CHAPTER 7

OXIDATION OF FATTY ACIDS BY PIGEON BREAST MUSCLE MITOCHONDRIA WITH TRIGLYCERIDES AS SUBSTRATE

Fatty acids are oxidized exclusively within one site in animal cells, the mitochondria (Schneider and Potter, 1949; Lehninger and Kennedy, 1949). In the preceding chapter was discussed the possibility of lipase playing a part in fat metabolism by splitting up the fat into fatty acids and glycerol before oxidation, because it is believed though not proved that this is the first step in the breakdown (Baldwin, 1954) and the final step in the synthesis of fat. It has been shown that during long and sustained muscular activity, the pigeon breast muscle utilizes fat as the chief fuel (George and Jyoti, 1958) . The lipids present in the muscle are chiefly in the form of glycerides, phospholipids and cholesterol (Deuel, 1955) and the fatty acids are mainly long chain fatty acids. It was shown by quantitative and histochemical methods that this muscle contains a lipase. The muscle lipase hydrolyses glycerides of short chain fatty acids more readily than those of long chain fatty acids. The pancreatic lipase also behaves in a like manner, but glycerides of long chain fatty acids are hydrolysed at a comparatively faster rate than by the muscle enzyme. Further the behaviour of these two enzymes towards inhibitors and activators are different in certain respects and it has been suggested that each of these enzymes

may be suited for the particular physiological environment in which it acts. Inorder to test this hypothesis it was decided to measure the relative amount of oxygen uptake in a manometric system having mitochondria and other cofactors, when triglycerides of short and long chain fatty acids and salts of the respective fatty acids are provided as substrates, with the assumption that any oxygen uptake in the flask containing triglycerides would indicate oxidation of fatty acids enzymically split from the glycerol moiety. The results obtained ,however, though not conclusive, gives a logical explanation for the difference in the beh - aviour of the muscle and the pancreatic lipase. Since the enzymes responsible for the oxidation of fatty acids are contained in the mitochondria, it was decided also to assay the lipolytic activity, if any, in the mitochondria. Concurrently some experiments were also done to find out the role of carbohydrates and intermediates of the tri - carboxylic acid cycle in the oxidation of glycerides, because this at present is a matter of great controversy.

According to Scholeffeld (1957),fatty acids inhibit the oxidation of pyruvate by rat kidney mitochondria. The long chain fatty acids and among them the even numbered ones are better inhibitors. Lassow and Chaikoff (1955) reported that carbohydrates spare long chain fatty acid oxidation. Knox et al (1948) and Green (1954,'55) believe that carbohydrates promote fatty acid oxidation by giving rise to ATP for activating the fatty acid and by providing the condensing partner, viz. oxalacetate, for active acetate formed during oxidation, a phemomenon called "sparking".

Recently Masoro and Felts (1958) supported the contention of Lassow and Chaikoff and further observed that for an optimal oxidation of fatty acids a phase of carbohydrate metabolism is essential and this effect does not involve the "sparking" action of the Krebs cycle intermediates. In this context my observations on the oxidation of fat are interesting.

Experimental

Isolation of mitochondria: Chappel and Perry (1953) isolated pigeon breast muscle mitochondria from homogenates in 8.5% sucrose. I followed the method of these authors with certain modifications. About 90 ml. of an 8.5% sucrose solution was chilled in a Waring blendor & surrounded it with a polyethylene bag, in a jacket, containing crushed ice, acetone and common salt. 10 gm. of the fresh muscle was introduced into it and blendorised for 3 min . At the end of this period the temperature of the homogenate was found to be about 5°C . To this was added 200 ml. chilled sucrose solution and thoroughly mixed for a few seconds and kept in ice bath till centrifugation was over. The homogenate was centrifuged first at 600 g. in a Servall refrigerated angle centrifuge for 30 min. The residue was discarded. The supernatant was again centrifuged at 3500 g. for 10 min. The sediment was resuspended in 10 ml. sucrose. Microscopic examination after staining with Janus Green B showed that the sample was a pure preparation

of mitochondria free from myofibrils. This mitochondrial suspension which contained approximately 10 mg. protein per ml. was used in the following study.

Lipase activity was determined by the manometric method described in the earlier chapters. Each flask contained 1.5 ml. 0.025 M $NaHCO_3$ and 1 ml. mitochondrial suspension in the main chamber and 0.5 ml. 4% tributyrin in 0.0148 M $NaHCO_3$, emulsified with "Tween 80", in the side arm. The gas phase contained a mixture of 95% N_2 and 5% CO_2. The whole system had a pH of 7.4 at 37°C. The substrate was tipped in after equilibration for 10 min. Sucrose is without effect on lipolytic activity.

Fatty acid oxidation was determined by the oxygen uptake in a manometric system. The experiments were performed at 37°C . The gas phase contained air. Each flask contained 4μM of $MgCl_2$, 4μM ATP, 1μM DPN, 0.5μM CoA , 0.5μM cytochrome c, 0.4 ml. 0.15 M phosphate buffer of pH 7.4, 15μM of sodium malate and 1 ml. of the mitochondrial suspension in the main chamber, 0.5 ml. of the substrate in the side arm and 0.2 ml. of 20% KOH and a roll of filter paper in the centre well. Total volume of the reaction mixture was 3.5 ml. Two triglycerides, tributyrin and triolein, and two fatty acids, butyric acid and oleic acid, were used as substrates. The triglycerides were emulsified in water with a small drop of "Tween 80"/20 ml. and the fatty acids were converted into the respective sodium salts by neutralising with NaOH. The 0.5 ml. of the substrate had a concentration of 20μM in the case of the triglycerides and 40μM in the

case of the fatty acid. The flasks were kept on ice slabs till they entered the water bath. The incubation was carried out for 2 hrs. The flasks and manometers were shaken at a speed of 100 oscillation /min. allowing an amplitude of about 4.5 cms. per oscillation. The substrate was tipped in after 5 min. of temperature equilibration, the levels adjusted and readings taken at intervals.

Results

The oxygen consumption by mitochondria in the different flasks is recorded in table 1.

Table 1

Oxidation of fatty acids by pigeon breast muscle mitochondria. QO_2, µl calculated per 10 mg. protein*

"Sparker"	Substrate				Blank, without substrate
	Butyrate	Oleate	Tributyrin	Triolein	
Malate	0.92	3.1	3.1	20.4	7.0
Lactate	2.45	1.2	-	21.8	8.1
Glycogen	1.80	3.26	1.0	25.6	3.6

* Average of three experiments

It can be seen that the oxygen consumption is highest in the flask containing triolein as substrate. A detailed analysis of the result obtained with triolein is given in table 2.

Table 2

Oxidation of triolein by pigeon breast muscle mitochondria

QO_2, l calculated per 10 mg. protein

'Sparker"	Blank, without fat (1)	Without sparker (2)	Complete system (3)	Corrected for blank (4)	Inhibition by sparker (5)	Due to sparker in the complete system (6)
Malate	7.0	27.5	27.4	20.4	7.1	nil
Lactate	8.1	27.5	29.9	21.8	5.7	2.4
Glycogen	3.6	27.5	29.1	25.5	2.0	1.6

Lipase activity of the mitochondria was found to be 14.6 μl CO_2/mg. protein/hr. on the average.

Discussion

As can be seen from table 1, the oxygen uptake is highest in the flask containing triolein as the substrate. This oxygen uptake is evidently due to the oxidation of fatty acid. The pH of the flask containing tributyrin was found to be about 5.8 at the end of the experiment, which suggests that being a short chain fatty acid , lipolytic activity is so rapid and the fall in pH is responsible for the nonoxidation of the fatty acid. On the other hand, the pH of the flask containing triolein was unchanged and since the oxygen consumption in the flask is very high, it is believed that oxidation of the long chain fatty acid takes place first and lipase comes in the picture only when the length of the carbon chain in the glyceride is reduced to 4or 2. This may be what actually takes place

in the tissue also. Such a mechanism avoids any large fluctuation inthe pH of the tissue, where active metabolism of fat is going on. The blocking of the hydrolysis of glycerides of long chain fatty acids might be a device brought about through necessity by the other cell constituents. This explains the reason why glycerides of long chain fatty acids are not so readily acted upon by the muscle lipase, as do the pancreatic lipase and supports the contention that each of these enzymes is particularly adapted for optimum activity in the physiological environment in which it acts.

The effect of glycogen and and the intermediates of carbohydrate metabolism on the oxidation of fatty acid is noteworthy. It appears that a"sparker" , as postulated by Knox et al and Green , is not necessary for the oxidation of fatty acid, because the addition of malate only inhibits the oxygen uptake (Table 2). The question arises as to how then the acetyl-CoA enters the Krebs cycle for further oxidation. The entry of the acetyl-CoA into the Krebs cycle may not be through citrate by condensing with oxalacetate. It is possible that the acetyl-CoA molecules condense by the reverse action of the succinate cleaving enzyme in the presence of the necessary cofactors, to form succinate and enter the Krebs cycle. The succinate cleaving enzyme has been shown to be present in skeletal muscles also(Seaman and Naschki, 1955). The inhibition of fatty acid oxidation as calculated by correcting for a blank containing only the "sparker" is highest when malate is added and least in the presence of glycogen. It may be

noted that Scholefield (1957) has reported inhibition of the oxidation of pyruvate by rat liver mitochondria. Longer chain fatty acids are more effective and even numbered are better inhibitors than odd numbered fatty acids. If it is assumed that the oxidation of the "sparker" is completely arrested in the experiments by fat, it can be seen that lactate and glycogen activates the oxidation of fat to a certain extent and that malate slightly inhibits and does not promote the oxidation of fat (Table 2). The inferences drawn are by no means conclusive and needs further experimental evidence. Nevertheless, considering all the available facts I venture to suggest that in tissues like the pigeon breast muscle which utilizes fat for energy, fat is oxidized in preference to carbohydrates as long as oxygen is available and when the supply of oxygen diminishes, anaerobiosis is resorted to and glycolysis takes place resulting in the production and accumilation of lactic acid which finally leads to fatigue.