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# HIGH ACTIVITY OF α-GLYCEROPHOSPHATE OXIDATION BY HUMAN PLACENTAL MITOCHONDRIA

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## Summary

Human term placental mitochondria oxidize α-glycerophosphate at an unusually high rate as compared to other substrates. The apparent $K_m$ both for oxidation and α-glycerophosphate dehydrogenase (EC 1.1.99.5) activity of DL-α glycerophosphate determined in a medium containing 2 mM EDTA and 5 mM $MgSO_4$ was approx. 0.7 mM. EDTA inhibited the α-glycerophosphate oxidation if the later was used at low concentrations. A subsequent addition of $MgSO_4$ or $CaCl_2$ restored the orginal activity. EDTA had no effect on mitochondrial respiration at high concentration of α-glycerophosphate. Possible physiological role of relatively high activity of human placental mitochondrial α-glycerophosphate dehydrogenase is discussed.

## Introduction

Recent studies have shown that human term placenta to be an metabolically active organ with an oxygen utilization of up to 55—110 ml per h [1]. Schreiner and Villee [2] first isolated mitochondria from term placenta and showed that these organelles yielded variable and generally low levels of respiration and phosphorylation. These authors suggested [2] that endogenous progesterone, known as an inhibitor of electron transport chain [3,4] and oxidative phosphorylation [5—8] is responsible for the low respiratory activity and phosphorylation. Klimek et al. [9] using bovine serum albumin throughout isolation and assay procedures were able to improve the P : O ratio of placental mitochondria, however, did not obtain any enhancement of respiratory activity. Recently Olivera and Meigs [10,11] described isolation and assay conditions

Abbreviation: CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

for oxidative phosphorylation of mitochondria from human term placenta. While studying the metabolism of human placental mitochondria we observed that these organelles oxidize α-glycerophosphate at a relatively high rate. High activity of α-glycerophosphate oxidation by homogenate from locust flight muscle was discovered by Zebe [12]. Further studies with mitochondria obtained from insect flight muscle [13—15], rat brown adipose tissue [16] and rat skeletal muscle [17,18] showed also relatively high activity of α-glycerophosphate oxidation. Estabrook and Sacktor [13] and Bücher and Klingenberg [19] suggested that the system of mitochondrial glycerophosphate dehydrogenase and NAD-linked cytosol dehydrogenase represents a physiological mechanism whereby reducing equivalents originating in the cytosol made available to the respiratory chain in the mitochondria. Hansford and Chappell [20] showed that the activity of α-glycerophosphate dehydrogenase (EC 1.1.99.5) of flight muscle mitochondria is markedly stimulated by very low $Ca^{2+}$ concentrations. It has also been shown that the lung [21] and brown adipose tissue [22] mitochondrial dehydrogenase possess similar properties. On the other hand Wikström et al. [23] reported that apparent $K_m$ for α-glycerophosphate oxidation in the bovine myometrial mitochondria was unaffected by calcium.

## Materials and Methods

*Isolation of mitochondria from human term placenta*

Human term placentas were prepared at 0—4°C within about 30 min of delivery. The blood was drained, membranes removed and the tissue obtained (mainly chorionic villi) was rinsed three times in 0.9% NaCl plus 5 mM EDTA + 10 mM Tris · HCl (pH 7.4) (about 600 ml per one placenta), twice in 0.25 M sucrose plus 5 mM EDTA plus 10 mM Tris · HCl (pH 7.4) (about 400 ml per one placenta). The tissue was passed through a meat grinder and suspended in isolation medium containing: 0.25 M sucrose, 5 mM EDTA, 10 mM Tris · HCl (pH 7.4) approx. 100 g tissue in 200 ml medium. The resulting mince was homogenized manually in a glass Potter-Elvehjem homogenizer with a teflon pestle. The homogenate was filtered through three layers of surgical gauze, centrifuged at 2300 × *g* for 1 min and the pellet discarded. The supernatant obtained was sedimented at 16 000 × *g* for 3 min. Mitochondria were suspended in about 70 ml of 0.25 M sucrose plus 10 mM Tris · HCl (pH 7.4). The suspension was centrifuged at 500 × *g* for 5 min, the pellet discarded and the supernatant was sedimented at 7000 × *g* for 10 min. The pellet so obtained was washed once with about 20 ml of 0.25 M sucrose plus 10 mM Tris · HCl (pH 7.4) and sedimented at 7000 × *g* for 10 min. The final pellet was suspended in 0.25 M sucrose plus 10 mM Tris · HCl (pH 7.4) to obtain about 20—30 mg mitochondrial protein per ml. The method yielded about 50 mg mitochondrial protein from 100 g of placental tissue. Rat liver and skeletal muscle mitochondria were prepared as described previously [24,25]. Rat kidney mitochondria were prepared in 0.25 M sucrose plus 10 mM Tris · HCl (pH 7.4) using centrifugation procedure as described by Johnson and Lardy [26].

Bovine heart mitochondria were prepared in the same medium as kidney using the centrifugation procedure described by Settlemire et al. [27].

Protein was estimated as described previously [28].

For electron microscopy, samples were fixed in 2% $OsO_4$ for 1 h, embedded in epon, and sectioned. Specimens were examined as described previously [29,30].

Oxygen uptake was measured polarographically in the conditions described under corresponding figures and tables.

α-Glycerophosphate dehydrogenase was assayed according to Bulos et al. [31] by measuring the rate of oxygen uptake in the reaction coupled with phenazine methosulfate reduction and its subsequent reoxidation in the medium described in Fig. 2b.

*Materials*

DL-α-Glycerophosphate (disodium salt, hexahydrate, grade X) succinate, pyruvate, DL-glutamate, ascorbate, citrate, DL-isocitrate, *cis*-aconitate, α-ketoglutarate were obtained from Sigma Chemical Co., carbonylcyanide *m*-chlorophenylhydrazone (CCCP) were from Calbiochem, L-malate, fumarate, cytochrome *c* were from Koch-Light, ADP from Lódź Poland, Sucrose-AnalaR and bovine serum albumin from B.D.H. All other compounds were of the highest purity available commercially. Sucrose solution was deionized by passing through a mixed-bed ion-exchange resin (Amberlit MB-3 BDH). Glass redistilled and deionized water was used throughout.

## Results

*Morphological and functional characteristics of human placental mitochondria*

Fig. 1 shows electron micrographs of mitochondria prepared by the method described above. It may be seen that the preparation was rich in mitochondria but contained also fragments of membranes and other organelles. Further purification of the mitochondrial fraction by differential centrifugation was not possible in the conditions described. Fig. 1 reveals also that the mitochondrial preparation is heterogeneous and two principal morphological forms may be seen, as has been reported previously by Klimek et al. [9]. As shown in Table I mitochondria of human placenta were able to oxidize pyruvate plus malate, glutamate and succinate, the respiration with these substrates being stimulated by ADP. These results suggest that mitochondria were able to generate ATP. The rates of respiration were comparable to those reported by Olivera and Meigs [10,11].

*Rates of substrate oxidation by human term placenta mitochondria*

The CCCP stimulated rate of oxygen uptake in the medium containing 10 μM cytochrome *c* was greatest with ascorbate and was also relatively high with α-glycerophosphate. Other substrates enumerated in Table II were oxidized at a slower rate. Pyruvate alone was metabolized poorly. Addition of low concentrations of L-malate or fumarate increased oxygen uptake with pyruvate as substrate. The oxidation of NADH was strongly enhanced by added cytochrome *c* and it was insensitive to rotenone and antimycin A (not shown here). This suggests that NADH-cytochrome *c* reductase associated with the outer mitochondrial membrane is responsible for NADH oxidation in the presence of cytochrome *c* [32].

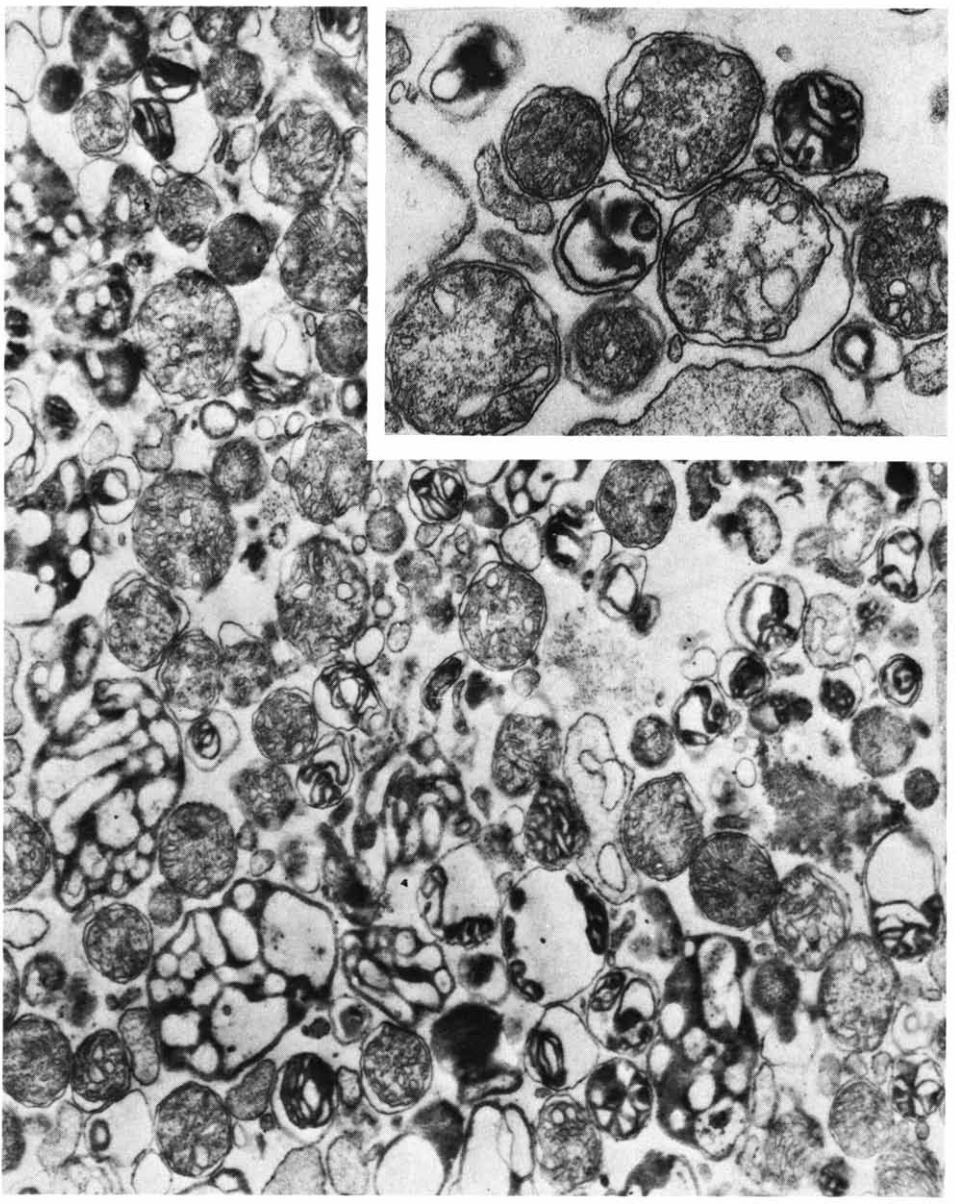

Fig. 1. Electron micrographs of isolated human placental mitochondria. Magnification, × 24 000, inset × 31 500.

Data presented in Table II indicate that mitochondria from human term placenta have an unusually high α-glycerophosphate dehydrogenase activity. The rate of oxygen uptake with α-glycerophosphate as substrate expressed as natoms/min per mg of mitochondrial protein was at the range 60—120, depending on placenta, however, predominantly the values approx. 90 have been obtained. Double reciprocal plots of the oxidation rate (Fig. 2a) and dehydrogenase activity (Fig. 2b) showed a linear relationship. The apparent $K_m$ for

TABLE I

RESPIRATORY RATES AND RESPIRATORY CONTROL OF HUMAN PLACENTAL MITOCHONDRIA

Respiration was measured with a Clark oxygen electrode in the medium containing: 15 mM KCl, 50 mM Tris · HCl (pH 7.4), 20 mM potassium phosphate buffer (pH 7.4), 5 mM $MgSO_4$, 2 mM EDTA, 10 μM cytochrome *c*, 0.5% bovine serum albumin and 8.2 mg mitochondrial protein. Assay temperature was 25°C.

| Substrate | | Oxygen uptake (natoms/min per mg protein) | | State 3 / State 4 |
|---|---|---|---|---|
| | | State 4 | State 3 | |
| Glutamate | 10 mM | 7.8 | 27.3 | 3.5 |
| Pyruvate | 1 mM | | | |
| + L-malate | 1 mM | 7.8 | 19.5 | 2.5 |
| Succinate | 10 mM | 13.0 | 44.2 | 3.4 |

State 3, 1 mM ADP was added.

TABLE II

RESPIRATORY ACTIVITY OF HUMAN TERM PLACENTA MITOCHONDRIA WITH VARIOUS SUBSTRATES

Respiration was measured with a Clark oxygen electrode at 25°C in 2.4 ml medium containing in Exp. 1: 15 mM KCl, 50 mM Tris · HCl (pH 7.4), 20 mM potassium phosphate buffer (pH 7.4), 5 mM $MgSO_4$, 2 mM EDTA, 1 μM CCCP, 10 μM cytochrome *c* and 8.6 mg mitochondrial protein (in the case of α-glycerophosphate oxidation 2—3 mg mitochondrial protein was used). Exp. 2: 15 mM KCl, 50 mM Tris · HCl (pH 7.4), 20 mM potassium phosphate buffer (pH 7.4), 5 mM $MgSO_4$, 2 mM EDTA, 1 mM arsenite, 1 mM ADP, 10 μM cytochrome *c*, 0.5 mM L-malate and 10.5 mitochondrial protein. Exp. 3: 40 mM KCl, 20 mM Tris · HCl (pH 7.4), 6 mM potassium phosphate buffer (pH 7.4) and 2.5 mg mitochondrial protein. Assay temperature was 25°C.

| Substrate | | Oxygen uptake (natoms/min per mg protein) |
|---|---|---|
| Exp. 1 | | |
| Succinate | 10 mM | 30.0 |
| DL-α-Glycerophosphate | 10 mM | 90.0 |
| Pyruvate | 10 mM | 5.9 |
| Pyruvate | 10 mM | |
| + L-malate (0.5 mM) | | 19.3 |
| Pyruvate | 10 mM | |
| + fumarate (0.5 mM) | | 17.0 |
| α-Ketoglutarate | 10 mM | 22.0 |
| DL-Glutamate | 10 mM | 19.3 |
| Ascorbate | 10 mM | 160.0 |
| Exp. 2 | | |
| Citrate | 2 mM | 10.0 |
| Isocitrate | 2 mM | 20.8 |
| *cis*-Aconitate | 2 mM | 18.5 |
| Exp. 3 | | |
| NADH | 0.2 mM | 14.0 |
| NADH | 0.2 mM | |
| + cytochrome *c* (10 μM) | | 100.0 |

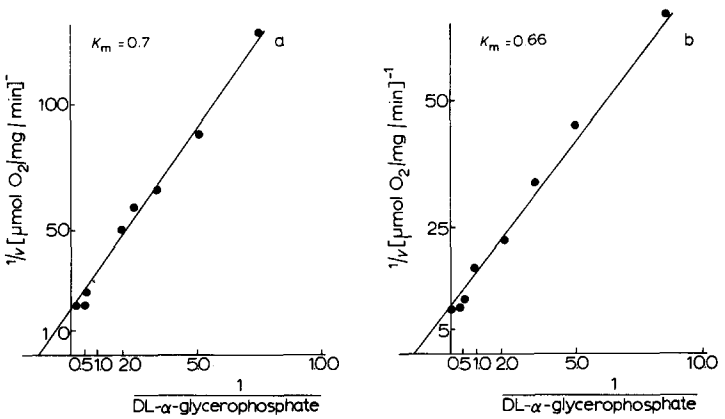


Fig. 2(a) Dependence of the rate of oxygen uptake on α-glycerophosphate concentrations. Experimental conditions as in Table II. Exp. I, except that α-glycerophosphate concentrations varied. (b) Dependence of the α-glycerophosphate dehydrogenase activity on α-glycerophosphate concentration. α-Glycerophosphate dehydrogenase was assayed by measuring the rate of oxygen uptake in reaction coupled with phenasine methosulfate reduction and its subsequent reoxidation in the medium containing 15 mM KCl, 50 mM Tris · HCl (pH 7.4), 20 mM potassium phosphate buffer (pH 7.4), 5 mM $MgSO_4$, 2 mM EDTA, 2 mM KCN, 0.35 mM phenasine methosulfate and 2.4 mg mitochondrial protein. The reaction was initiated by the addition of variable concentration of α-glycerophosphate. Assay temperature was 25°C.

DL-α-glycerophosphate oxidation in the conditions described in Table II, Exp. 1, was approx. 0.7 mM.

Estabrook and Sacktor [13] showed that the oxidation of α-glycerophosphate by isolated blow-fly flight muscle mitochondria is inhibited by EDTA and that this inhibition is reversed by the addition of $Mg^{2+}$ or $Ca^{2+}$. Therefore, we studied the effect of $Ca^{2+}$ and EDTA on α-glycerophosphate oxidation by human placental mitochondria. As shown in Fig. 3 oxidation of 1 mM α-glycerophosphate was immediately inhibited by the addition of 2 mM EDTA. The subsequent addition of $MgSO_4$ or $CaCl_2$ restored the orginal activity almost completely. $MgSO_4$ added in this conditions before EDTA was without effect on α-glycerophosphate oxidation; subsequent addition of EDTA affected only slightly respiration. In the case when 10 mM α-glycerophosphate was oxidized 2 mM EDTA had only a slight effect on respiration. Ethyleneglycol-bis-(β-

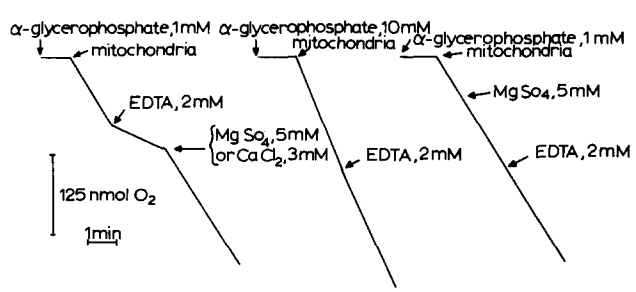


Fig. 3. Effect of EDTA and divalent cations on α-glycerophosphate oxidation by human placental mitochondria. Reaction was carried out in the medium containing: 250 mM sucrose, 20 mM Tris · HCl (pH 7.4), 1 μM CCCP and 2.5 mg mitochondrial protein. Other additions were as indicated on the figure. Assay temperature was 25°C.

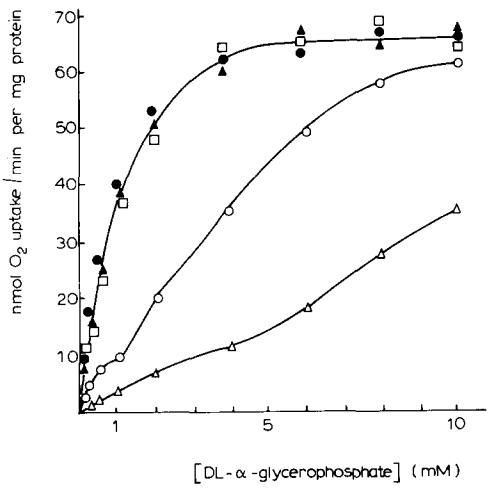


Fig. 4 Dependence of the α-glycerophosphate dehydrogenase activity on α-glycerophosphate concentration. α-Glycerophosphate dehydrogenase was assayed as in Fig. 2b in medium containing: 120 mM KCl, 20 mM Tris · HCl (pH 7.4), 2 mM KCN, 0.35 mM phenasine methosulfate and 2.4 mg mitochondrial protein. ●———●, control; ▲———▲, 0.2 mM $CaCl_2$; ○———○, 0.1 mM EDTA; △———△, 0.5 mM EDTA; □———□, 0.1 mM EDTA plus 0.8 mM $CaCl_2$. Assay temperature was 25°C.

aminoethylether)-*N*,*N*'-tetraacetic acid (EGTA) inhibited α-glycerophosphate oxidation similarly to EDTA (not shown here). $Ca^{2+}$ at the range of concentrations 0.1—8 mM was without effect on α-glycerophosphate oxidation by human term placental mitochondria both in low (1 mM) and high (10 mM) concentrations of substrate if EDTA was omitted (not shown). Detailed studies of the effect of EDTA and $Ca^{2+}$ on α-glycerophosphate dehydrogenase activity are presented in Fig. 4. The apparent $K_m$ for α-glycerophosphate, was unaffected by $Ca^{2+}$ and was increased by addition of EDTA. Addition of 0.8 mM $CaCl_2$ in the presence of 0.1 mM EDTA completely restored the orginal activity.

## Discussion

Little information has been available on the oxidative metabolism of α-glycerophosphate by human placental mitochondria. While preparing this paper Olivera and Meigs [11] demonstrated the presence of α-glycerophosphate dehydrogenase activity in human placental mitochondria. It is evident from the results of the present study that human term placental mitochondria have an unusually high activity of α-glycerophosphate dehydrogenase both as compared to other mitochondrial dehydrogenases (Table II) and α-glycerophosphate dehydrogenase from various tissues. It amounted to 182 natoms 0/min per mg protein when determined in the conditions described in Fig. 2b. In the same conditions rat skeletal muscle, rat liver, rat kidney and bovine heart mitochondria showed α-glycerophosphate activity of 290.0, 15.6, 34.0 and 6.2 natoms 0/min per mg protein respectively. Comparison of α-glycerophosphate oxidation and pyruvate plus malate oxidation by human placental and rat skeletal muscle mitochondria indicates that in the case of rat skeletal muscle mitochondria pyruvate plus malate was oxidized with a higher rate than α-glycerophos-

phate (150 natoms 0/min per mg protein with α-glycerophosphate and 250 natoms 0/min per mg protein with pyruvate plus malate) whereas in placental mitochondria α-glycerophosphate was oxidized at a rate about 4—5 times greater than pyruvate plus malate. Oxidation of α-glycerophosphate by human placental mitochondria was unaffected by amytal or rotenone (even if the inhibitors were used at high concentrations) but was strongly inhibited by antimycin A and cyanide (not shown). This indicates that oxidation of α-glycerophosphate by those organelles involves the flavoprotein dehydrogenase. When α-glycerophosphate was the substrate ferricyanide reduction catalysed by mitochondria from human term placenta was not inhibited by antimycin A. Under conditions where succinate was the substrate antimycin A inhibited ferricyanide reduction in about 80% (not shown). These results are in agreement with the data reported by Klingenberg and Buchholz [33] and are consistent with the α-glycerophosphate dehydrogenase being localised on the outer surface of the inner mitochondrial membrane. In this respect oxidation of α-glycerophosphate by human placental mitochondria resembles the oxidation of this substrate by mitochondria from other tissues. Similarly to blow-fly flight muscle mitochondria oxidation of α-glycerophosphate at low concentration is inhibited by EDTA and this inhibition is reversed by $Mg^{2+}$ or $Ca^{2+}$. However, differences between the $Ca^{2+}$ action on the oxidation of α-glycerophosphate by blow-fly flight muscle and human placental mitochondria have been observed. $Ca^{2+}$ in the case of blow-fly flight mitochondria stimulated α-glycerophosphate oxidation by lowering $K_m$ for α-glycerophosphate. In the case of human placental mitochondria no effect of $Ca^{2+}$ on α-glycerophosphate oxidation was observed. It seems that two interpretations of this finding are possible: (i) α-glycerophosphate dehydrogenase from placental mitochondria like α-glycerophosphate dehydrogenase from the myometrial mitochondria [23] is not sensitive to $Ca^{2+}$, (ii) endogenous $Ca^{2+}$ is responsible for maximal stimulation of the enzyme activity. The last hypothesis is most probable as it is consistent with the inhibitory effect of EDTA or ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*'-tetraacetic acid. To solve this problem further studies are taking place.

At the present time the question arises what is the physiological role of high activity of α-glycerophosphate dehydrogenase in human placental mitochondria. The α-glycerophosphate shuttle is one of several proposed mechanisms for the transfer of reducing equivalents from the cytosol to mitochondria in intact cell [34]. Olivera and Meigs [11] recently suggested that this system is also operative in human placenta. Low activity of NAD-linked α-glycerophosphate dehydrogenase in human placental cytoplasmic fraction (lower than 5 nmol NADH formed/min per mg protein determined in the conditions described by Lee and Lardy [35]) suggests that the shuttle in the placenta may be not as effective as in blow-fly flight muscle. Mitochondrial flavoprotein dehydrogenases (succinate and acyl-CoA dehydrogenases) from adrenal cortex [36] and porcine ovarian lutea [37] generate NADPH which is supporting hydroxylations. It is reasonable to think that the same functions are being carried out by α-glycerophosphate dehydrogenase in human placental mitochondria. On the other hand placenta is storing triglycerides synthetized either from fatty acids or by lipogenesis from glucose and other substrates [38]. Since α-glycerophos-

phate is an important substrate for the synthesis of triglycerides, a possible role of mitochondrial dehydrogenase as an important regulatory factor in the control of lipid synthesis and lipid metabolism in placenta or in foetus-placenta unit is also worthy consideration.

However, the physiological significance of the relatively high activity of α-glycerophosphate dehydrogenase in human placental mitochondria requires further investigation.

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