J. Fac. Fish. Anim. Husb.
Hiroshima Univ. (1968), 7: 235~241

Oxidative Phosphorylation in Mitochondria from Rats Fed a Vitamin E-Deficient Diet

Hiroshi NAITO

Department of Food Chemistry and Technology, Faculty of Fisheries and Animal Husbandry, Hiroshima University, Fukuyama

(Fig. 1; Tables 1-3)

A number of reports have been presented mentioning some inhibitory effects of vitamin E-deficiency on oxidative phosphorylation[1].

These results, however, were quite controversial, probably due to differences in the experimental conditions, chiefly those in response of different tissues and animals.

MARZIUS obtained a decreased oxidative phosphorylation from vitamin E-deficient rabbits diaphragm but not from liver mitochondria[2].

However, MC CAY and Caputto[3] as well as ZALKIN and TAPPEL[4] reported that P/O ratio in liver mitochondria from dystrophic vitamin E-deficient rabbit decreased.

On the other hand, little is known about the similar studies using mitochondria from vitamin E-deficient rats.

Since the rat is known to be less sensitive to the vitamin deficiency, particularly to the incidence of developing muscular dystrophy, as compared with other experimental animals, any significant change in oxidative phosphorylation may not better be demonstrated than in rabbits.

However, ZALKIN and TAPPEL believe that lipid peroxidation damages some sensitive membranes responsible for the impared respiration and possibly for damaged oxidative phosphorylation, which was actually found in liver mitochondria of rabbits rather than in dystrophic muscles[4].

SCHWARZ reported that liver homogenate from vitamin E-deficient rats induced by feeding the *Torula* yeast diet caused marked respiratory decline when α-ketoglutarate or succinate was added as a substrate[5].

It seems, therefore, to be worth investigating whether or not any changes take place in oxidative phosphorylation in rat mitochondria prepared when certain known symptoms of the deficiency have become evident.

In this connection, the authors attempted to elucidate this phenomenon by using mitochondria of liver and heart and submitochondrial particles from rats fed on vitamin E-deficient diet containing 20% casein as a protein source.

EXPERIMENTAL

Weanling male albino rats from Wister strain were placed on the vitamin E-free diet which comprizes the following constituents[6], glucose 65.4 %, casein 20 % (vitamin-free), Salts 446[7] 4.0 %, choline chloride 0.1 %, vitamin mixture[8] 0.5 % and lard (vitamin E-free, Distillation Products Indust.) 10 %. The rats had free access to the diet and to drinking water.

Experiments were made up of three different groups raised under similar feeding duration which differed from five to eleven weeks.

The animals were well in appearances, although their growth rate was slightly slower than that of the tocopherol-supplemented group, but the symptoms of vitamin E-deficiency could be observed by increasing creatine excretion and fatal toxicity of methyl linoleate hydroperoxide (MLHP) which led to death by severe haemolysis within about half an hour after the intravenous injection of purified 10 mg hydroperoxide (peroxide value 2,200)[9][13] in 1 m*l* of mixture of ethanol and 16 % Tween 80 (1:9); while neither severe haemolysis nor fatality occured in the tocopherol-supplemented control group.

After the definite period of feeding, ranging from five to eleven weeks, rats were killed by decapitation and tissue was taken for the preparation of liver[10] and heart mitochondria[4], and liver digitonine particles[12]. Details of this were presented in our previous report[13].

Oxygen consumption was measured by the conventional WARBURG method. The reaction mixture for liver mitochondria and digitonine particles contained the following substances in μmoles: 65 Tris (pH 7.4), 25 phosphate (pH 7.4), 0.03 cytochrome c (Sigma), 3 ATP, 0.2 NAD (Sigma), 15 NaF, 30 $MgCl_2$ and 25 glucose and 20–24 KM units of hexokinase (Sigma, type 3). Two hundred mg fresh liver equivalent mitochondria or 5 mg protein containing digitonine particles were added.

0.25 M sucrose was added too, to make a final volume of 3.0 m*l*. For heart mitochondria the following constituents in μmoles, in a final volume of 3.0 m*l*, were used; 40 Tris (pH 7.4), 25 phosphate (pH 7.4), 50 KCl, 3 ATP, 45 NaF, 30 $MgCl_2$ and 25 glucose and 25 KM units hexokinase. Three to 5 mg protein equivalent mitochondria in 0.4 M sucrose, containing 5×10^{-7} M EDTA, were used. In each experiment, 50 μmoles of succinate, β-hydroxybutyrate or glutamate or 30 μmoles of α-ketoglutarate and 10 μmoles of malonate were added as a substrate.

After 7 minutes at 30° of shaking, glucose and hexokinase were tipped from a side arm of the vessel and oxygen uptake was measured during 30–37 minutes at 5 minute intervals.

The reaction was stopped by adding 5 % trichloracetic acid and aliquotes were taken for analysis of inorganic phosphate by the method of LOWRY[14].

The amount of oxygen consumed during 30 minutes was expressed as μatoms oxygen per mg protein. The latter was measured by using FOLIN's phenol reagent[15].

Thiobarbituric acid (TBA)-reactants formed were measured by the method of

CORWIN[16] using fresh mitochondrial suspension in 0.25 M sucrose, and values were expressed as optical density at 532 mμ per mg mitochondrial protein.

RESULTS AND DISCUSSION

Table 1 shows the observed oxygen uptake in 30 minutes and P/O ratios by fresh mitochondria from vitamin E-deficient and from vitamin E-supplemented rats after nine weeks of feeding.

Table 1. Oxidative phosphorylation by liver mitochondria from vitamin E-deficient and -supplemented rats (Experiment 1; Nine weeks of feeding on deficiency)

Supplemented*1			Deficient		
No. of rats	Oxygen uptake $\frac{\mu atoms}{mg\ protein}$	P/O	No. of rats	Oxygen uptake $\frac{\mu atoms}{mg\ protein}$	P/O
16	*Succinate* 2.34	1.68±0.48*2	13	*Succinate* 2.05	1.54±0.36*2
14	*β-Hydroxybutyrate* 1.53	2.61±0.63	14	*β-Hydroxybutyrate* 1.47	2.50±0.44
10	*Glutamate* 2.01	2.63±0.47	8	*Glutamate* 1.87	2.52±0.30
No. of rats	Creatine/Creatinine		No. of rats	Creatine/Creatinine	
4	0.22±0.03		4	0.77±0.18	
Toxicity of MLHP *3					
No. of rats	No. of death		No. of rats	No. of death	
6	0		8	8	

*1, Two mg dl-α-tocopherol acetate was orally administered once a week.
*2, Average and its standard error.
*3, See in the text.

The values from succinate, β-hydroxybutyrate or glutamate did not change by vitamin E-deficiency; even the symptoms were able to be known by the development of creatinuria and the aforementioned fatal toxicity of MLHP, which had been injected into the tail vain.

Similar results in oxidative phosphorylation are presented in Table 2, which shows values from both liver and heart mitochondria and from liver digitonine particles isolated from the rats fed for eleven weeks. In all the trials, measured P/O ratio was not altered in the deficient groups when those substances were used, whereas the addition of α-ketoglutarate together with malonate seemed to cause a certain decline in oxygen uptake, but not in P/O ratio, during 30 minutes of the reaction.

Table 2. Oxidative phosphorylation by liver mitochondria and digitonine particles and by heart mitochondria (Experiment 2; Eleven weeks of feeding on deficiency)

	Supplemented*1			Deficient		
	No. of rats	O_2uptake $\frac{\mu atoms}{mg\ protein}$	P/O	No. of rats	O_2uptake $\frac{\mu atoms}{mg\ protein}$	P/O
Liver mitochondria		*Succinate*			*Succinate*	
	5	2.59±0.35	1.72±0.25	5	2.20±0.31	1.36±0.68
		β-Hydroxybutyrate			*β-Hydroxybutyrate*	
	4	0.92±0.18	2.62±0.31	4	0.97±0.21	2.62±0.35
		α-KG+Malonate			*α-KG+Malonate*	
	5	0.98±0.29*2	2.83±0.29	4	0.59±0.19*2	2.81±0.22
Digitonine particles	No. of trials*3			No. of trials*3		
		β-Hydroxybutyrate			*β-Hydroxybutyrate*	
	4	0.93±0.12	2.10±0.20	4	0.84±0.25	1.76±0.31
Heart mitochondria	No. of trials*3			No. of trials*3		
		Succinate			*Succinate*	
	4	1.14±0.43	1.47±0.45	4	1.38±0.72	1.32±0.26

*1, dl-tocopherol was intraperitoneally injected once a week (two mg in 1.0 m/ of 1:9 ethanol-16% Tween 80).

*2, Values are significant ($P<0.3$).

*3, Figures represent the number of trials, each comprized pooled tissues from 10 rats for the preparation of digitonine particles and 5 for that of heart mitochondria.

Table 3. Respiratory decline with α-ketoglutarate accompanying lipid peroxidation of mitochondria from rats on vitamin E-deficiency (Experiment 3: Five weeks of feeding on deficiency)

	Supplemented*1			Deficient		
	No. of rats	O_2uptake $\frac{\mu atoms}{mg\ protein}$	P/O	No. of rats	O_2uptake $\frac{\mu atoms}{mg\ protein}$	P/O
Liver mitochondria		*β-Hydroxybutyrate*			*β-Hydroxybutyrate*	
	4	1.38±0.09	1.96±0.35	4	1.59±0.21	2.34±0.24
		α-KG+Malonate			*α-KG+Malonate*	
	11	1.06±0.23*2	3.18±0.47	11	0.58±0.19*2	3.92±0.82
		TBA-reactants (O. D. 532 mμ/mg protein)				
Liver mitochondria	10	0.0066±0.0032*3		10	0.0362±0.0156*3	
Muscle mitochondria	3	0.0240±0.0070		3	0.0686±0.0083	

*1, Two mg of dl-tocopherol was orally fed once a week.

*2, $P<0.01$

*3, $P<0.02$

These phenomena were reexamined in experiment 3, which showed a highly significant decrease in oxygen uptake, along with the development of lipid peroxidation even in the earlier period of the deficiency.

The respiration decline depending upon α-ketoglutarate is also shown in Fig. 1.

From these results, it can be assumed that vitamin E-deficiency may not neces-

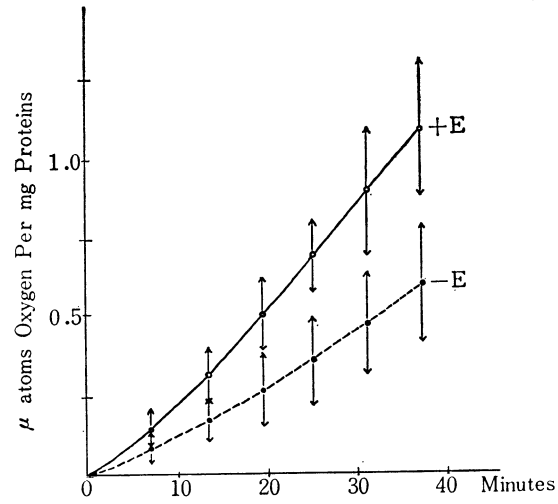

Fig. 1. Comparison of α-ketoglutarate oxidation by liver mitochondria in the presence of molonate from rats fed a vitamin E-deficient diet with those from a vitamin E-supplemented diet. (Experiment 3) The arrows indicate the standard errors.

sary cause any observable changes in oxidative phosphorylation, even though several signs of the deficiency could be detected by creatinuria, lipid peroxidation as well as toxic effect of MLHP.

ZALKIN and TAPPEL[4] suggested in their experiments using vitamin E-deficient rabbits that lipid peroxidation may possibly be involved in the mechanism of damaged oxidative phosphorylation, since TBA-reactans in isolated liver mitochondria were significantly increased, although these differences between deficient and supplemented groups were not significant in muscle mitochondria.

It was found in our previous studies[13] that the addition of MLHP to the incubation media containing normal rat mitochondria caused uncoupled oxidative phosphorylation which could be prevented by the simultaneous inclusion of α-tocopherol or of some antioxidants.

It was also found, in our unpublished data, that MLHP lowered ATP-Pi exchanges and increased ATP-ase activity.

These results may suggest that vitamin E-deficiency produces hydroperoxide free-radicals in lipid constituents of mitochondria and directly attacks enzyme sites responsible for oxidative phosphorylation. Therefore, lipid peroxide formed during vitamin E-deficiency as could be seen by increasing TBA-reactants is a probable

uncoupler in the respiratory chain, although attempts to observe consistent effect of intravenously injected MLHP on mitochondrial respiration have failed, because this substance seemed not to reach into the respiration sites but was merely trapped within blood stream to produce severe haemolysis.

Respiratory decline, specifically related with α-ketoglutarate, seems to be one of the characteristics of vitamin E-deficiency as is described by CORWIN[16]. By his assumption, this decline was primarily due to the accumulation of oxaloacetate in the presence of NAD, which can be reversed by α-tocopherol, and secondarily to an inhibition at the site of lipoyl dehydrogenase in the system of α-ketoglutarate dehydrogenase.

However, the system used in our studies was something different from that of CORWIN, since the mitochondrial preparation which had been isolated from non-necrotic vitamin E-deficient rat liver did not significantly decrease succinate oxidation in the presence of NAD, although ZALKIN and TAPPEL observed some significant damages in succino oxidase system in the rabbit[4]. On the other hand, relative intactness of succinate oxidation is supported by our previous studies using MLHP as a potent *in vitro* uncoupler[13], which revealed relatively less stability in the respiratory activity in NADH dependent substrate systems than that of succinate.

Moreover, MLHP inhibited preferentially phosphorylation rather than oxidation, in the case using α-ketoglutarate.

Therefore, changes in α-ketoglutarate oxidation observed in our present results are to be explained by other mechanisms than those proposed by ZALKIN and TAPPEL[4] using dystrophic rabbit, by CORWIN[16] using prenecrotic rat liver and by our previous *in vitro* lipid peroxidation studies[13].

Further studies are necessary to allow definite conclusion to be drawn about the possible role of vitamin E and lipid peroxidation on α-ketoglutarate oxidation and oxidative phosphorylation.

REFERENCES

1) BOYER, P.D.: The Enzymes, **3**, 353 (BOYER, P. D., H. LARDY and K. MYRBÄCK ed.), Academic Press, N. Y. (1960).
2) MARZIUS, C.: Proc. 3rd. Intern. Congr. Biochem. Brussels, **1**, (1955).
3) MC CAY, P., and R. CAPUTTO: Fed. Proc., **15**, 311 (1956).
4) ZARKIN, H., and A. L. TAPPEL: Arch. Biochem. Biophys., **88**, 113 (1960).
5) SCHWARZ, K.: Vitamins and Hormones, **20**, 463 (1962).
6) DRAPER, H. H., and B. C. JOHNSON: J. Nutrition, **65**, 161 (1958).
7) MAMEECH, M. S. and B. C. JOHNSON: J. Agric. Food Chem., **6**, 920 (1958).
8) DRAPER, H. H., S. GOODYEAR, K. D. BAABEE and B. C. JOHNSON: Proc. Soc. Expt. Biol. Med., **93**, 186 (1956).
9) KOKATNUR, M. G., J. C. BERGAN and H. H. DRAPER: Anal. Biochem., **12**, 325 (1965).
10) SCHNEIDER, W. C., and G. H. HOGEBOOM: J. Biol. chem., **183**, 123 (1950).
11) AZZONE, G. F., and F. CARAFOLI: Exptl. Cell Res., **21**, 456 (1960).
12) WADKINS, C.L., and A.L. LEHNINGER: in Method in Enzymol., **6**, 265 (COLOWICK, S.P., and N. O. KAPLAN ed.,) Academic Press, N. Y., (1962).

13) NAITO, H., B. J. JOHNSON and B. C. JOHNSON: Proc. Soc. Exptl. Biol. Med., **122**, 545 (1966).
14) LOWRY, O. H., and J. A. LOPEZ: J. Biol. Chem., **162**, 421, (1946).
15) LOWRY, O. H., N. J. ROSENBOUGH, A. L. FARR and R. J. RANDALL: J. Biol. Chem., **193**, 265 (1951).
16) CORWIN, L. M.: Arch. Biochem. Biophys., **97**, 51 (1962).

摘　　要

ラットを離乳直後からビタミンE欠乏食で5-11週間飼育し，欠乏の徴候発現を確認したのち肝ミトコンドリアおよびそのジギトニン標品また心筋ミトコンドリアを調製し，数種のトリカルボン酸を基質として酸化的リン酸化を測定した結果，E欠乏によってとくに変化が認められなかった。しかしE欠乏ミトコンドリアではα-ケトグルタル酸の酸化能が減退した。