C H A P T E R - I

ROLE OF PHOSPHATE AND ORGANIC ANIONS ON BRAIN MITOCHONDRIAL GLUTAMINASE ACTIVITY

It is well known that glutaminase (L-glutamine-amido-hydrolase, E.C. 3.5.1.2) of brain or kidney is activated by various inorganic and organic anions (25, 26, 27). Glutaminase in kidney is a mitochondrial enzyme (25, 26). It has been suggested that guinea pig brain mitochondrial glutaminase is an allosteric enzyme (27, 138).

Although glutaminase is inhibited by both the products of its action viz. ammonia and glutamic acid (28), the latter substance is more likely to attain in vivo the concentration required for effective inhibition than the former.

Considering these activation and inhibition phenomena Sayre and Roberts (11) postulated that glutaminase has two cationic sites and one -SH catalytic site. Glutamine was assumed to be attached to one of the cationic site through its α-carboxylic group and its amino group was thought to be linked to phosphate which again was bound to the other cationic site. In this way phosphate activation and glutamate inhibition of glutaminase was explained. It was postulated that inhibitory effect of glutamate was not only due to competition with inorganic phosphate but also to lowering of the pH of the inner space of mitochondria (28). Accumulation of large amount of inorganic phosphate also leads to lowering of the pH of inner space. For this reason succinate relieves this inhibition much better than does inorganic phosphate.

Under the conditions used in the present experiments, glutaminase is practically inactive in the absence of anionic activator, such as phosphate. On this basis phosphate might be described as "coenzyme" of glutaminase (27). The term "coenzyme", however, does not imply any specific involvement as other organic anions can replace phosphate to a limited extent. This is supposed to be due to an allosteric mechanism involving a second active site other than substrate binding site (27).

RESULTS

Activity of glutaminase in different organs of rat and in different subcellular fractions of the rat brain

Glutaminase is found to be mostly located in mitochondrial fraction when treated under conditions used for the experiments summarized in Table I. The specific activity of glutaminase in brain mitochondria has been found to be higher than that in kidney mitochondria which is again much higher than that in liver mitochondria. Among the different subcellular fractions examined, the activity observed in synaptosomal and myelin fractions might be due to contamination of mitochondria in those fractions (Table-I). Actually neuronal mitochondria are heterogeneous in their biochemical and functional characteristics as well as particle size (14, 15).

Table - I

Glutaminase activity in brain, liver and kidney

Organ	Cell fraction	Specific activity*
Brain	Homogenate	12.8 ± 1.6
Brain	Mitochondria	94.8 ± 7.2
Brain	Synaptosome	22.1 ± 1.4
Brain	Myelin	5.5 ± 0.8
Kidney	Mitochondria	55.8 ± 5.1
Liver	Mitochondria	6.1 ± 0.4

Fig. 1. Effect of pH on glutaminase activity in rat brain mitochondria.

Each reaction mixture contained 0.005 M L-glutamine; 0.02 M tris-HCl buffer (pH range 7.0 to 9.4); 0.02 M Na-phosphate buffer (pH range 7.0 to 9.4); 0.5 ml of mitochondrial suspension in 0.32 M sucrose containing 1 mg of protein and distilled water to a final volume of 3 ml. Incubation time was 60 minutes at 37°C.

Values reported are based on average determination of five separate experimental preparations.

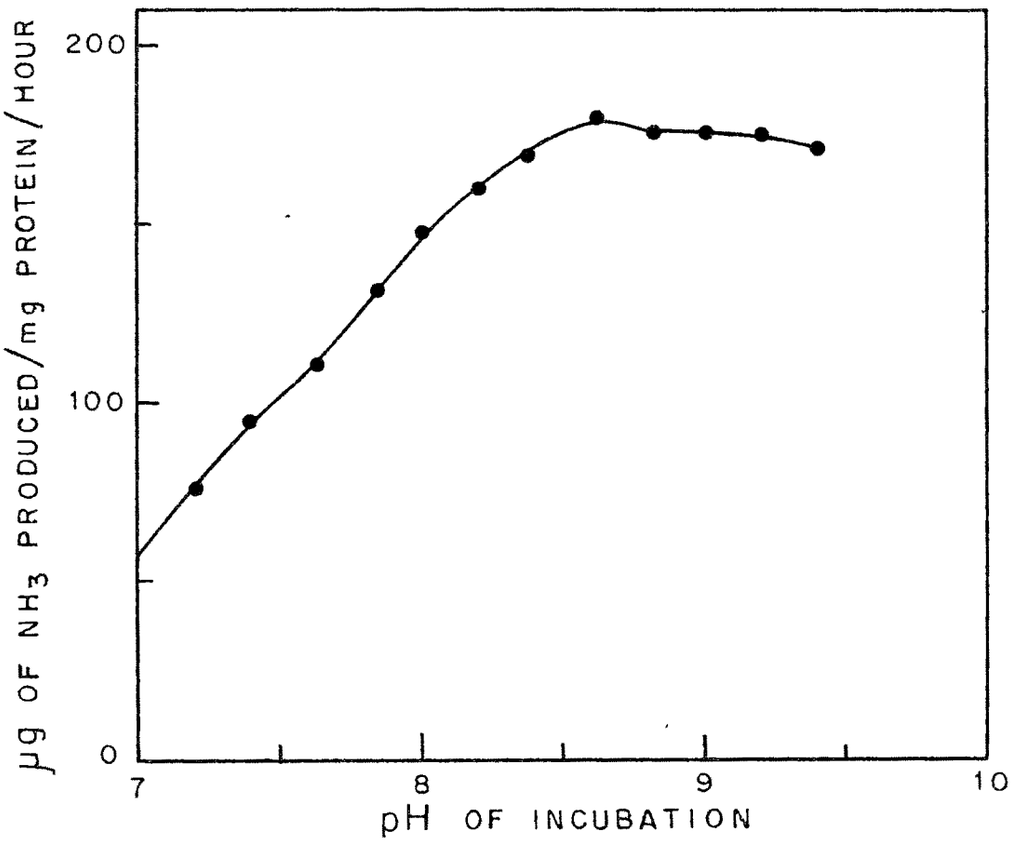
200
100
0
7
8
9
10
µg OF NH3 PRODUCED/mg PROTEIN/HOUR
pH OF INCUBATION

Fig. 1

Fig. 2. Effect of L-glutamine and phosphate on the activity of brain mitochondrial glutaminase.

In one series (● — ●) effect of 0.02 M phosphate on glutaminase activity was noted in the presence of different concentration of L-glutamine (0 to 0.07 M). In a second series (○ — ○) effect of 0.005 M L-glutamine on glutaminase activity was noted in presence of different concentration of phosphate (0 to 0.14 M). Other additions include 0.02 M tris-HCl buffer, pH 7.4 and 0.5 ml of mitochondrial suspension in 0.32 M sucrose containing 1 mg of protein and distilled water to a final volume of 3 ml. The reaction mixture was incubated at 37°C for 60 minutes.

Activity = μg of ammonia formed per mg of protein/hour.

Values reported are based on average determination of five separate experimental preparations.

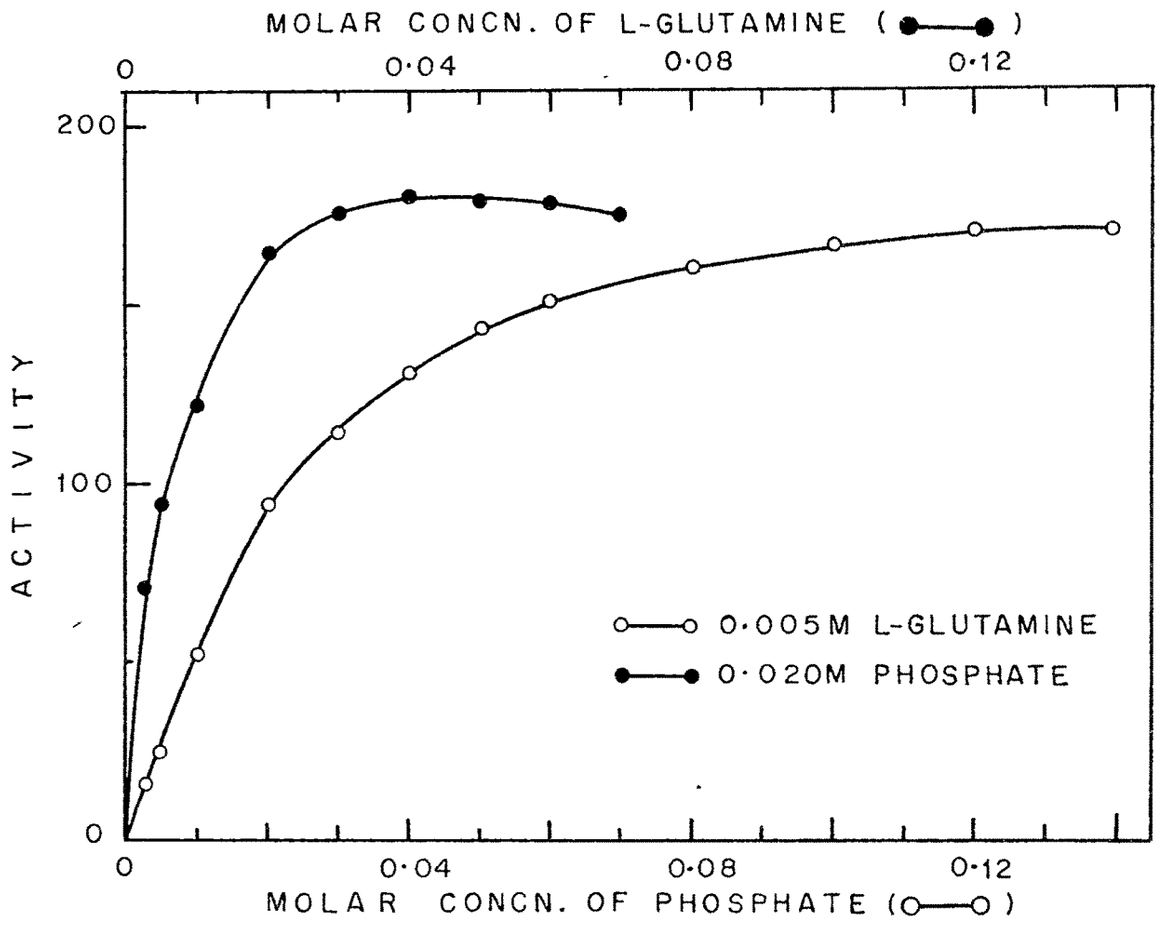
MOLAR CONCN. OF L-GLUTAMINE (● — ●)
0
0·04
0·08
0·12
200
100
0
ACTIVITY
0·005M L-GLUTAMINE
0·020M PHOSPHATE
0
0·04
0·08
0·12
MOLAR CONCN. OF PHOSPHATE (○ — ○)

Fig. 2

The incubation mixture contained 0.005 M L-glutamine; 0.02 M Tris-HCl buffer, pH 7.4; 0.02 M Na-phosphate (pH 7.0); 0.5 ml of respective fraction equivalent to 50 mg tissue in 0.32 M sucrose and distilled water to a final volume of 3 ml. The reaction mixture was incubated at 37°C for 1 hour.

Glutaminase activity is expressed as μg of ammonia formed per mg of protein per hour of incubation.

*Means ± S.D. from five separate experimental preparations.

Effect of pH on brain mitochondrial glutaminase activity

Glutaminase activity is generally catalyzed over a wide pH range, with optimal activity between pH 8.5 to 9.0 (Fig. 1). In the present investigation, the enzyme activity, however, was not determined in that pH range since alkalinity of the medium plays a great role on activation of mitochondrial glutaminase (Fig. 9). Enzyme activity was determined at pH 7.4, which is close to physiological pH.

Role of phosphate and L-glutamine on glutaminase activity

Glutaminase activity gradually increased, either when increasing amount of phosphate was being added to incubation mixture containing constant amount of L-glutamine or when increasing amount of L-glutamine was being added to incubation mixture containing constant amount of phosphate, upto a certain state of activity. Both the curves were typical hyperbolic form of saturation curve. In Fig. 2, it is shown that approximately half maximum activity was reached when either 0.02 M phosphate

Fig. 3. Effect of preheating on glutaminase activity associated with whole brain homogenate.

Curve A : Native homogenate in tris-HCl buffer, pH 7.4 plus no phosphate.

Curve B : Preheated homogenate in tris-HCl buffer, pH 7.4 (at 50°C for 5 minutes) plus no phosphate.

Curve C : Native homogenate in tris HCl buffer, pH 7.4 plus 0.02 M phosphate, pH 7.4.

Curve D : Preheated homogenate in tris-HCl buffer, pH 7.4 (at 50°C for 5 minutes) plus 0.02 M phosphate, pH 7.4.

Curve E : Preheated homogenate in mixed tris-HCl and phosphate buffer at pH 7.4 plus no phosphate.

Assay was conducted at 37°C. Preincubated and native material were kept in the ice bath. A certain aliquot of each of the above preparation was taken for incubation so that final incubation mixture contained components of equal concentrations. In cases of (B) and (D) aliquot contained 0.3 ml of 0.2 M tris-HCl buffer and 0.5 ml of homogenate. In case of (E) aliquot contained 0.3 ml of 0.2 M tris-HCl buffer, pH 7.4 and 0.3 ml of 0.2 M phosphate buffer pH 7.4 and 0.5 ml homogenate. 1 ml of homogenate in 0.32 M sucrose is equivalent to 100 mg of tissue. Distilled water was added to make the volume 2.7 ml. The reaction was initiated by the addition of 0.3 ml of L-glutamine solution to a final concentration as designated on the abscissa of Fig. 3. Incubation time was 60 minutes at 37°C.

Activity is expressed as μg of ammonia formed per mg of protein per hour.

Values reported are based on average determination of five separate experimental preparations.

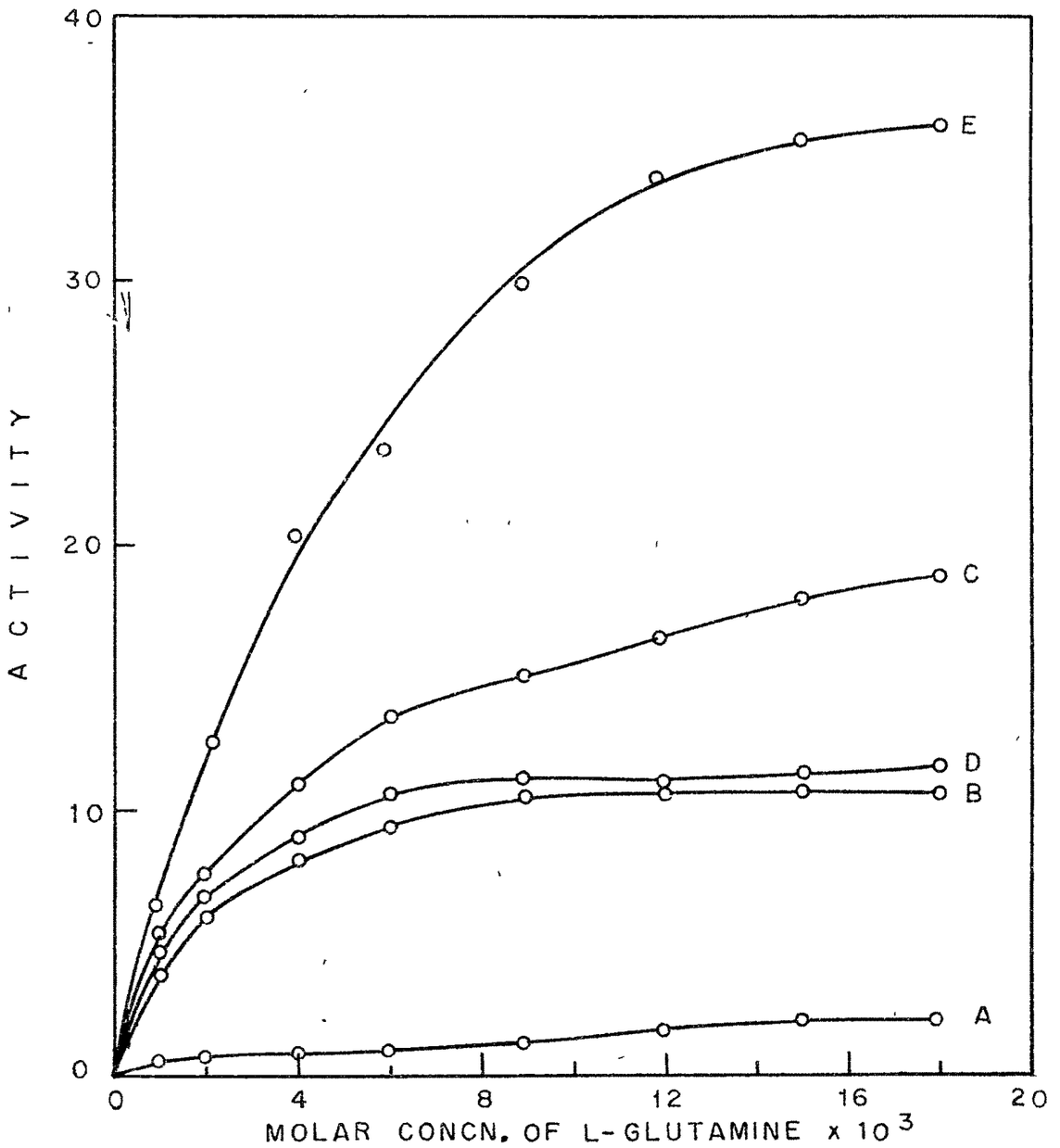
40
30
20
10
0
ACTIVITY
E
C
D
B
A
0
4
8
12
16
20
MOLAR CONCN. OF L-GLUTAMINE x 10 3

Fig. 3

and 0.005 M L-glutamine or 0.02 M L-glutamine and 0.005 M phosphate were present in incubation. It shows both phosphate and glutamine play equally important role in the regulation of glutaminase activity.

Effect of preheating on glutaminase activity associated with whole brain homogenate

Comparing the curves A with B and C with E, it seems preheating itself has got an activation effect on glutaminase activity. But comparison of the curves C and D, indicates that preheating partially destroys glutaminase activity. Again comparing the curves C and E, it can be said that phosphate has got a protective effect against denaturation due to preheating of glutaminase activity as well as produces additional stimulatory effect. Partial destruction of enzyme in absence of added phosphate might be due to little endogenous phosphate which is not sufficient to stabilize the whole amount of enzyme under incubation. However, this little endogenous phosphate may be responsible for activity of glutaminase observed with homogenate preparation (Fig. 3). Actually purified mitochondrial preparation shows no activity in absence of added phosphate or anionic activator, under the conditions used in the present experiments (Table - III).

Fig. 4. Effect of preheating on glutaminase activity associated with brain mitochondria.

Measured amount of mitochondrial suspension in 0.32 M sucrose was preheated at 50°C for 5 minutes with measured amount of Na-phosphate buffer, pH 7.4; so that 0.8 ml of preheated aliquot contained 0.5 ml of mitochondrial suspension containing 1 mg of protein and 0.3 ml of phosphate buffer, which when made upto a final volume of 3 ml contained the phosphate concentration as designated on the abscissa of Fig. 4. A similar preparation which was not subjected to heating, acted as native enzyme source. The reaction was initiated by the addition of 0.3 ml each of 0.2 M tris-HCl buffer, pH 7.4 and 0.05 M L-glutamine. The reaction mixture was incubated at 37°C for 60 minutes.

Activity = μg of ammonia formed per mg protein per hour of incubation.

Vertical line (I) = $\pm$ S.D. from five separate experimental preparations.

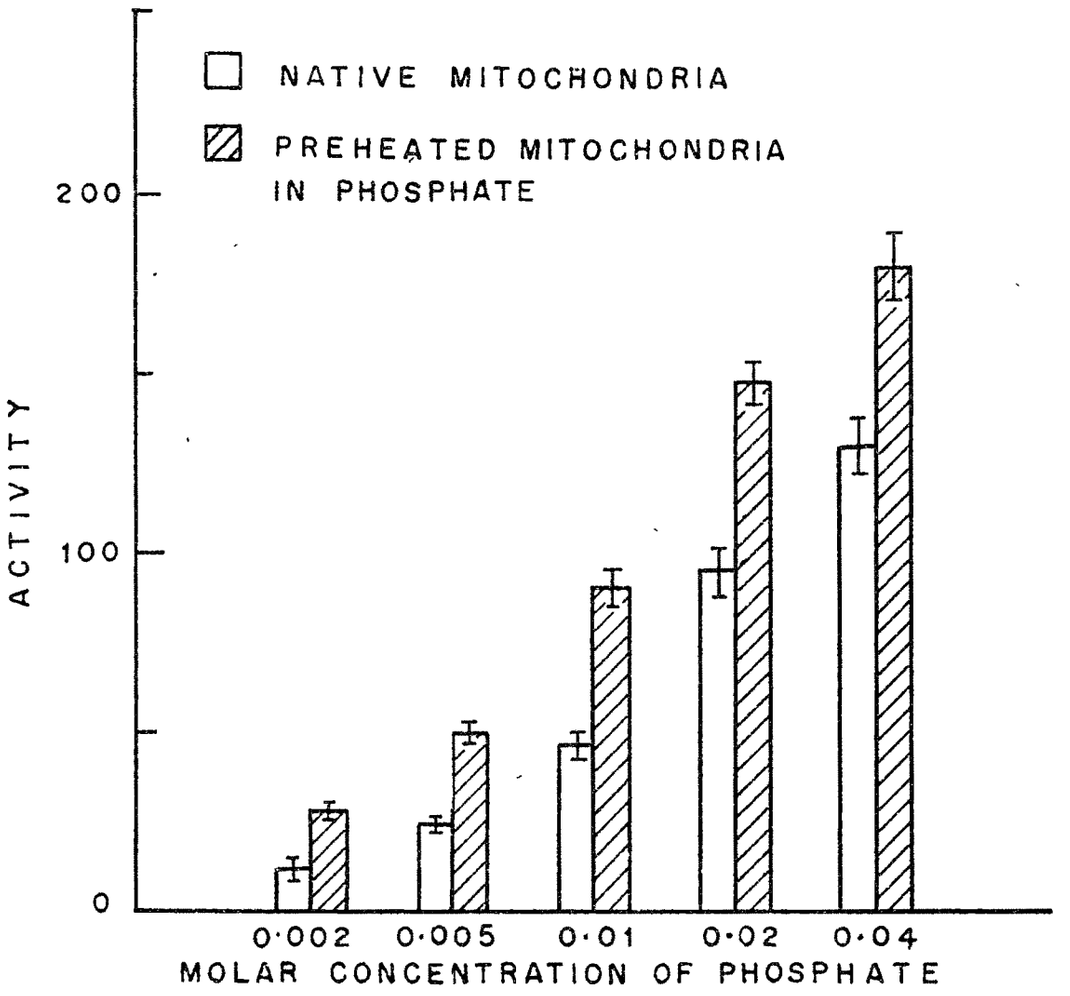
NATIVE MITOCHONDRIA
PREHEATED MITOCHONDRIA IN PHOSPHATE
ACTIVITY
200
100
0
0·002
0·005
0·01
0·02
0·04
MOLAR CONCENTRATION OF PHOSPHATE

Fig. 4

Fig. 5. Effects of mono-, di-, and tri-carboxylic acids on brain mitochondrial glutaminase activity.

The incubation mixture contained 0.005 M L-glutamine, 0.02 M tris-HCl buffer (pH 7.4); 0.02 M sodium salt of carboxylic acids and phosphate (pH 7) in respective incubation mixture, 0.5 ml of mitochondrial suspension containing 1 mg of protein in 0.32 M sucrose and distilled water to a final volume of 3 ml. Incubation time was 60 minutes at 37°C.

Percent activity is calculated on the basis of mean activity of five separate experimental preparations.

Effect of preheating on glutaminase activity associated with brain mitochondria

Preincubation of mitochondria at 50°C for 5 minutes with 0.01 M phosphate produces more than 100% activation of glutaminase activity as compared to its activity in native mitochondria containing same concentration of phosphate in incubation (Fig.4). Percent activation on preheating diminished as activity with higher concentrations of phosphate are being compared.

Effects of mono-, Di-, and tri-carboxylic acids on glutaminase activity

Studies on the effects of various organic acids on glutaminase activity show that tri-carboxylic acid is more effective than di-carboxylic acids which in turn are much more effective than monocarboxylic acids (Fig.5). Again among phosphate and citrate ions, though both are trivalent anion, activity in presence of phosphate is much higher than that in the presence of citrate, when activity in presence of 0.02 M of each is considered. This perhaps indicates the possible role of proximity of negative charges in an ion in regulation of glutaminase activity. When activity in presence of equimolar amount of dicarboxylic acids were considered, the order found to be oxalate> malonate> succinate (Table - II). This indicates as the size of the ion increases, its role regarding regulation of glutaminase activity decreases.

Table - II

Comparison of the effects of oxalate, malonate and succinate on brain mitochondrial glutaminase activity

Concentrations	Glutaminase activity*		
	Oxalate	Malonate	Succinate
Nil	0.2 ± 0.03	0.2 ± 0.03	0.2 ± 0.03
0.02 M	53.2 ± 2.70	24.2± 1.80	9.6 ± 0.50
0.04 M	110.0 ± 6.20	48.8 ± 2.60	19.6 ± 1.10
0.08 M	172.8 ± 8.00	73.0 ± 4.5	29.0 ± 2.00
0.16 M	188.0 ± 9.3	80.8 ± 3.5	33.5 ± 1.20

The incubation mixture contained 0.005 M L-glutamine; 0.02 M tris-HCl buffer, pH 7.4; sodium salts of carboxylic acids (final concentrations 0.02 M, 0.04 M, 0.08 M and 0.16 M in respective incubation mixture); 0.5 ml of mitochondrial suspension containing 1 mg of protein in 0.32 M sucrose and distilled water to final volume of 3 ml. Incubation time was 60 minutes at 37°C.

Activity = μg of ammonia/mg of protein/hour.

*Means ± S.D. from five separate experimental preparations.

DISCUSSION

Under the conditions used in the present investigations for the determination of glutaminase activity, it was found that activity in brain mitochondria is higher than that in kidney mitochondria which in turn is higher than that in liver mitochondria. Optimum pH of brain glutaminase was found between pH 8.5 and 9. Glutaminase in mitochondria is practically inactive in the absence of anionic activator. Slight residual glutaminase activity obtained in preheated homogenate preparation can possibly be due to the presence of endogenous phosphate and other anions.

With mitochondria as enzyme source, it was found that product of substrate (glutamine) concentration and phosphate concentration remains unaltered at half maximum velocity (Fig.2). This is an indication of equivalent role played by phosphate and glutamine when enzyme activity is considered. Preheating of mitochondria with phosphate indicate that it protects the enzyme from inactivation. Thus phosphate can be entitled as both a stabilizer and a "coenzyme" of glutaminase. Preheating with phosphate also produces activation of glutaminase with respect to native mitochondria incubated with same concentration of phosphate. Thus phosphate could be supposed as an agent which increases the accessibility of substrate to enzyme. Alteration of

permeability of substrate into mitochondria in this situation might happen.

Among the carboxylic acids, tricarboxylic acid was found to be more potent than dicarboxylic acids which in turn are better activator than monocarboxylic acids. The effectiveness of 0.02 M citrate was found to be 74% with respect to that with 0.02 M phosphate at the level of glutaminase activity. Among dicarboxylic acids, oxalate is better activator than malonate which in turn is better activator than succinate (Table - II). In case of latter two acids, carboxylic groups are spaced by $-CH_2-$ and $-CH_2-CH_2-$ respectively, whereas in oxalate carboxylate anions are adjacent. In phosphate ion also negative charges are in closer proximity than citrate, where negative charges on carboxylic groups are held apart by structural skeleton $-CH_2-\overset{|}{C}(OH)-CH_2-$. This makes the citrate less effective than the phosphate (Fig.5). Thus "coenzyme" role of phosphate might be carried out by citrate and other carboxylic acids to different extent. These facts suggest the existence of a regulatory site on the enzyme which is modified differentially by different anionic regulator.
