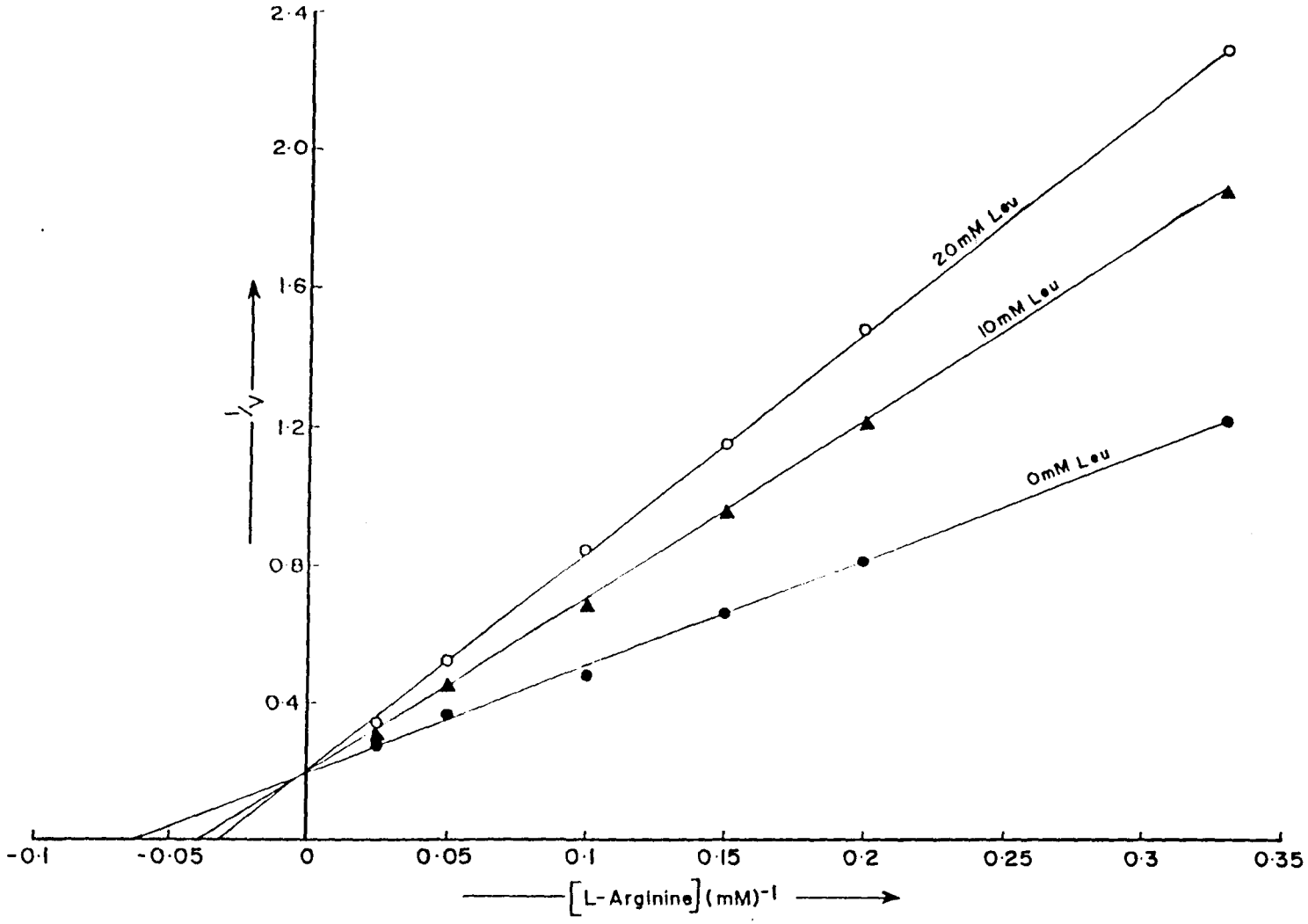


Fig. 23 **Lineweaver-Burk plot for determination of the nature of inhibition of L-leucine on the activity of arginase purified from the liver of H. fossilis.**

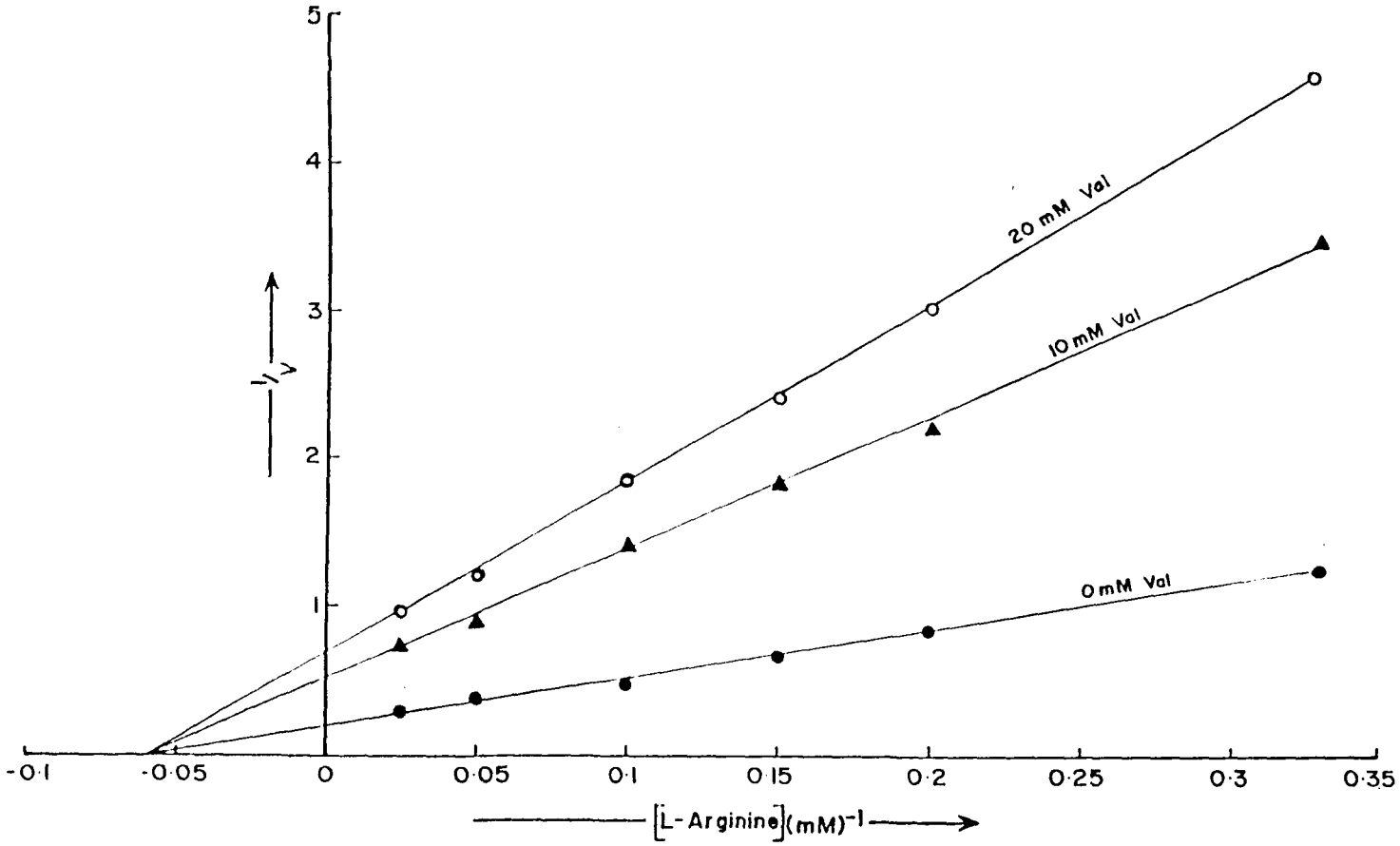


Fig. 24 Lineweaver-Burk plot for determination of the nature of inhibition of L-valine on the activity of arginase from the liver of H. fossilis.

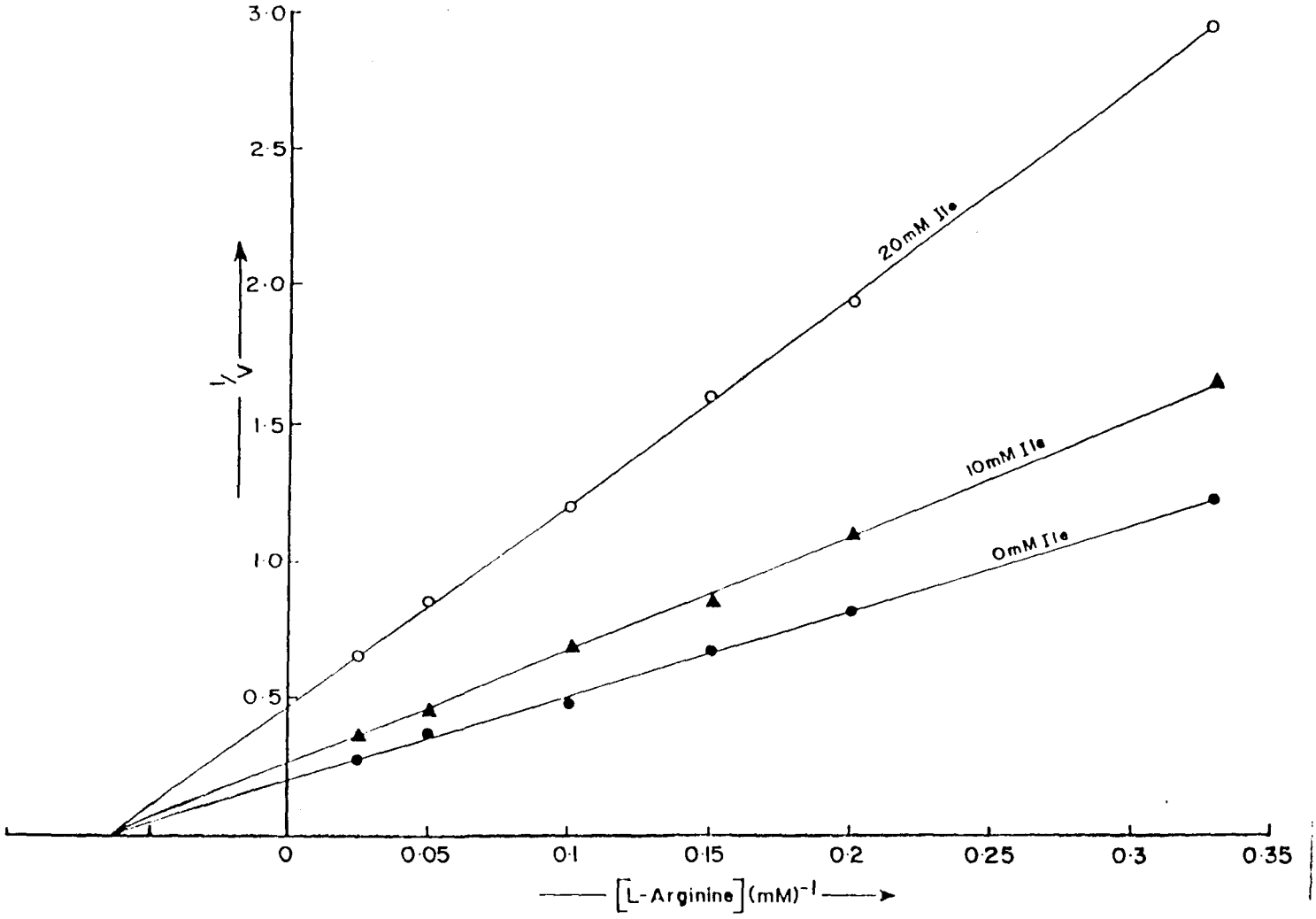


Fig. 25 Lineweaver-Burk plot for determination of the nature of inhibition of L-isoleucine on the activity of arginase purified from the liver of H. fossilis.

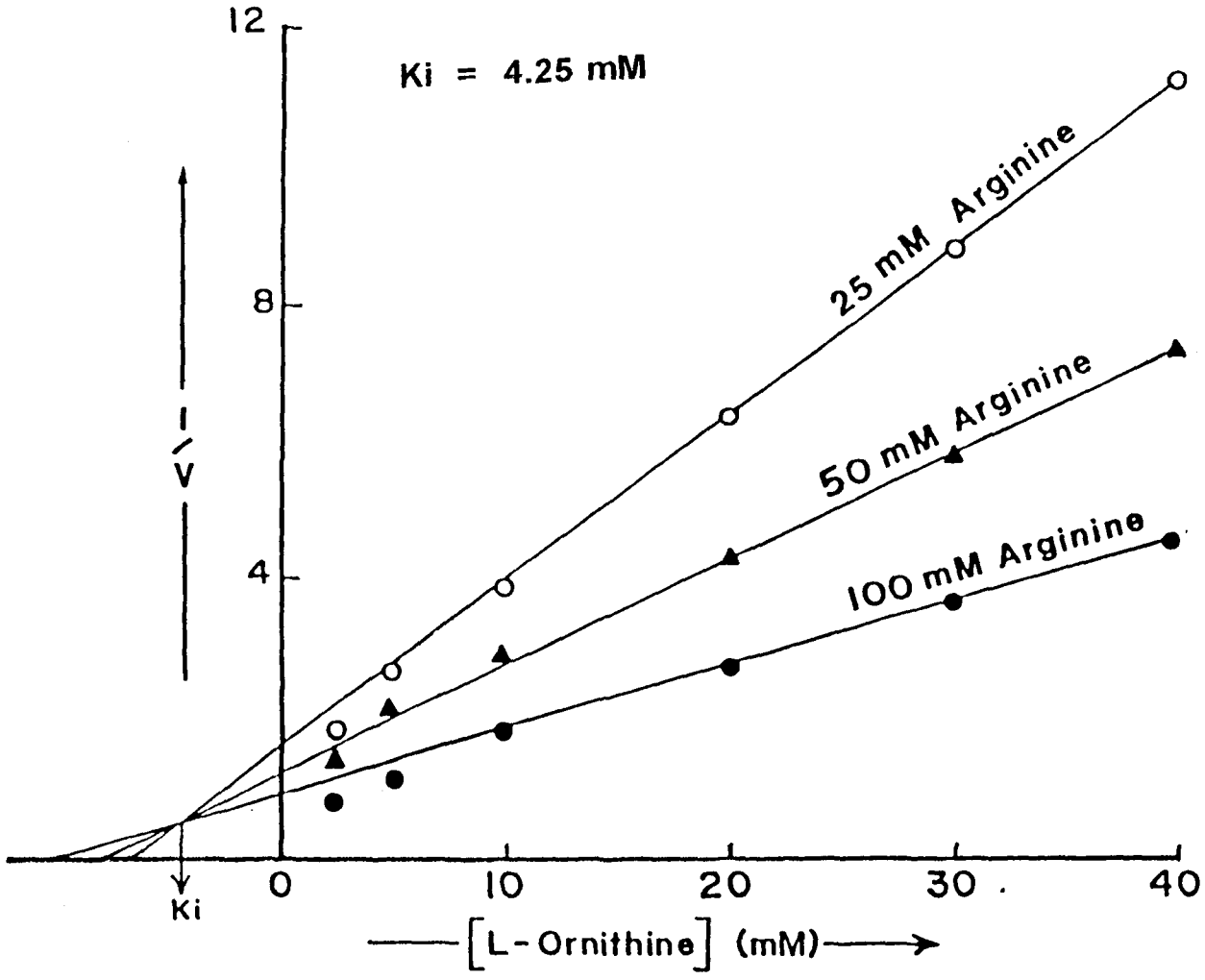


Fig. 26 Dixon plot for determination of inhibitor constant (Ki) of L-ornithine for arginase purified from the liver of H. fossilis.

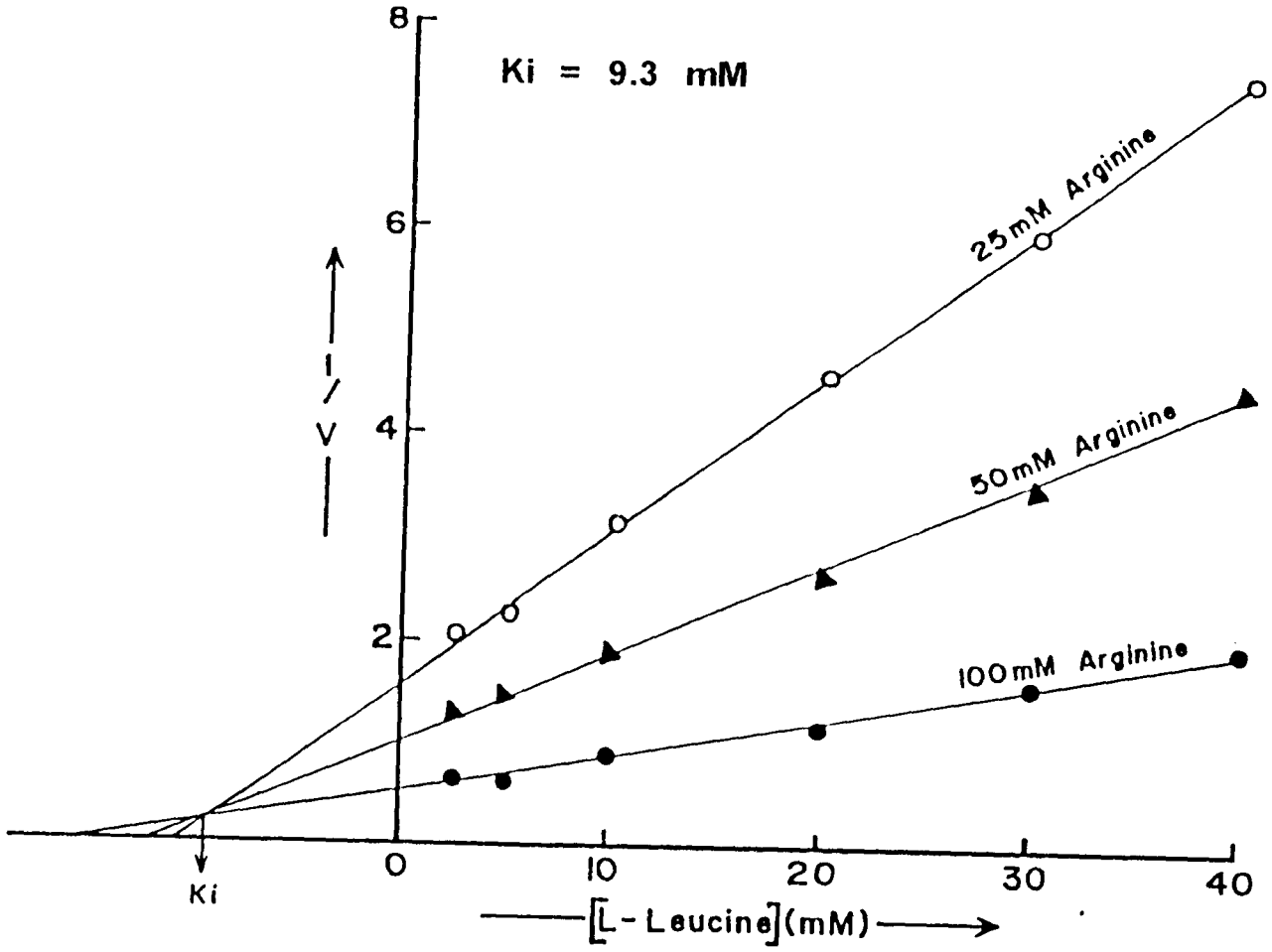


Fig. 27 Dixon plot for determination of inhibitor constant (Ki) of L-leucine for arginase purified from the liver of H. fossilis.

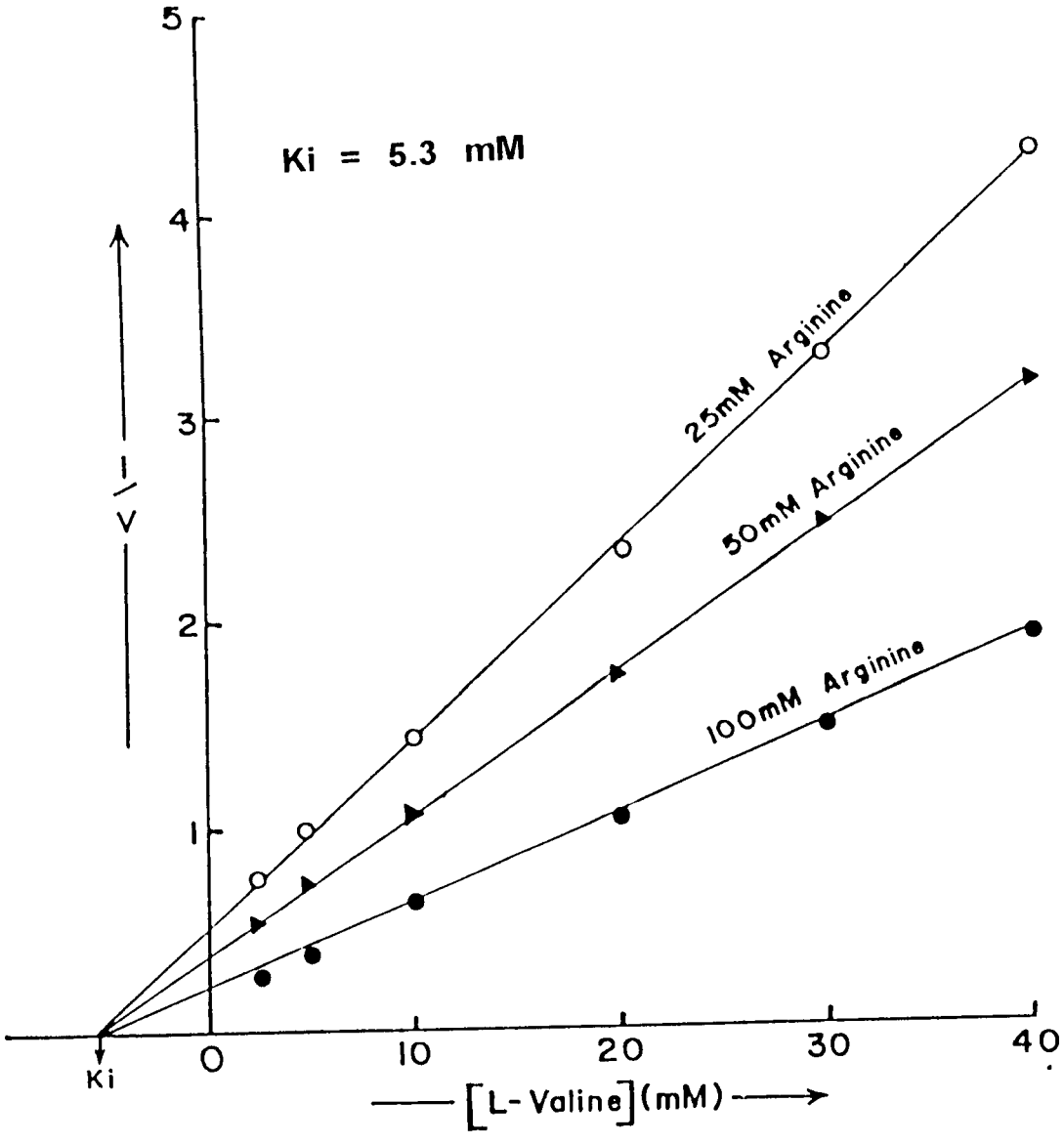


Fig. 28 Dixon plot for determination of inhibitor constant (Ki) of L-valine for arginase purified from the liver of H. fossilis.

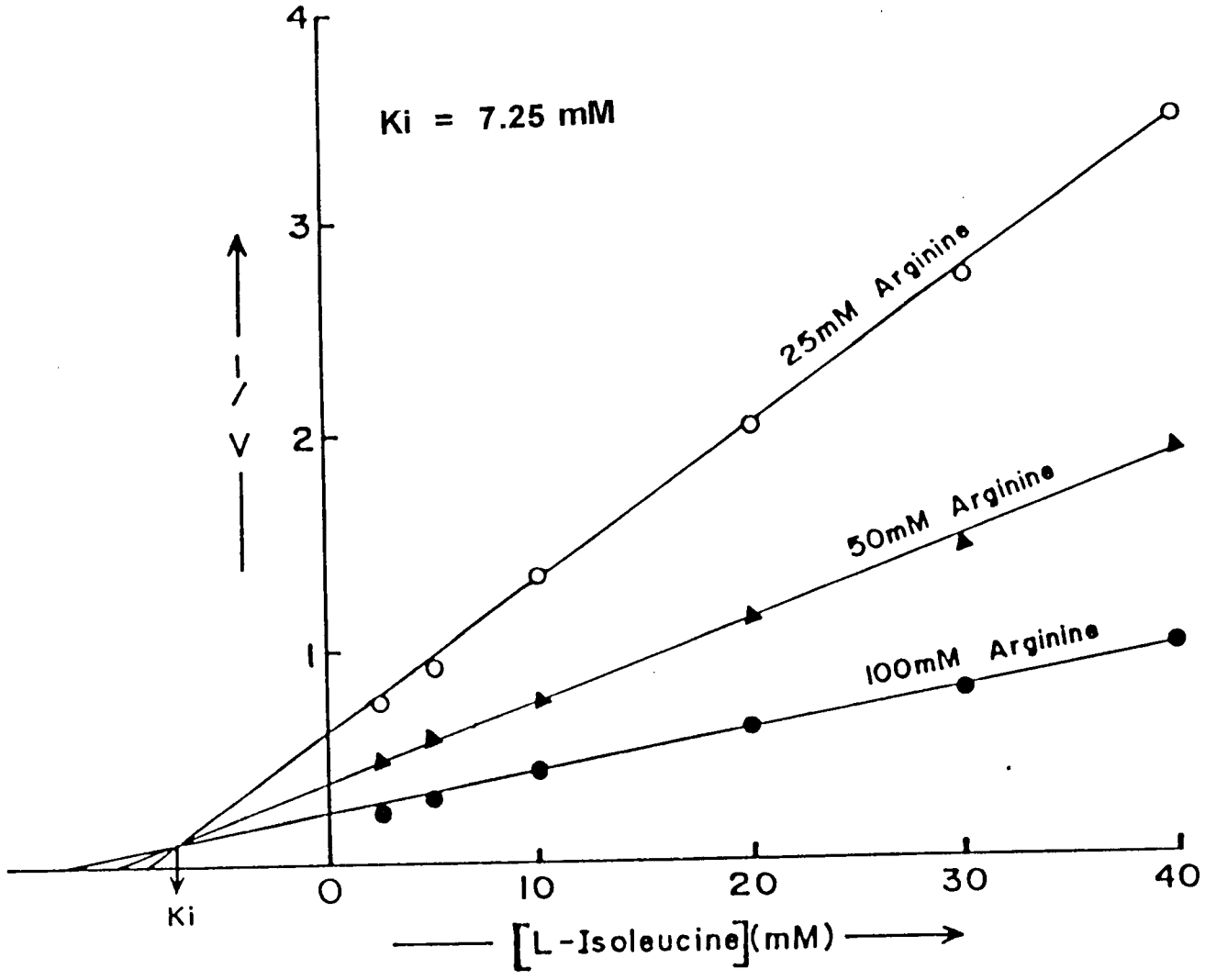


Fig. 29 Dixon plot for determination of inhibitor constant (Ki) of L-isoleucine for arginase purified from the liver of H. fossilis.

DISCUSSION

Induction of ureogenesis in perfused liver of H. fossilis by infusing different concentrations of ammonium chloride:

Although ammonia is a toxic substance in exclusively aquatic animals, it is the main nitrogenous end product of the catabolism of amino acids, purine and pyrimidine bases (Campbell, 1991; Randall and Wright, 1987; Wood, 1993; Anderson, 1994a). Presence of ammonia in aquatic medium at higher level has been known to be toxic to ammoniotelic freshwater fishes. The 48 hr LC_{50} value of ammonia for Cyprinus carpio was 15 mg/l (Dabrowska and Wlasow, 1986). The rate of mortality with 24 hr exposure at 8 mg and 40 mg/l ammonia was reported to be 50% for trout, Salmo gairdneri, (Olson and Fromm, 1971) and 10% for goldfish (Schenone et al, 1982) respectively. The 24 hr LC_{50} value for ureotelic alkaline lake Magadi tilapia O. a. grahami was found to be 0.75 mM ammonia (Walsh et al, 1993). Aquatic frog, Xenopus leavis, did not survive beyond 1 hr at 10 mM NH_4Cl (Janssens, 1972). The mudskipper, P. cantonensis has been reported to tolerate upto 15 mM NH_4Cl (Iwata, 1988).

However, the freshwater air-breathing teleost, H. fossilis has been reported to tolerate and survive well in 75 mM NH_4Cl for 28 days (Saha and Ratha, 1986;1990;1994) which is very high compared to any freshwater teleost and also aquatic amphibia. This indicated the presence of efficient physiological and biochemical mechanisms for detoxification of excess ammonia in this fish. The

induction of o-u cycle enzymes and enhanced ureogenesis has been reported when this fish was exposed to 50 mM NH_4Cl for 28 days (Saha and Ratha, 1994) where the presence of a functional o-u cycle in this fish was reported earlier (Saha and Ratha, 1987; 1989). However, the threshold level of ammonia load needed in the liver to cause induction of ureogenesis was not determined. In the present experiment different concentrations of NH_4Cl starting from very low to high was infused in perfused liver (the most ureogenic tissue) and the induction of o-u cycle enzymes and the rate of urea synthesis with relation to ammonia load *in vivo* was studied to find out the minimum threshold level and the optimum level to cause maximum induction of ureogenesis.

At low rate of ammonia infusion i.e., upto 0.29 μmol/g liver/min for 60 min, there was no significant increase of ammonia level in perfused liver (Table 1). The excretion of ammonia into the effluent was also found to be negligible (about 25% of the total added) indicating that most of the ammonia infused into the liver was getting converted to either urea, or may be to some non-essential amino acids (not determined in the present study) (Table 2). Out of the total ammonia uptake about 80 to 90% was converted to urea at a lower rate of ammonia addition without causing any significant induction of any of the o-u cycle enzymes (Tables 3 and 4; Figs. 5-8). The physiological level of activity of the enzymes of the o-u cycle in this ureogenic teleost must be enough to manage the accumulation of toxic ammonia *in vivo* initially along with other detoxification process to a certain level. In alkaline lake Magadi

tilapia, O. a. grahami 3 fold increase of urea excretion was noticed without causing any induction of o-u cycle enzymes when this fish was exposed to 0.5 mM NH_4Cl (Randall et al, 1989; Walsh et al, 1993). This indicated that ammonia accumulation in the liver remained lower than the threshold level to cause any induction of the o-u cycle enzymes when exposed to such a low ambient ammonia as has been noticed in the perfused liver of H. fossilis, although urea excretion rate was enhanced significantly in both the cases. Similar type of response in urea excretion rate has been reported in the gulf toadfish, O. beta when exposed to higher ambient ammonia (Walsh et al, 1990). It could be also possible that other ammonia utilization pathway such as synthesis of non-essential amino acids might be efficient to manage accumulated ammonia at lower concentrations. Significant accumulation of ammonia in perfused liver was seen with the addition of ammonia at the rate of 0.58 µmol/g liver/min onwards followed by further increase at a still higher rate of ammonia addition (Table 1). The accumulation of ammonia in liver seems to be a saturable process as has been noticed in the present study that at both 2.8 and 5.55 µmoles/g liver/min ammonia addition the accumulation of ammonia in perfused liver was nearly the same (Table 1). Earlier studies by Saha and Ratha (1994) where the whole fish was exposed to different concentrations of NH_4Cl also indicated similar effects.

The uptake of ammonia which is due to both accumulation and also conversion of either urea or some other non-toxic compounds was also a saturable process in

the liver of H. fossilis (Fig. 3). This indicates that there is a maximum limit of tolerance of toxic ammonia level in vivo, and this has been reported in this fish to be tissue specific (Saha and Ratha, 1994). The rate of formation of urea-N from ammonia was found to be again a saturable process reaching a Vmax of 0.4±0.02 µmol/g liver/min with the addition of NH_4Cl at the rate of 1.18 µmoles/g liver/min (Table 2; Fig. 4). Initially at a lower rate of addition of ammonia, most of the ammonia got converted to urea-N and there was a linear increase of urea-N release into the effluent with the increase of addition of ammonia indicating that urea-N formation is directly related to ammonia load till it reaches the Vmax. At a higher ammonia load, the rate of urea formation became independent of ammonia (substrate) load. This was similar to an earlier report when the whole animal (H. fossilis) was exposed to 25, 50 and 75 mM NH_4Cl and the induced rate of excretion of urea was found to be almost same in all the concentrations (Saha and Ratha, 1988; 1991).

Significant and maximum induction of all the o-u cycle enzymes except ARG was obtained with the addition of NH_4Cl at the rate of 0.58 µmol/g liver/min where the level of ammonia in perfused liver raised from 12.75±1.65 to 18.1±2.01 µmoles/g wet wt. after 60 min of infusion. At a still higher rate of addition of NH_4Cl the level of ammonia in the liver increased further, but no further increase of o-u cycle enzymes was noticed. Hence, an increase of ammonia level in the liver from the physiological level of 12.75 µmoles/g wet wt. to a threshold level between 15 to

18 μmoles/g wet wt. is sufficient to cause induction of o-u cycle enzymes in this fish. There was no further increase of urea-N excretion into the effluent even at a higher rate of NH_4Cl addition (Fig.4). The maximum induction of activity of about 200% was seen in case of CPS (ammonia dependent) which is considered to be the rate limiting enzyme of the o-u cycle (Jackson et al, 1986; Nener, 1988), and this rate of induction was directly related with the induction of urea-N excretion.

The Lahontan cutthroat trout (Oncorhynchus clarki henshawi), which has been uniquely adapted to the highly alkaline waters (pH 9.4) of Pyramid lake, Nevada, showed a two fold increase of urea excretion rate, although there was no induction of o-u cycle enzymes in the liver of this fish when exposed to a media of pH 10 (Wilkie et al, 1993). However, they found significant increase of one of the uricolytic enzymes, allantoicase, at pH 10 and suggested to be one of the probable causes of elevated urea excretion. The tilapia, Oreochromis nilotica, endemic to neutral water (Wood et al, 1989) and the rainbow trout, Oncorhynchus mykiss (Wilkie and Wood, 1991), both of which are thought to lack the o-u cycle, also increased urea production upon exposure to high environmental pH. However, in the present study, while perfusing the liver of H. fossilis the pH of the media containing different concentrations of NH_4Cl was kept constant (pH 7.4). Therefore, the induction of ureogenesis under higher ammonia load which has been noticed here cannot be the effect of pH change of the media. However, the effect of pH on ureogenesis in H.

fossilis needs to be studied separately.

Significant increase of urea excretion rate in ureogenic toadfish, Opsanus beta was reported with high levels of external ammonia or exposure to air for extended periods of time (Walsh et al, 1990). Physical confinement and also crowding has been reported to initiate a switch to ureogenesis within a day or two in this toad fish, which was accompanied by a significant increase in glutamine synthetase activity (Walsh et al, 1994). It was concluded that these effects in toadfish are not due to increase in ammonia concentrations or to air exposure, but due to variations in environmental habitat or stress related effects (Walsh et al, 1991; Anderson, 1994a). However, in case of amphibious fish, H. fossilis it is clear from this perfusion experiment that higher ammonia load in the liver beyond a threshold level causes enhanced ureogenesis via induced o-u cycle.

Isoenzymes of CPS (I, II and III) in the liver and kidney of H. fossilis :

Three isoenzymic forms of CPS (I, II and III) have been reported in various animals and micro organisms. CPS I uses ammonia and CPS II and III use glutamine as nitrogen donating substrate. However, CPS I and III require NAG as a co-factor for enzymatic activity, whereas CPS II does not. CPS I and III have been shown to be responsible for arginine or urea synthesis where as CPS II is responsible for pyrimidine biosynthesis (Ratner, 1973; Jackson et al, 1986; Campbell and Anderson, 1991; Anderson, 1994a).In most of the cases reported, either CPS I or CPS III (responsible for arginine or urea synthesis) is present in a single vertebrate species along with CPS II (responsible for pyrimidine synthesis). There are reports of occurrence of both CPS I and III in a single species in only a few invertebrates (Tramell and Campbell, 1971). However, all the three isoenzymic forms of CPS (I, II and III) were found in both liver and kidney of H. fossilis. The presence of a functional o-u cycle with significantly higher levels of all the o-u cycle enzymes including CPS in both liver and kidney tissues of H. fossilis were reported earlier (Saha and Ratha, 1987; 1989). But the studies made by Saha and Ratha (1987; 1989) did not clarify the isoenzymic forms of CPS present in this fish. Depending upon the presence of isoenzymic forms of CPS in different groups of vertebrates a hypothetical phylogenic tree have

been proposed by Devaney and Powers-Lee (1983). The presence of all the three isoenzymes both in the liver and kidney of H. fossilis is unique. The presence of CPS III in vertebrates was reported for the first time in a freshwater teleost, Micropterus salmoides (largemouth bass) (Anderson, 1976) followed by in elasmobranchs (Anderson, 1980; Casey and Anderson, 1982) and in toadfishes, coelacanth, carp, trout, bowfin and in midshipman (Mommsen and Walsh, 1989).

Presence of both CPS I and III in the liver and kidney of H. fossilis is puzzling. Earlier studies from this laboratory have shown that CPS (ammonia dependent) along with other o-u cycle enzymes could be regulated by various environmental factors such as higher ambient ammonia, dehydration and hyper-osmotic stress (Saha, 1986; Saha and Ratha, 1986, 1991, 1994). In the present investigation, it was seen that infusion of NH_4Cl at a concentration of 1 and 2 mM in perfused liver induced CPS I activity significantly to about 145% and 150% respectively along with significant synthesis of urea from ammonia (Table 7). In another set of experiment, when glutamine (1 mM and 2 mM) was infused in the perfused liver of H. fossilis there was also significant formation of urea from glutamine via CPS III along with significant induction of CPS III activity (Table 7). Therefore, it is clear from this perfusion experiment that both CPS I and CPS III in the liver of this fish are very much functional and both involve in ureogenesis. Although both CPS I and III isoenzymes are present in the liver of H. fossilis, CPS I

appears to be more efficient than CPS III in this fish. However, further studies needs to be conducted to confirm this.

Except certain indirect evidences by Anderson (1980), there was no definite report of the presence of carbamyl phosphate synthetase II (glutamine dependent and NAG independent) activity in fish which is responsible for pyrimidine synthesis. Presence of CPS II activity was reported for the first time in the spleen of dog fish (S. acanthias) by Anderson (1989). He, however, could not detect the activity of CPS II along with aspartate carbamyl transferase, another enzyme of pyrimidine pathway, in the liver of dogfish and suggested that pyrimidine synthesis in dogfish takes place in extrahepatic tissues only. Cao et al. (1991), however, could detect CPS II activity in the liver of few teleost species, M. salmoides (large mouth bass), crappies, bluegills (sunfish family), and a North American bullhead (Ameiurus family). After this first definite report, the present study in H. fossilis, is the second definite report of existence of CPS II in any teleost liver and kidney (Tables 5 and 6). The synthesis of pyrimidine in the bass liver has been confirmed further due to the presence of some other enzymes, related to pyrimidine synthesis other than the CPS II (Cao et al., 1991). Even though it seems quite possible in H. fossilis liver and kidney, further enzymatic studies are necessary to confirm this.

Sub-Cellular localization of CPS isoenzymes (CPS I, II and III) and other o-u cycle enzymes :

There was 70-120% and 80-165% increase in the activity of CPS I, CPS III, OTC and ARG in liver and kidney of H. fossilis respectively when the tissue homogenates were treated with Triton X-100 (0.5%) for 30 min (Tables 8 and 9). No significant alterations of activity was noticed in case of ASS and ASL in both the tissue homogenates treated with Triton X-100 (Tables 8 and 9). Triton X-100 was used to release mitochondrial enzymes in homogenate Chakravorty et al, 1989; Das et al,1991). Therefore, it is indicated that CPS I, CPS III, OTC and ARG are localized in the mitochondria, and ASS and ASL are localized in the cytosol in both the tissues of H. fossilis. This was further confirmed after separating the mitochondrial and cytosolic fraction by differential centrifugation, and studying their activities in different fractions with different marker enzymes (Tables 10 and 11). It is interesting that the enzymes of o-u cycle are distributed both in the cytosolic and mitochondrial fractions in the ureogenic, ureotelic and ureo-osmotic species. However, this distribution shows wide range of variations among different groups of vertebrates.

Ammonia generated or transported inside the mitochondria is detoxified to citrulline in presence of mitochondrial ammonia and NAG-dependent CPS (CPS I) and OTC

in ureotelic species (Ratner, 1973; Cohen, 1976; Jackson et al, 1986) (Fig. 30). Citrulline is then transported out of mitochondria for ultimate conversion to urea and ornithine by three other o-u cycle enzymes namely ASS, ASL and ARG, located in the cytosol. Ornithine enters the mitochondria to complete the cycle. GS is a cytosolic enzyme in hepatic and renal tissues of ureotelic mammals and amphibians (Wu, 1963 a,b; Katunuma et al, 1970). Therefore, the presence of only CPS I and lack of CPS III in the mitochondria are justified in mammals and amphibians with relation to urea synthesis. In the mitochondria of liver and kidney of ureo-osmotic marine elasmobranchs and toad fish, O. beta, the presence of GS (Webb and Brown, 1976, 1980; Anderson, 1980, Casey and Anderson, 1982; Smith et al, 1987, Mommsen and Walsh, 1989), CPS III (Anderson, 1980; Casey and Anderson, 1982; Anderson and Casey, 1984; Mommsen and Walsh, 1989) and ARG (Casey and Anderson, 1982; Mommsen and Walsh, 1989) has been reported (Fig. 31). Therefore, in this case, it is arginine instead of ornithine which is transported into the mitochondria with a modified transporter system.

The sub-cellular distribution pattern of the nitrogen metabolizing enzymes in the liver and kidney of H. fossilis (Fig. 32) was different from the ureotelic species and resembled more with the ureo-osmotic elasmobranchs. In addition to the possibility of assimilation of ammonia via CPS I (ammonia and NAG-dependent), there is also a possibility of assimilation of ammonia via glutamine by

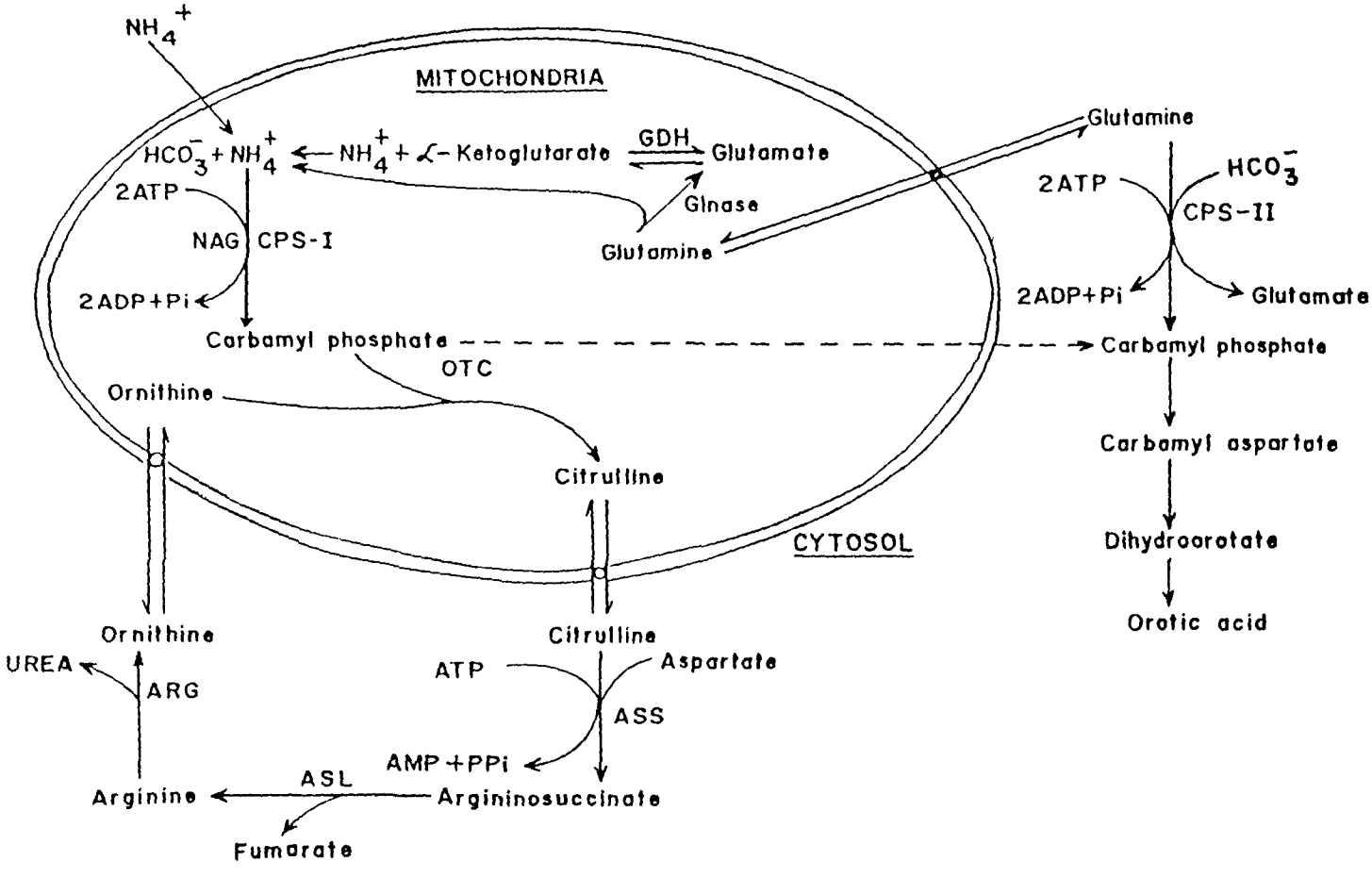


Fig.30 Urea synthesis in mammals and amphibia

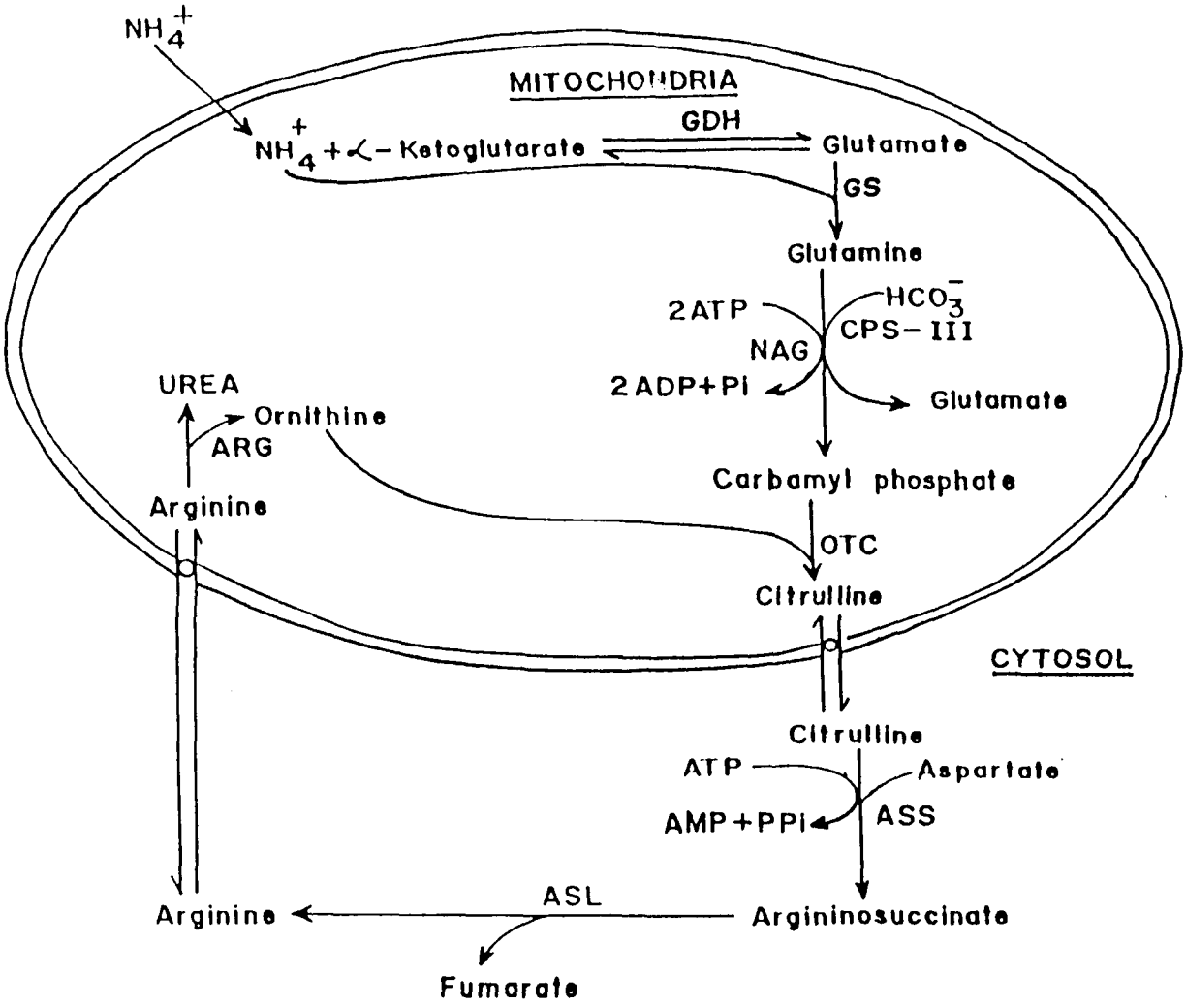


Fig. 31 Urea synthesis in elasmobranchs

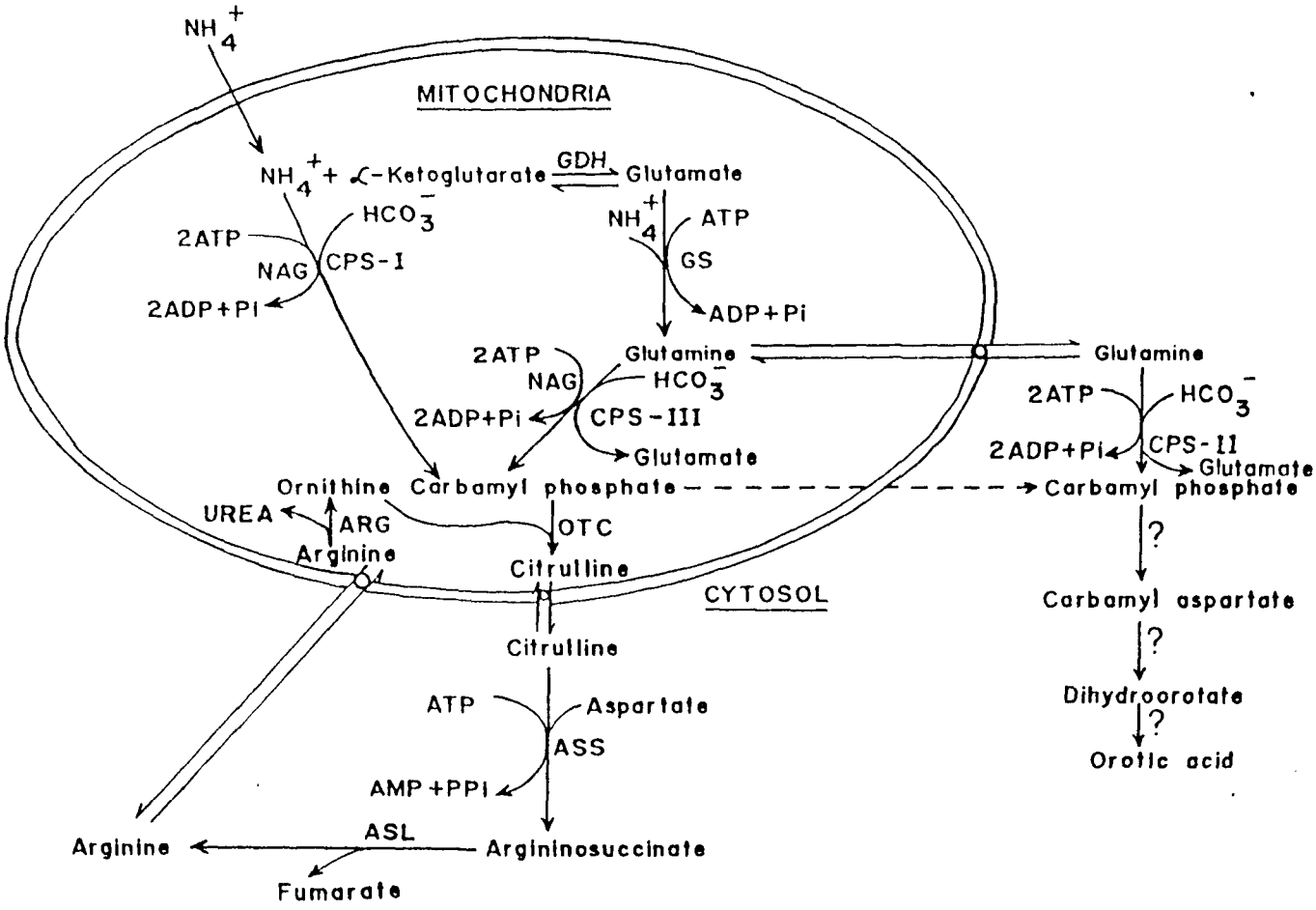


Fig. 32 Urea synthesis in H. fossils (Liver & Kidney)

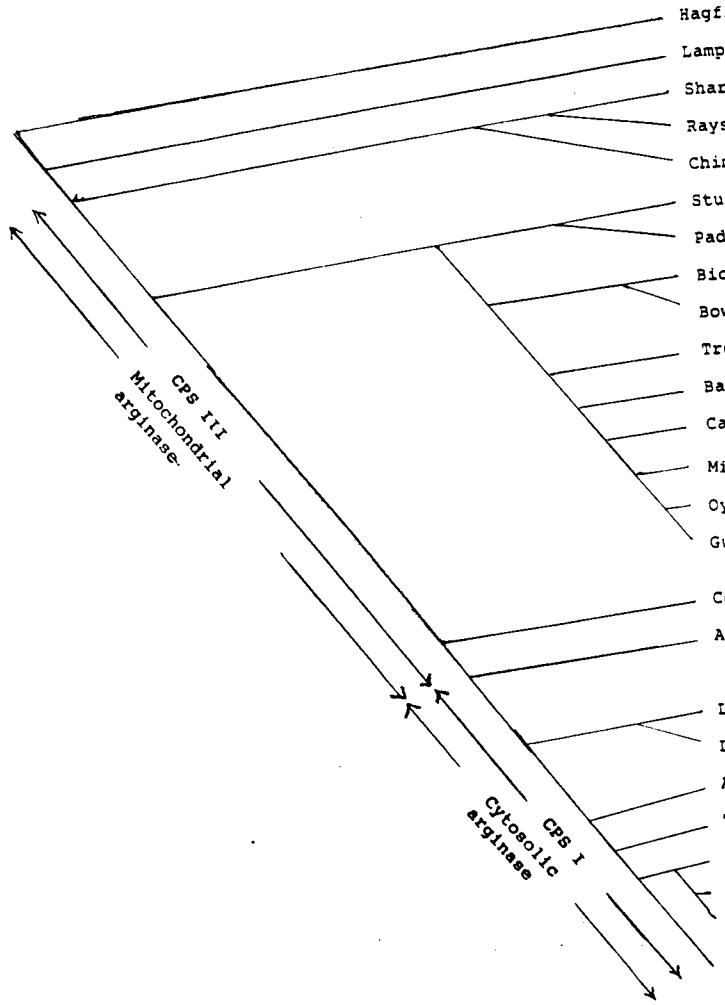
CPS III (glutamine and NAG-dependent) for carbamyl phosphate and urea synthesis in the mitochondria of liver and kidney of H. fossilis. This is possible due to the presence of comparatively higher level of glutamine synthetase (GS) (Chakravorty et al, 1989), CPS I and CPS III activities localized together inside the mitochondria (Fig. 32). Another difference with relation to the ureotelic species is that arginine formed in the cytosol by cytosolic enzymes ASS and ASL has to be transported into the mitochondria for ultimate conversion to urea and ornithine, since ARG is localized within the mitochondrial matrix. As a result, ornithine is directly available inside the mitochondria for the synthesis of citrulline by the mitochondrial OTC in the liver and kidney of H. fossilis (Fig. 32).

ARG in some cases has been reported to be associated with rat liver mitochondria due to non-specific binding. The enzyme was solubilized by increasing of the ionic (KCl) concentration in the homogenate (Rosenthal et al, 1956; Soberon and Palacois, 1976; Skrypeck-Osiecka et al, 1980). The homogenizing media containing 0.1 M KCl used in the present study did not release the arginase activity. Therefore, the mitochondrial localization of arginase observed in the liver and kidney of this freshwater teleost is not an artifact due to non-specific binding.

Presence of both mitochondrial CPS I and CPS III in liver and kidney of H. fossilis is unusual and may have

some physiological significance with relation to their adaptation to very high ambient ammonia (upto 75 mM NH_4Cl) reported from our laboratory (Saha and Ratha, 1986; 1990; 1994). This fish has both the possibilities of assimilating ammonia to urea i.e. one by directly entering to o-u cycle by CPS I and another by assimilating ammonia first to glutamine by the mitochondrial GS and then part of it entering to o-u cycle via CPS III.

Presence of both CPS I and CPS III in air-breathing teleost, H. fossilis has also some evolutionary significance. Depending upon the presence of the type of CPS (I or III), Mommsen and Walsh (1989) proposed a cladogram and suggested that urea synthesis within the vertebrate line is a monophyletic trait that has undergone biochemical and functional changes in the course of vertebrate evolution. They also suggested that only minor biochemical changes need be postulated to explain this development : (i) the switch from CPS III to CPS I; (ii) the replacement of mitochondrial arginase by a cytosolic counterpart and (iii) adjustment of mitochondrial transporter specificity from arginine to ornithine. With the addition of the unique results on the presence of all the three isoenzymes of CPS, mitochondrial GS and arginase, and functional o-u cycle in the liver and kidney of H. fossilis, the cladogram of Mommsen and Walsh (1989) has been modified (Fig. 33). Due to the presence of both CPS I and CPS III with GS in the mitochondria of liver and kidney the freshwater air-breathing fish H. fossilis has been placed between coelacanth and lungfishes (Fig. 33). It



Vertebrate	Urea Synthesis function	CPS type	ARG	Glutamine synthetase	References
Hagfish (Eptatretus stonti Myxeniformes)	No		M	C	Mommsen & Walsh, 1989
Lampreys	No				"
Sharks (Elasmobranchii)	Yes(1)	III	M	M	Anderson, 1980; Shankar & Anderson, 1985
Rays, skates (Raja erinacea)	Yes(1)	III	M	M	Anderson, 1980; Mommsen & Walsh, 1989
Chimaera (Hydrolagus colliei, Holocephali)	Yes(1)	III	M	M	Anderson, 1980; Ritter et al, 1987; Mommsen et al(unpublished data) Brown & Brown (1985)
Sturgeon (Chondrostei)	No				"
Paddlefish (Chondrostei)	No				Mommsen & Walsh, 1989
Bichir (Polypterus spp., Holostei)	No	III	M		"
Bowfin (Amin calva, Holostei)	No	III	M	C	"
Trout (Salmo gairdneri,	No		M	C	Casey & Anderson, 1983, Cao et al, 1991
Bass (Micropterus salmoides)	No	III	M	C	Vellas & Serfaty, 1974
Carp (Cyprinus carpio, Cyprini)	No	III	M	M	Anderson, 1980
Midshipman (Porichthys notatus, Batrachoididae)	No	III			Mommsen & Walsh, 1989
Oyster toadfish (Opsanus tau, Batrachoididae)	Yes(3)	III	M	M	"
Gulf toadfish (O. beta, Batrachoididae)	Yes(3)	III	M	M	"
Coelacanth(Latimeria chalumnae, Coelacanthiformes)	Yes(1)	III	M		Present work; Saha,1986; Chakravorty et al,1989; Dkhar et al,1991;Saha & Ratha, 1994
Air-breathing catfish (Heteropneustes fossilis, Siluridae)	Yes (1,2,4)	I,III	M	M	Mommsen & Walsh, 1989
Lungfish (Protopterus aethiopicus, Dipnoi)	Yes(2,1)	I	C	C	"
Lungfish (Protopterus annectens, Dipnoi)	Yes(2,1)		C	C	Marshall & Cohen, 1961; Janssens & Cohen, 1968
Amphibians	Yes(2)	I	C	C	Vorhaben & Campbell, 1977; Campbell Smith, 1985
Turtle, tortoises	Yes(2)	I		M	"
Snakes, lizards	No	I		C	"
Crocodiles	No			M	Grisolia , 1976; Jackson et al, 1986
Birds	No			M	
Mammals	Yes(2)	I	C	C	
Hibernators Ruminants	Yes(2,3)	I	C	C	

Fig. 33 Urea synthesis by the o-u cycle in vertebrates. Function of urea: 1- osmolyte; 2- ammonia detoxification; 3- nitrogen cycling between liver and gut; 4- ureogenic; M- mitochondrial; C- cytosolic; ARG- arginase; CPS- carbamyl phosphate synthetase

was suggested (Saha and Ratha, 1987; 1989), while reporting for the first time the presence of a functional o-u cycle in this fish, that this air-breathing freshwater semi-aquatic catfish is either primitive or relatively more advanced than the present day freshwater teleosts. With this present knowledge of occurrence of both CPS I and CPS III isoenzymes in H. fossilis, we can easily say that this fish is more advanced. However, arginase is still retained inside the mitochondria, and therefore, further studies are necessary to establish the true evolutionary position of these freshwater air-breathing semi-aquatic catfishes (Silurideae family).

Annual variation of o-u cycle enzymes in the liver and kidney of H. fossilis:

Annual breeders such as fish including H. fossilis, have been known to show variations in their chemical composition and metabolism in different tissues throughout a year usually coinciding with their reproductive cycle in nature. Variation in the temperature and light intensity and duration have been reported to be the major guiding external forces for regulating annual cycles, particularly of reproduction. However, in the present study, the variation in the activity of o-u cycle enzymes through out a year could also be seen in the liver and kidney tissues of H. fossilis, which were maintained in the laboratory

under controlled conditions of temperature, light and feeding schedule.

H. fossilis spawns during the beginning of monsoon season (June-July). The annual reproductive cycle is divided into mainly 4 phases- pre-spawning, spawning, post-spawning and resting phase (Sundararaj, 1959; Sundararaj and Goswami, 1969). December to February (winter months) are resting period, March to May pre-spawning, June to August spawning and September to November are post-spawning period. The pre-spawning and spawning phase show enormous physiological and biochemical activities related to provide molecular ingredients and energy for reproduction. This event is known to be influenced by various hormones primarily steroids whose levels increase during pre-spawning phase reaching a peak during spawning period. In the present study the activity of the enzymes of o-u cycle, which is not directly related to reproduction but have a role to play during active protein/amino acid metabolism, showed a very close correlation to the variation in the steroid level reported in H. fossilis (Lamba et al, 1983). Hormones such as steroids, thyroxine and glucagon have known to induce the activity of o-u cycle enzymes in other animals (Lamers and Mooren, 1981; Husson and Vaillant, 1982; Husson et al, 1983; 1985; 1987; Marti et al, 1988; Hayase et al, 1991). The levels of steroid hormones has been reported to be higher during the pre-spawning and spawning periods and lower during the post-spawning period in fishes (Liley, 1969; Sundararaj, 1959; Sundararaj and

Goswami, 1969) as well as in amphibians (Wallace, 1985; Kwon et al, 1993 ; Kwon and Ahn 1994)

A similar pattern of annual variation in GDH activity, which is another important enzyme of nitrogen metabolism, has also been reported in H. fossilis (Das, 1991). The GDH activity in reductive amination direction has been reported to be maximum in summer and minimum in winter in both liver and kidney. In contrast, the GDH activity in oxidative deamination direction has been reported to be maximum in winter and minimum in summer. Higher rate of production and accumulation of ammonia in summer in this fish could be the cause of induction of GDH activity (reductive amination) to reduce ammonia toxicity in summer as this enzyme has also been reported to get induced under hyper-ammonia stress (Das, 1991). This would also favour the synthesis of glutamate and then to some other amino acids by transamination which could be correlated with increased requirement of amino acids and proteins for vitellogenesis in the liver during the pre-spawning phase in fishes (Schmidt et al, 1965; Campbell and Jalabert, 1979; de Vlaming et al, 1980; Wiegand et al, 1982; Wallace, 1985). A large amount of protein synthesis and degradation takes place in various tissues of teleosts for egg development and maturation. The o-u cycle have also been useful in the synthesis of arginine, ornithine, carbamyl phosphate besides utilizing excess of ammonia and carbon dioxide and converting aspartate to fumarate. Therefore, this multifunctional cycle might have been useful during the

pre-spawning and spawning period of the fish for the large scale metabolism and protein turnover, and the enzymes might have been induced by the increased levels of steroids.

The levels of different hormones in rat liver, cultured hepatocytes and hepatoma cell lines have been reported to alter both the synthesis and degradation of proteins, o-u cycle enzymes activity and also different metabolic pathways of nitrogen metabolism (Morris, 1992). For example, glucagon is known to stimulate proteolysis and inhibition of protein synthesis in rat liver (Häussinger and Lang, 1991; Stoll et al, 1992). This reflects the induction or inhibition of the enzymes related to nitrogen metabolism depending on the accumulation of metabolites so as to maintain the nitrogen equilibrium. Therefore,with the change in the levels of different metabolites, activities of different pathways could also vary.

Annual variation of ARG activity has also been reported in the liver of another air-breathing freshwater fish, C. batrachus (Singh and Singh, 1988) which coincided with the variation of ARG activity during gonadal maturation phase in fishes (Love, 1980). The rate of citrulline synthesis as a result of glucagon infusion in perfused rat liver is reported to vary during different months indicating that CPS and OTC activities show annual variation (Bryla et al, 1977). Besides the differential levels of steroid hormones, the intracellular

concentrations of amino acids and ions could be the contributing factors for the observed differences in enzyme activities during different months of the year. With the present results obtained, it is not yet clear whether the induction of the o-u cycle enzymes in summer is due to the synthesis of new enzyme proteins or activation of pre-existing inactive enzyme molecules. Further immunological studies and also studies relating to the levels of different mRNA's coding for the o-u cycle enzymes in both the tissues are required to know exactly the reason of alterations of activity through out the year.

Purification of hepatic arginase and its physico-chemical properties :

Purification of arginase:

The protocol followed for purification of hepatic arginase of H. fossilis yielded 36% recovery of activity and 306 fold of purification (Table 16). The fold of purification and percentage recovery obtained for arginase from other species showed a wide range of variation. The fold of purification ranged from 41 in human lung (Rao et al, 1976) to 1,76,000 in human erythrocytes (Ikemoto et al, 1989) and the percentage recovery ranged from 18 (rat liver), (Tarrab et al, 1974) to 60% (bovine liver), (Visco et al, 1987) depending on the various steps used for

purification.

Acetone precipitation which resulted in an increase in specific activity of arginase from mammalian and other sources (Hirsch-Kolb et al, 1970; Visco et al, 1987; Peiser and Balinsky, 1982; Ikemoto et al, 1989; Tarrab et al, 1974) was unsuitable for hepatic arginase from H. fossilis was more labile to acetone precipitation. Along with decrease in total protein concentration, there was a drastic loss of enzyme activity (data not presented). Therefore, acetone precipitation step was avoided for purification of hepatic arginase of H. fossilis. Heat treatment resulted about two fold increase in specific activity and 1.35 times increase in percentage recovery of activity after ammonium sulphate fractionation. Activation of arginase at a higher temperature has been reported in case of mammalian arginase (Schimke, 1964; Hirsch-Kolb et al, 1970; Kedra-Luboinska et al, 1988). Mohamed and Greenberg (1945) suggested that the activation of arginase by heat treatment involves the transformation of an inactive protein proarginase to its active form. 2-mercaptoethanol was used during the heat treatment step for human erythrocyte arginase by Beruter et al, (1978) and for rabbit liver arginase by Vielle-Breitburd and Orth (1972). They suggested that 2-mercaptoethanol could help in stabilizing the disulphide bond of arginase during heat treatment. Sulfhydryl groups have been reported to be responsible for interacting with manganese since elimination of manganese leads to exposition of certain

sulfhydryl groups which were otherwise inaccessible to sulfhydryl group modifying reagents (Fuentes et al, 1994). Mora et al (1966) found that 2-mercaptoethanol protected arginase from chicken liver and Neurospora crassa from denaturation during dialysis. Therefore, in case of H. fossilis liver arginase also 1 mM 2-mercaptoethanol was always taken in the suspending buffer media after ammonium sulphate fractionation to protect the enzyme from denaturation.

The appearance of 17% activity of arginase in the void volume while washing the DEAE-Sephacel column with buffer could indicate the presence of another isoenzyme of arginase in the liver of this fish. Arginase isoenzymes have been reported in several tissues such as liver, kidney, sub-maxillary gland, lung as well as erythrocytes of different animals (Herzfeld and Raper, 1976; Reddi et al, 1975; Kedra-Luboinska et al, 1988; Gasiorowska et al, 1970; Venkatakrishnan and Reddy, 1983; Singh and Singh, 1990). However, this could also be a degradation product of a single enzyme species. Further investigations in this connection are required to clarify this point.

Molecular weight of arginase:

The apparent molecular weight of hepatic arginase of H. fossilis as determined by gel filtration was found to be 81,000 which was almost in the range of molecular weight reported for arginase from other sources. The molecular

weight of arginase was 1,20,000 in rat and mouse (Hirsch-Kolb et al, 1970; Spolarics and Bond, 1988), 1,10,000 in rabbit (Vielle-Brietburd and Orth, 1972), 76,000 in aquatic frog Xenopus laevis (Peiser and Balinsky, 1982), 80,000 in the tadpole of Rana esculenta (Porembska, 1973), 1,05,000 in elasmobranch, Squalus acanthias (dogfish) (Casey and Anderson, 1982) and 87,000 in an air-breathing teleost, Clarias batrachus (Singh and Singh, 1990). Significant differences in molecular weight of arginase from ureotelic species with that of uricotelic species and that of uricotelic species have been reported (Hirsch-Kolb et al, 1970; Mora et al, 1965a,b). Mora et al (1965a,b), suggested that the molecular weight of arginase was correlated with the mode of nitrogen excretion in animals. Arginase has also been reported to undergo association-dissociation reaction in the fat body of some insects (Reddy and Campbell, 1969). However, such studies on arginase purified from H. fossilis have not been done. $MnCl_2$ was used throughout the purification steps as it gives better stability to the enzyme (Hirch Kolb et al, 1970). However, the role of this metal ion on the association-dissociation of arginase is not yet known. The molecular weight (81,000) of hepatic arginase of H. fossilis was very close to the hepatic arginase of amphibians, X. laevis and R. esculenta and air-breathing teleost, C. batrachus.

Effect of temperature and thermal stability of arginase:

The temperature optima of purified hepatic arginase of

H. fossilis was found to be 50 °C and the peak was quite sharp (Table 17, Fig. 16). The temperature optima of arginase from C. batrachus (Singh and Singh, 1990), G. maculatus (Carvajal et al, 1987) and sea mollusc, Concholepas concholepas (Carvajal et al, 1984) were found to be 40, 42 and 43°C respectively. Campbell (1966) reported the optimum temperature to be 55°C for rat and snail arginase. The variation in temperature optima of an enzyme in different species is usually correlated to their habitat and temperature. However, the differences in the temperature optima of hepatic arginase from C. batrachus and H. fossilis which usually live in similar habitat can not be explained at this stage.

Purified hepatic arginase started loosing its activity with increasing time specially at temperatures higher than 35°C when pre-incubated at different temperatures (Table 18; Fig. 17). The activity was completely lost after 30 min of pre-incubation at 60 °C and 95% after 60 min of pre-incubation at 55 °C. Pre-incubation of the purified enzyme in presence of substrate (L-arginine) and co-factor (Mn^{2+} ion) individually or in combination at 55 °C, protected the enzyme from thermal denaturation (Table 19, Fig. 18). However, presence of both L-arginine and Mn^{2+} showed synergistic effect in protecting arginase against thermal denaturation studied at 55 °C for 60 min. Purified hepatic arginase incubated at 55 °C for 60 min retained only about 5% of the enzyme activity. But in presence of Mn^{2+}, L-arginine, and both Mn^{2+} and L-arginine together retained

30%, 41% and 58% of the enzyme activity after 60 min at 55 °C. The protection was much better within 30 min with both the substrate and co-factor showing 90% enzyme activity against less than 50% without any addition. The result of the present study indicated that both the substrate and metal ion binding sites are involved in providing better protection to the enzyme against thermal denaturation. The functional enzyme-substrate-cofactor complex renders a more stable conformation to the enzyme.

Effect of pH on arginase activity:

The pH optima of 9.5 for hepatic arginase of H. tossilis reported in the present study resembled with the pH optima reported for arginase from teleosts, M. gayi (Carvajal et al, 1989) and G. maculatus (Carvajal et al, 1987), marine elasmobranch, S. acanthias (Casey and Anderson, 1982) and a freshwater air-breathing teleost, C. batrachus (Singh and Singh, 1990). Arginase has long been reported to exhibit an unusual pH optimum of 9.5 (Roholt and Greenberg, 1956). Mammalian arginase has a broader pH range of 9.3 to 10.5 (Hirsch-Kolb et al, 1970). Kuhn et al (1991) reported that the pH dependence of the .pre-incubation or activation stage was mainly confined to the alkaline side, whereas the pH-dependence at the assay stage lies between 6.5 and 9.5. Although the pH dependence of arginase has not been studied separately for the effect of activation/assay stage, however, if the above mentioned observation is applicable for liver arginase of H.

fossilis, it could be suggested that a stable conformation resulting from the binding of Mn^{2+} to the enzyme molecule is more favourable at alkaline pH. A drastic reduction of arginase activity on both sides of the pH optimum indicated that under acidic condition, protonation of the amino groups of the amino acid residues in the enzyme molecule occurred, whereas above pH 10, ionization of the carboxyl groups occurred. In other words it could be possible that the zwitterion of arginine rather than the univalent cation is the one that is catalytically attacked by the enzyme. The physiological significance of alkaline pH optima for arginase is still obscure, although one possibility could be an adaptational strategy for survival in alkaline environment as well. The narrow pH range obtained for liver arginase of H. fossilis, in contrast to mammalian arginase having a broader pH range, could be correlated to the physiological significance of the o-u cycle in this fish. In H. fossilis, although the o-u cycle plays a significant role in ammonia detoxification (Saha and Ratha, 1987; 1989; 1991; 1994), but it could also serve for the synthesis of arginine. In this connection, it may be mentioned that a narrow pH range would prevent an indiscriminate hydrolysis of arginine which is an essential amino acid for growth and for the formation of proline as in other fish tissues (Hird et al, 1986; Walton et al, 1986).

Effect of amino acids:

As early as 1945, Hunter and Downs reported the inhibition of bovine liver arginase by different L-amino acids and not their D-isomeric forms. They proposed that inhibition of arginase was mainly dependent on the chain length and branching of the carbon chain of amino acids. In the present study it was found that at 25 mM concentration L-ornithine (68%), L-isoleucine (63%), L-valine (61%) and L-leucine (60%) caused maximum inhibition of arginase purified from the liver of H. fossilis followed by L-threonine (49%), L-histidine (49%), L-aspartate (49%) and L-lysine (47%), (Table 21). A similar pattern of inhibition has been reported for arginase from earthworm Pheretima communissima and rat liver (Iino and Shimadate, 1986). However, L-alanine, L-glutamate, L-cysteine, L-serine, L-tyrosine, L-glycine, L-tryptophan and L-methionine showed less rate of inhibition on arginase activity (Table 21). It also appears from the results that amino acids having four to six carbon atoms and also without branched chains acted as better inhibitors for arginase. The inhibition of arginase by heterocyclic amino acid L-proline (26%) is of particular interest in the present study. In mammalian species, proline is converted to ornithine as suggested by Smith et al ,1967). Hunter and Downs (1945) have also reported the inhibition of bovine liver arginase by proline. It is, therefore, tempting to speculate that the inhibition of arginase by proline may be of regulatory significance involving a feedback mechanism.

It also seems likely that in the tissues of fish, arginine would be converted to proline as suggested by Hird et al, (1986).

Effect of metal ions:

Metal ions play an important role in maintaining the catalytic activity of the enzyme as well as contributing to the stability of the tertiary and quaternary structure of the enzyme (Greenberg et al, 1956). Among various metal ions studied it became clear that Mn^{2+} is the most suitable co-factor for arginase enzyme (Table 22). Of the other metal ions tested, Ca^{2+}, Mg^{2+} and Co^{2+} were also found to activate arginase activity. The optimum concentration of Mn^{2+} and most other metal ions was found to be 5 mM since at higher concentrations (10 and 20 mM) the induction rate was either decreased or unchanged. Activation of arginase by Ca^{2+}, Co^{2+}, Cd^{2+} and Ni^{2+} has also been reported for mammalian arginase as well as arginase from C. concholepas (Hirsch-Kolb et al, 1971 ; Baret et al, 1972; Farooqui et al, 1978; Carvajal et al, 1984). Divalent cations are well-known activators of arginase activity (Mohamed and Greenberg, 1945). It has been shown that several of these metal ions have a different degree of effectiveness as activators of arginase extracted from different sources (Reddy and Campbell, 1969). Purified arginase from H. fossilis liver was markedly sensitive to inhibition by Zn^{2+} and also by the two heavy metals Hg^{2+} and Ag^{2+}. This was similar to the inhibitory effects reported for bovine liver arginase and

hepatic arginase from M. gayi (Mohamed and Greenberg, 1945; Carvajal et al, 1989). Monovalent cations, on the other hand, did not alter the activity of hepatic arginase from H. fossilis in all the three concentrations studied like that reported in kidney arginase from the bullfrog Rana catesbiana (Carlisky et al, 1972).

Activation of arginase by Mn^{2+} involves a conformational change in the enzyme molecule. Experimental evidences have been provided to show that the activation of arginase by Mn^{2+} is associated by the aggregation of the monomeric sub-units to form the oligomeric structure (Carvajal et al, 1971; Vielle-Breitburd and Orth, 1972). Therefore, activation depends upon a measurable reaction between the enzyme and the ion activator, and not between the substrate and ion. The process of activation might be assumed to be purely catalytic since prolonged dialysis of the enzyme against Mn^{2+}-free buffer leads to inactivation of the enzyme. A similar result has been presented by Hirsch-Kolb et al (1970) for mammalian arginase who suggested that neutral or acidic arginase (on the basis of pI values) do not bind Mn^{2+} ion tightly, thereby losing the metal ion very easily.

Mohamed and Greenberg (1945) suggested that the activation process by divalent cations involves the reversible transformation of an inactive protein pro-arginase to arginase according to the following reaction :

$$\text{Proarginase} + Mn^{2+} \longrightarrow \text{Arginase-M}$$

A general formulation of the role of the protein bound metal ion in reacting with the substrate was first given by Hellerman (1937) and a substrate-orienting function was assigned by Thoai et al (1953). It is apparent that hepatic arginase might be regulated by various divalent cations in vivo and more than one type of cations might be involved in this regulation process. However, the interaction of these regulatory ions for H. fossilis hepatic arginase if any, has to be found out.

Kinetics:

The Km value of hepatic arginase of H. fossilis was found to be 10 to 12 mM for arginine, which lies within the range of the Km value reported for ureotelic species of 5-20 mM (Hirsch-Kolb et al, 1970; Iino and Shimadate, 1986; Jenkinsen and Grigor, 1994). The Km value obtained for hepatic arginase of H. fossilis resembled also with many teleosts such as 9.1 mM at pH 7.5 and 11.5 at pH 9.5 in G. maculatus (Carvajal et al, 1987), 10.3 mM at pH 7.5 in M. gayi (Carvajal et al,1989) and 12.5 mM in C. batrachus (Singh and Singh,1990). However, arginase from uricotelic species had higher Km values in the range of 100 to 200 mM (Mora et al, 1965b). Dixon and Webb (1964) suggested that the same enzyme, but from different sources show many similarities in their kinetic properties, because they presumably function via the same catalytic mechanism. The activity of hepatic arginase from H. fossilis decreased when the arginine concentration exceeded 0.1 M (Fig. 20).

A similar pattern of substrate inhibition has been reported for arginase from the marine crustacean, Carcinus maenas (Hanlon, 1975), Neurospora crassa (Carlisky et al, 1968), shark liver (Campbell, 1961) and X. laevis (Peiser and Balinsky, 1982) and some vertebrates (Tarrab et al, 1974; Carlisky et al, 1972). Arginase of ureo-osmotic marine dogfish, S. acanthias liver has a lower Km value of 1.2 mM (Casey and Anderson, 1982). Urea synthesis in dogfish takes place mainly for osmoregulation (Smith, 1936; Yancey and Somero, 1980; Casey and Anderson, 1982; Yancey et al, 1982; Brown and Brown, 1985; Anderson, 1981), whereas, in ureotelic species (Campbell, 1991; Hochachka and Somero, 1973; Nener, 1988; Powers-Lee and Meister, 1988) and ureogenic species (Saha and Ratha, 1987, 1989, 1991, 1994) urea synthesis takes place mainly for metabolic ammonia detoxification. In H. fossilis, it could be possible that apart from ammonia detoxification, the urea cycle also serves for the synthesis of arginine which would be retained and utilized for energy metabolism due to higher Km of arginase in contrast to marine elasmobranch, S. acanthias, where the low Km value for arginine favours the formation of urea from arginine and thereby, retention of urea as an osmolyte.

Nature of inhibition by amino acids and Ki values:

The nature of inhibition of arginase by amino acids particularly by ornithine, leucine, valine, proline, isoleucine and lysine have been reported for several

mammalian species (Hunter and Downs, 1945; Paik et al, 1978; Reddy and Baby, 1976; Rao et al, 1973; Kaysen and Strecker, 1973; Carvajal et al, 1982). Bond (1973) reported that amino acids such as valine, leucine, cysteine and isoleucine could protect bovine liver arginase against proteolytic inactivation as long as their concentration remained within the physiological range. In case of hepatic arginase of H. fossilis, ornithine and leucine were found to be competitive inhibitors. Ornithine which is one of the product of arginase activity was reported to be a competitive inhibitor for uricotelic arginase and a non-competitive inhibitor for ureotelic arginase by some workers (Mora et al, 1965b, 1966; Muszynska et al, 1972). This difference in the nature of inhibition by ornithine was suggested to distinguish between uricotelic and ureotelic arginase. However, many workers found ornithine to be a competitive inhibitor for arginase in ureotelic (Hunter and Downs, 1945; Campbell, 1966; Gasiorowska et al, 1970; Glass and Knox, 1973; Rao et al, 1973), uricotelic lizards (Baby and Reddy , 1980) and ammoniotelic teleosts like G. maculatus (Carvajal et al, 1987) and C. batrachus (Singh and Singh, 1990). Carvajal et al(1982) reported that human liver arginase exhibited a hyperbolic kinetics at pH 7.5 in presence of ornithine and sigmoidal kinetics at pH 9.5. This pH dependent transition from hyperbolic to sigmoidal kinetics indicated an allosteric interaction at pH 9.5. The nature of inhibition of arginase by amino acids seemed to be different depending on enzyme source, the levels of amino acids, and physiological conditions.

The synthesis of urea by rat liver in vitro has been reported to be inhibited by leucine. This effect has been attributed to the stimulation of glutamate dehydrogenase which resulted in increased glutamate synthesis from ammonia (Mendes-Mourao et al, 1975,Das, 1991). It also could be that the proximity of interaction of certain amino acids which affected the enzyme activity might alter the conformation of the enzyme active site preventing the substrate to bind at lower substrate concentration. The Ki values obtained for ornithine, leucine, valine and isoleucine in the present study were 4.25, 9.3, 5.3 and 7.25 mM respectively. Rao et al (1973) have reported Ki value of 4.4. 2.9 and 5.3 mM for ornithine, leucine and valine for sheep liver arginase. Campbell (1966) reported a Ki value of 1.34 mM for ornithine from bovine and rat liver arginase. It is apparent that out of the 4 amino acids studied, isoleucine binds less tightly to the enzyme molecule as compared to ornithine. The differences in the nature of inhibition observed for hepatic arginase from H. fossilis and in other species could be correlated to the differences in the experimental conditions such as pH and activators which have been reported to alter the kinetics of inhibition of arginase by amino acids (Rao et al, 1973; Carvajal et al, 1982; Carvajal and Cederbaum, 1986).

CONCLUSION

Conclusion:

The induction of ureogenesis via o-u cycle under hyper-ammonia stress is one of the major ammonia detoxifying pathway to reduce its toxicity in vivo in this unique freshwater air-breathing teleost, Heteropneustes fossilis which has been shown to tolerate a very high ambient ammonia (upto 75 mM (NH_4Cl). The increase of ammonia level in the liver (the most ureogenic tissue) from the physiological level (12.75 µmoles/g wet wt.) to a threshold level between 15 to 18 µmoles/g wet wt. is sufficient enough to cause maximum induction of o-u cycle enzymes and ureogenesis in this fish. Presence of both the isoenzymes, CPS I and CPS III both in the liver and kidney tissues of H. fossilis is unique which is normally not present together in vertebrates in a single species. This may have some evolutionary significance with addition to their physiological significance to tolerate a very high ambient ammonia. All the o-u cycle enzymes both in the liver and kidney of H. fossilis show annual variation in their activity having maximum activity in summer and minimum in winter. This variation of activity has got direct correlation with their annual breeding cycle. The molecular weight of hepatic arginase of H. fossilis shows similarities with the arginase of ureotelic and ureosmotic species. Higher Km value of arginase for arginine obtained for hepatic arginase of this fish suggested that this fish favours more storage of arginine an essential

amino acid for synthesis of new proteins rather than degradation. The narrow pH range of hepatic arginase activity obtained in this fish also favours the same hypothesis.

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