CHAPTER 9

AN *IN VITRO* STUDY OF THE OXIDATION OF TRIGLYCERIDES BY THE PIGEON BREAST MUSCLE MITOCHONDRIA AND WHOLE HOMOGENATE

It is fairly well established that the flight muscles of birds indulging in long and sustained activity utilize fat as the major fuel for energy as already stated in the previous chapter. It is also known that fatty acids are metabolized by a series of extrahepatic tissues including the homogenized pigeon breast muscle (Weinhouse, Millington, and Volk, 1950), and slices of the skeletal muscles (Geyer, Matthews, and stare, 1950; Wertheimer and Ben-Tor, 1952). On the basis of the observations by Lehninger (1946) and by Chernick, Masoro, and Chaikoff (1950), Goldman *et al* (1950) postulate that the oxidation of higher fatty acids must be a normal occurrence in muscle. Since mitochondria are the principle locale for carrying out all of the reactions of fatty acid oxidation (Schneider, 1948; Kennedy and Lehninger, 1948 and 1949) the occurrence of lipase in these particles of the pigeon breast muscle may be attributed to the fact that this enzyme plays a significant role in splitting neutral fats into fatty acids and glycerol before β-oxidation starts (Chapter 8). The appreciable concentration of lipase in the mitochondrial fraction (Chapter 8) of the pigeon breast muscle homogenate , indirectly lends support to the fact that the neutral fats are not oxidized directly but they are first broken down to fatty acids and glycerol before β-oxidation comes into the picture. Thus, lipase could be expected to play a major role in

the first step of the breakdown of fat. It also appears possible that the concentration of this enzyme, in the flight muscles of birds, indulging in sustained flight, should be an index of the extent of the utilization of fat. This contention is supported by the fact that the presence of lipids in the muscles are chiefly in the form of glycerides, phospholipids and cholestrol (Deuel, 1957) and that animal fat usually contains only small amounts of free fatty acids while the newly synthesized fatty acids are immediately esterified with glycerol to triglycerides through the reversal of lipase action (Kleinzeller, 1948). Thus it appears that inspite of having considerable data on the oxidation of fatty acids by mitochondria, comparatively very little is known about the details of the process of the oxidation of triglycerides or neutral fats, which as a matter of fact should be a normal process in vivo not only in muscle but in all other tissues which are capable of utilizing fat. In the present investigation, therefore, an attempt has been made to find out whether or not and if so to what rate and extent the two triglycerides, tributyrin and triolein are oxidized in vitro by the pigeon breast muscle mitochondria as well as the whole homogenate.

Material and Methods

Mitochondria from the pigeon breast muscle homogenate were isolated by the conventional differential centrifugation methods using a refrigerated centrifuge (Chapter 8). The medium for the isolation of mitochondria used was 0.25 M sucrose solution. Microscopic examination after staining with Janus Green B showed that the sample was a pure preparation of

mitochondria free from myofibrils. The homogenate used in the study was also prepared in 0.25 M sucrose solution. Since a possible role of the Krebs cycle intermediates in supporting fatty acid oxidation is now apparent (Knox et al, 1948; Kennedy and Lehninger, 1952; Judah and Rees, 1953; Lardy and Wellman, 1953; Green, 1954 and 1955), malate was used as 'sparker' in the present study of the oxidation of triglycerides in vitro. Some preliminary experiments were also tried without using any 'sparker'.

The in vitro oxidation of triglycerides (tributyrin and triolein) was determined by the oxygen uptake in a manometric system. The experiments were performed at 37° C. The gas phase contained air. Each Warburg flask contained 0.6 ml. of 0.1 M phosphate buffer of pH 7.4, 0.15 ml. of 0.15 M $MgCl_2$, 0.2 ml. of 0.0225 M ATP, 0.15 ml. of 0.00045 M cytochrome c, 0.2 ml. of 0.015 M DPN, 0.2 ml. of 0.0075 M coenzyme A, 0.2 ml. of 0.15 M Na-malate, 0.8 ml. of mitochondrial suspension or the whole homogenate of the muscle and 0.5 ml. of tributyrin or triolein in different concentrations. Thus the total volume of the reaction mixture was 3.0 ml. This gave a final concentration of 20 µM of phosphate buffer, 7.5 µM of $MgCl_2$, 1.5 µM of ATP, 0.0225 µM of cytochrome c, 1 µM of DPN, 0.5 µM of coenzyme A and 10 µM of Na-malate per flask. The final concentrations of the tributyrin used were 1 µM and 5 µM whereas the triolein 1 µM. The centre well contained 0.2 ml. of 20 % KOH and a roll of filter paper. This incubation mixture which contained all the necessary cofactors, a sparker,

a particular triglyceride under study and mitochondria or whole homogenate as the case may be, hitherto will be referred to aa the 'complete system'. The triglycerides were emulsified in water in a waring blendor for nearly 30 seconds. The flasks were kept on ice slab till they were transferred to the water bath. The flasks and the manometers were shaken at a speed of 100 oscillations per minute allowing an amplitude of about 4.5 cm. per oscillation. After an equilibration period of 10 minutes, the levels were adjusted and the readings taken at an interval of 15 minutes for 1 hour.

The lipase activity of the mitochondrial suspension as well as the whole homogenate was determined manometrically by the method adopted from Martin and Peers (1953) in a bicarbonate carbon dioxide buffer system of pH 7.4 at 37° C. using the Warburg apparatus as mentioned in chapter 4. The protein contents of the mitochondrial suspension as well as the homogenate used for oxidation were determined by the micro-Kjeldahl steam distillation method (Hawk et al, 1954).

Results and Discussion

The results obtained for the oxidation of the triglycerides (tributyrin and triolein) at different concentrations used, by the mitochondria as well as the whole homogenate of the pigeon breast muscle are summarized in Table 1. These results clearly indicate that both the triglycerides used at different concentrations under the conditions of the experiment, were not oxidized in vitro but on the contrary inhibited malate oxidation to a certain extent as indicated in

Table 2. The lipase activity of the mitochondria used for the study was found to vary from 36 to 59 µl CO_2/mg. protein per hour whereas the protein content of the mitochondrial suspension used for the oxidation was approximately 1.5 to 2 mg. per flask.

Table 1

Oxidation of triglycerides (tributyrin and triolein) by pigeon breast muscle mitochondria and whole homogenate..The 'Complete system' as mentioned in the text contains all the necessary cofactors, sparker, fat and the enzyme preparation either in the form of mitochondria or homogenate.

Expt.	Final concentrations of the triglycerides used	µl O_2 uptake/hour			
		Complete system	No fat	No sparker	No fat no sparker
1	Tributyrin 5 µM	12.37	29.43	1.95	5.0
2	,, 1 µM	11.68	17.98	-	-
3	Triolein 1 µM	12.59	29.43	3.43	5.0
4	,, 1 µM	10.30	17.98	-	-
5*	Tributyrin 1 µM	20.02	21.25	-	-
6*	Triolein 1 µM	10.30	21.25	-	-

* Oxidation due to whole homogenate

As mentioned earlier <u>in vivo</u> the oxidation of neutral fats must be a normal process in the muscle under study since the mitochondrial fraction of this muscle contains an appreciable concentration of lipase (Chapter 8) which should play a

significant role in splitting the fat into fatty acids and glycerol. This indirectly lends support to the view that the triglycerides cannot be oxidized directly but they are to be first hydrolyzed to fatty acids and glycerol prior to β-oxidation. It seems therefore that fatty acid esters and phospholipids are oxidized *in vivo* though there is no evidence that they are oxidized as such which is most unlikely since the enzyme preparations are rich in estrase activity.

Table 2

Showing the percentage of inhibition of the malate oxidation by the various concentrations of the triglycerides used

Final concentration of the triglycerides used for oxidation	Inhibition of the oxidation of malate with different preparations of the muscle	
	Mitochondria	Whole homogenate
Tributyrin 1 µM	45 %	Nil
,, 5 µM	58 %	-
Triolein 1 µM	65 %	50 %

The main reason for the failure in demonstrating the oxidation of triglycerides by mitochondria or the whole homogenate, *in vitro* may perhaps be explained on the assumption that the free fatty acids in the system under study are liberated in such a way that it is difficult to maintain the pH of the incubation medium under the conditions of the experiment. It was interesting to note that the tributyrin

at a final concentration of 1 μM, however, did not inhibit malate oxidation when the whole homogenate was used (Table 2) which perhaps indicates that the inclusion of some additional factors might be necessary in the incubation mixture in order to demonstrate the oxidation of triglycerides in vitro. This points to the suggestion that the factor responsible for the demonstration of the oxidation of tributyrin in vitro though may be present in the homogenate, for any net oxidation in vitro the addition of this factor is essential. The oxidation of triglycerides in vitro could not be achieved by either the pigeon breast muscle mitochondria or the whole muscle homogenate under the conditions of the method employed. Nevertheless, the possibility of the oxidation of neutral fats through β-oxidation after hydrolysis could be considered as a normal process in vivo since there is no evidence that they are oxidized directly.