# GENERAL DISCUSSION

In the rat brain, mitochondria are found to be the richest source of glutaminase (Table - I). Under the conditions used in the present experiments, glutaminase is practically inactive in the absence of anionic activator such as phosphate and carboxylic acids (Table - III). In homogenate, however, little activity of glutaminase is observed in the absence of phosphate when high concentration of substrate (glutamine) is used (Fig. 3). This activity is supposed to be due to the presence of endogenous anionic groups. Thus phosphate can be called "coenzyme" of glutaminase. Phosphate can be replaced by organic mono-, di-, or tri- carboxylic acid, though they are less potent than phosphate as a coenzyme (Fig. 5). Among the carboxylic acids, glutaminase activity in presence of citrate is found to be higher than that in presence of dicarboxylic acids which in turn is higher than activity in presence of monocarboxylic acids. Again, among dicarboxylic acids, glutaminase activity in presence of oxalate shows higher value than that in presence of malonate which in turn produces higher activity than that in presence of succinate. Thus proximity of negative charges in anions of dicarboxylic acids is an important regulator of glutaminase activity. Since negative charges in phosphate ion much in closer proximity than that in citrate, higher activity was observed in presence of phosphate than that of citrate, in equivalent concentrations.

Mitochondria preheated with different concentration of phosphate at 50°C for 5 minutes activates glutaminase to different extent with respect to the activity observed in native mitochondria in presence of similar phosphate concentration. But preheating in absence of phosphate, however, produces irreversible damage of enzyme activity. So, phosphate is also a stabilizer of glutaminase in mitochondria. Both phosphate and glutamine play equally important role in the regulation of glutaminase activity (Fig.2). It seems that phosphate increases accessibility of substrate to enzyme either at the level of mitochondrial membrane permeation or accelerate enzyme substrate binding phenomenon.

The permeation of substrate and phosphate molecule inside mitochondria seems to be essential since chaotropic agents, which are known to destabilize native structure of mitochondria, produce inhibitory effect on native mitochondrial glutaminase activity, but produce little inhibitory effect on mitochondria preincubated with phosphate (Fig. 14). That an irreversible damaging action at the level of mitochondrial morphology is produced by chaotropic agents, seems possible by the fact that swelling agents, which are shown to activate mitochondrial glutaminase, cannot counteract completely inhibitory effect of those agents. Digitonin and DOC produces damaging action of glutaminase when higher concentration of either of those agents was used in incubation. Activity of

glutaminase however increases gradually with added digitonin or DOC at the initial phase (Fig. 11). Thus bell-shaped activation curves obtained with the surface active agents indicate that though digitonin or DOC produces activation of glutaminase by increasing permeation of mitochondrial membrane, mitochondria looses its structural integrity when higher concentration of those agents were used. Wojtczak and Zaluska (100) showed using electron micrographs of rat liver digitonin treated mitochondria, that gradual detachment of outer membrane occurs. In this connection, it may be noted that mitochondrial swelling agents were found to produce inhibition of glutaminase activity when higher concentrations of those agents were used (Fig. 8). Persons et.al. (80) showed that swelling of mitochondria is accompanied by distended and disrupted outer membrane. Thus mitochondrial structural integrity to a limited extent is essential for enzyme activity.

Wojtczak and Lehninger (46) showed that mitochondrial swelling agents can be divided into two groups according to their power of formation of U-factor on incubation with mitochondria. U-factor has been shown to be equivalent to fatty acid particularly oleic acid (46). Among them, $Ca^{++}$ and thyroxine represent the swelling agents which produce fatty acid on incubation with mitochondria. GSH induced swelling of mitochondria was not accompanied by fatty acid production.

Since BSA binds with fatty acids, effect of BSA was investigated on glutaminase activity in presence of different swelling agents. BSA was found to counteract completely, $Ca^{++}$ and thyroxine induced activation of glutaminase, but almost without effect on the activation produced by GSH. It may be stated that though mitochondrial swelling agents can activate glutaminase, their mechanism of activation does not mediate through a common pathway. It seems likely that thyroxine and $Ca^{++}$ induced swelling is mediated via the production of free fatty acids and conversion of them into bound state by BSA in reaction mixture, completely counteract the activation process. Activation by thyroxine and $Ca^{++}$ may be attributed to their direct or indirect effect on mitochondrial phospholipase A, which is associated with outer membrane and responsible for the production of fatty acids. Thus permeation inside mitochondria can be controlled by regulating phospholipase A. But GSH induced activation cannot be explained in this way, though percent activation produced by optimum amount of GSH is comparable to that produced by thyroxine and higher than that produced by $Ca^{++}$. Thus if anionic molecules are entitled as primary regulator of glutaminase, then swelling agents can be called secondary regulator of glutaminase activity.

Since thyroxine is known to be an uncoupler of oxidative phosphorylation, comparative studies between thyroxine and 2:4-DNP, another well-known uncoupling agent, were made at the level of glutaminase activity. Results indicate that thyroxine and 2:4-DNP influence glutaminase activity in opposite directions to each other. The former activates glutaminase to the extent of 125% whereas the latter inhibits it to the extent of 48% (Table - V). Hence it can be stated that uncouplers of oxidative phosphorylation do not as a rule activate glutaminase. As the possible role of fatty acid in the activation of mitochondrial glutaminase was indicated as a result of activation induced by thyroxine and $Ca^{++}$, the effect of individual fatty acid was investigated. Maximum percent activation produced by oleic, palmitic and myristic acids are 44%, 57% and 43% respectively, in case of adult rat brain mitochondria. Thus maximum percent activation produced by fatty acids is very close to that produced by optimum amount of $Ca^{++}$, but much less than that produced by optimum amount of thyroxine. Thus thyroxine induced activation may be mediated through a differnt pathway than that of fatty acid and $Ca^{++}$ induced activation process. With optimum amount of phosphate in incubation mixture, mitochondrial glutaminase become less sensitive to thyroxine induced stimulation.

The state of activity produced by optimum amount of phosphate can, however, be attained by suboptimum amount of phosphate in presence of thyroxine. Thus though thyroxine itself

has got no stimulatory effect on mitochondrial glutaminase, it might however increase the permeability of phosphate to enzyme site. Thus the extent to which glutaminase activity is stimulated in presence of 0.12 M phosphate was found to be equivalent to that in presence of 0.02 M phosphate plus $2 \times 10^{-4}$ M thyroxine (Table - IV). Activation process is again found to be governed by pH of the medium and at optimum pH state, mitochondrial glutaminase becomes almost insensitive to thyroxine induced stimulation. Thus thyroxine and alkalinity of the medium act in same direction as regards to activation of glutaminase. Hence, like thyroxine, alkalinity of the medium may regulate mitochondrial membrane structure thus enhancing permeation process.

Among the fatty acids tested, oleic acid has got speciality in regulation of glutaminase activity. It is effective at very low concentration as compared to that of palmitic and myristic acid, which require at least 100 fold concentration to produce similar activation. Mitochondrial glutaminase of young rat brain subjected to in vitro aging processes, becomes less sensitive to oleic acid induced activation with time. Again, it has been shown that brain mitochondria of adult rat are less sensitive to oleic acid induced activation as compared to that of young rat, at the level of glutaminase activity (Fig. 15). It might be stated that similar type of modification of mitochondrial morphology occur when mitochondria are aged in vitro, and the

changes undergone in them with developmental process.

Enzymatic studies on the guinea pig brain revealed that an adult level, in most of the brain enzyme activity, was attained in the later part of gestation period, whereas rat attained adult stage at least three weeks after birth, in that respect (106). The development of the brain is reflected in an increase of many cellular constituents and enzymes (122,124,125). In the present investigation, glutaminase activity of rat brain mitochondria in presence of phosphate and other anionic molecules is found to increase with development. Another important observation is that adult mitochondrial glutaminase is less sensitive to fatty acid induced activation whereas young rat brain mitochondrial glutaminase is very much sensitive (Fig. 15). Thyroxine, $Ca^{++}$ and GSH, however, do not produce any remarkable difference of activation between young and adult rat brain mitochondrial glutaminase. It has been reported that maturation of brain is characterized by a progressive increase in the long chain fatty acids of cerebrosides and sulphatides in the rat (137). Metabolism of fatty acid is expected to be higher in adult rat as a result adult rat brain mitochondria become less sensitive to fatty acid induced change. Thus mitochondrial structural state is the most important regulator of neuronal glutaminase activity. Mitochondrial structural state undergoes various change by <u>in vitro</u> addition of various physiological substances as well as <u>in vivo</u> maturation with development and aging.

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