DISCUSSION

DHODase is the enzyme that catalyze the conversion of dihydro-orotate (DHO) to orotic acid (OA) and transfered 2 electrons to a simple electron transport system in mitochondria (Fig.16).It is found predominantly in the particulate fraction of P.falciparum and other species of Plasmodium (Gutteridge et al.,1979; Gero et al.,1981a,1984; Prapanwattana et al.,1989). The previous reports showed that the enzyme activity was not solubilized by freezing and thawing or sonication, suggesting that it is a membrane - bound enzyme. The enzyme has been shown to be similar in mechanism and properties to the mammalian enzyme (Gutteridge et al.,1979; Gero et al., 1981a). In the mammalian system, the enzyme is also particulate and its location is on the outer surface of the innermembrane of mitochondria (Keppler and Holstege, 1982). P.falciparum DHODase may, therefore, be located on mitochondrial membrane as suggested by previous reports, since the parasite does possess mitochondria (Langreth et al, 1978; Divo et al., 1985b; Slomiany and Prensier, 1986). By contrast, the enzyme capable of catalyzing the conversion of DHO to OA in some protozoa, e.g., the Kineto-plastida (Trypanosoma spp., Leishmania spp.) and Crithidia spp., is shown to be localized in soluble fraction and was different with respect to mecha-nism and properties from mammalian enzyme (Gutteridge et al., 1979; Ham-mond and Gutteridge, 1982; Pascal et al., 1983; Tampitag and O' Sullivan, 1986), and a similar enzyme has been found in Escherichia coli (Larsen and Jensen, 1985). In Giardia lamblia, the enzyme involved in conversion of DHO into OA, as well as other enzyme in pyrimidine de novo biosynthetic pathway, could not be detected (Landmark and Jarroll, 1982).

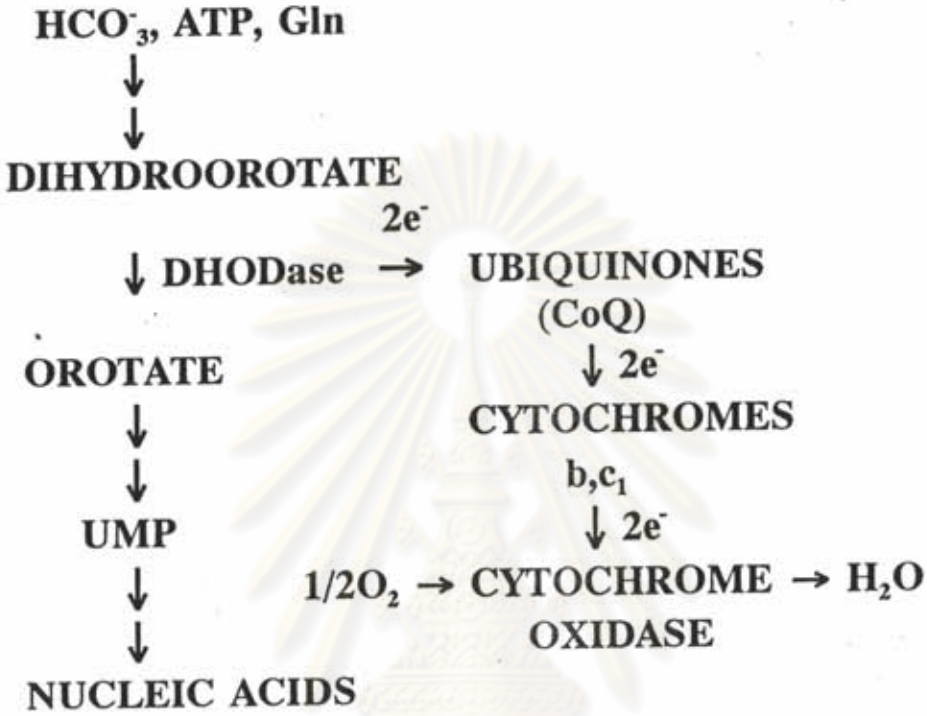

FIGURE 16 : Proposed conversion of dihydroorotate to orotate (Sherman, 1984)

The specific activity of DHODase in crude homogenate and detergent solubilization fraction in each experiment was found to be varied in different storage time. It was noted that the longer storage time of the enzyme, the lower specific activity (Table 1).

The specific activity of DHODase in crude homogenate of P.falciparum shown here was much higher than in detergent solubilization fraction (Table 1). The total activity in crude extract may due to the activity of other dehydrogenase enzymes which can reduce DCIP, an electron acceptor, without using L-DHO as substrate. However, the strain variation of the parasite used, the method of enzyme preparations, the method of assay (specificity of the method), and the relative distribution of the morphological forms of the parasite could also be contributing factors to the observed differences.

Besides DHODase, the malaria parasite also contains other enzymes for completing the pyrimidine de novo biosynthetic pathway (Walsh and Sherman, 1968; Van Dyke et al., 1970; O'Sullivan and Kelly, 1980; Hill et al., 1980; Gero et al.,1981 a,b; Reyes et al., 1982; Gero et al.,1984), but no enzymes of pyrimidine salvage pathway could be detect (Reyes et al., 1982). It is now well established that the intraerythrocytic malaria parasite is unable to salvage preformed pyrimidines from its environment and relies exclusively on de novo pathway for its pyrimidine requirement (Sherman, 1984).

In mammals and other higher eukaryotes,the first three enzymes of pyrimidine biosynthesis, carbamylphosphate synthase II (CPS II), aspartate transcarbamylase (ATCase), and dihydroorotase (DHOase, 4,5-dihydroorotate amidohydrolase) are carried by a molecular weight 240000 Da multifunctional protein (Kelly et al, 1986; Jones, 1980). There is little information as to the nature of these enzymes in parasitic protozoa. The third enzyme of the pathway, dihydroorotase, has been purified in P.berghei

and Crithidia fasciculata (Krungkrai et al., 1990). The fourth enzyme of the pathway, dihydroorotate dehydrogenase (DHODase), has been characterized in C. fasciculata, Trypanosoma brucei (Pascal et al.,1983) P.berghei (Krungkrai et al., 1991). Orotate phosphoribosyl transferase (OPRTase) and orotidine 5'-phosphate decarboxylase (ODCase) catalyzes the final step of de novo synthesis of UMP in mammalian cells, and exist as a bifunctional protein (Jones, 1980),but in P.falciparum,the most important cause of human malaria, these proteins have been described as discrete entities (Rathod and Reyes, 1983).

It appears that P.falciparum is shown to contain all the enzyme required for the biosynthesis of UMP (Gero et al.,1981b; Reyes et al,1982; Gero et al., 1984). In P.berghei the highest activity being observed for ATCase and lowest for CPS II and DHODase (Hill et al., 1981). In keeping with the findings of Gero et al.(1981b), the P.falciparum enzyme is somewhat lower than P. berghei (Gero et al, 1984).

P.falciparum DHODase was also shown to be the stage-dependent enzyme. The highest specific activity was observed in the trophozoite stage parasite . As was found in the previous report of Gero et al.(1984) that the enzymes responsible for the synthesis of UMP in P.falciparum increase during parasite growth and reaching a maximum at approximately 27 h just prior to schizogony (at late trophozoite stage).The activities reflect the total amount of enzyme protein present, and follow or somewhat similar pattern as observed for DNA synthesis by Newbold et al. (1982).

The DHODase activity has been shown to be linked to the respiratory chain in mammalian cells (Jones, 1980; Keppler and Holstege,1982).

This appears to be the case for P.berghei and P.knowlesi (Gutteridge et al., 1979; Gero et al., 1981a), and could be related to the finding that malaria-infected red cells showed ability to enhance oxygen uptake (Scheibel et al., 1979). The previous reports showed that inhibition of pyrimidine de novo biosynthetic enzyme would result in highly effective antimalarial activity. For example, inhibition of DHODase by menoctone and agents in the hydroxynapthoquinone series (analogs of ubiquinone) that interfere with the respiratory chain linked to the DHODase (Gero et al., 1981a, 1984; Hudson, 1984; Hudson et al., 1984; Hammond et al, 1985). In addition, orotate analogs, 5-fluoroorotic acid (FOA) showed inhibitory effect to P.falciparum DHODase (Table 6) with 50% inhibition at 0.16 mM. In P.berghei DHODase, it showed competitive inhibition with a Ki of 35 μM (Krungkrai et al., 1991) and L-OA, the product of the enzymatic reaction, was a competitive inhibitor of DHO oxidation with a Ki of 30 μM (Krungkrai et al., 1991).

Inhibition of orotate phosphoribosyltransferase and orotidylate decarboxylase, the last two enzyme in pyrimidine de novo pathway, by pyrazofurin (Scott et al., 1986). Thus, all of these results can be explained by the inability of malarial parasite to take up preformed pyrimidines to relieve the blockade. It is rather unlikely that such an effect would cause an almost immediate arrest of nucleic acid, and even more so, of protein synthesis. In the latter case, existing cellular pools of m-RNA and t-RNA should suffice, at least temporarily for protein synthesis before the shortage of pyrimidines would affect this process. This is especially true for the ring stage parasite with low levels of nucleic acid and protein synthesis (Gritzmacher and Reese, 1984; Inselburg and Banyal, 1984; De Rojas and Wasserman, 1985; Waki et al., 1985).

The rodent malaria parasite P.berghei is chosen as a suitable source for DHODase characterization because of its relative ease of culture and because it has proved a reliable model of the human pathogen in related studies of malaria biochemistry (Krungkrai et al., 1991). On purification to apparent homogeneity, like P.falciparum DHODase in this experiment, P.berghei DHODase is found to be membrane-bound and active in monomeric form with an estimated molecular mass of 55 kDa (Krungkrai et al., 1991). The Km value for L-DHO of DHODase in P.falciparum experiment was 88.7±24.1 μM whereas the Km value for L-DHO of P.berghei DHODase was 7.9±2.5 μM (Krungkrai et al., 1991) and of P.falciparum was 11.0±1.2 μM (Gero et al., 1984).These differences might be due to the very low enzyme activity of DHODase obtained in this experiment that result in the unexpected high Km value for L-DHO.

The malarial DHODase are found to be extremely labile. In this experiment the cell-free extracts of P.falciparum DHODase were stored at $-20^{\circ}C$ (freezer) and $-196^{\circ}C$ (liquid nitrogen). Even in the presence of protease inhibitors at $-20^{\circ}C$ the DHODase activity was decreased by more than 80% in the first 24 h. Whereas the enzyme activity in the same protease inhibitors used at $-20^{\circ}C$ but stored at $-196^{\circ}C$ was quite stable, the activity was decreased about 2% in the first 24 h. As previous reports on cell-free extracts of P.berghei DHODase, in the presence of protease inhibitors at $4^{\circ}C$, the activity was decreased by more than 50 % overnight (Krungkrai et al., 1991)

In conclusion, the enzyme DHODase from P.falciparum, a human malaria, has been purified to near homogeneity by using detergent solubilization and followed by anion-exchange and affinity chromatography. The physical properties and kinetic behaviors are not different to the well characterized enzyme in P.berghei, a rodent malaria. These characteristics of the enzymes from both malaria parasites will be used for antimalarial agents development.