CHAPTER 11

LIPASE AND SUCCINIC DEHYDROGENASE ACTIVITY OF THE PARTICULATE FRACTIONS OF BREAST MUSCIE HOMOGENATE OF THE MIGRATORY STARLING STURNUS ROSEUS IN THE PRE-MIGRATORY AND POST-MIGRATORY PERIODS

Considerable evidence has accumulated in the recent years in support of the view that fat forms the chief fuel for energy during long and sustained muscular activity (Weis-Fogh, 1952; George and Jyoti, 1953; 1955; 1957; Bing, 1956). It has also been shown that migratory birds store up fat in the body prior to migration (Odum and Perkinson, 1951; Odum and Connell, 1956; Farner, 1955; MaGreal and Farner, 1956; Merkel, 1958) and that fat is reduced during migration (Williamson, 1952; 1955). The pectoralis muscle of flying birds and other highly active muscles such as the breast muscle of the bat and the flight muscles of insects and the vertebrate heart muscle have been shown to contain a high concentration of a fat splitting enzyme lipase (George and Scaria, 1956; 1957; George, Susheela and Scaria, 1958; George, Vallyathan and Scaria, 1958; George and Bhakthan, 1960; George and Iype, 1959). The presence of high concentrations of lipase in these muscles is therefore of special significance.

The breast muscle of flying birds also contain high concentrations of the oxidative enzyme succinic dehydrogenase (SDH) (George and Talesara, 1961a). The higher SDH activity (George and Talesara, 1961a) and an increased storage of fat in the liver (Naik, 1963) and the breast muscle (Chapter 8)

have been noted in the pre-migratory period of the Rosy pastor. Recent studies of George and Talesara (1962a) and George and Iype (1963a) on the lipase activity of the particulate fractions of the pigeon breast muscle and the pigeon heart have indicated the importance of lipase and its possible role in the breakdown and the synthesis of neutral fats. Their studies also suggest that neutral fats are not oxidized directly under in vitro conditions but only after hydrolysis by the lipase in the muscle. Though the presence and the significance of lipase in the flight muscles of birds is realized, the exact localization and distribution of this enzyme in the subcellular fractions has not been so far attempted in any migratory bird.

The present study was therefore undertaken to explore the part played by lipase and succinic dehydrogenase in different cellular particulates in the degradation and utilization of fat and in the synthesis of fat from fatty acids. These studies were extended to post-migratory (December) and pre-migratory (April) periods.

Material and Methods

Rosy pastors were collected in the post-migratory and the pre-migratory periods. Birds were shot with an air rifle and collected before dawn (4 to 4.30 A.M.) and in the evenings between 6 to 7 P.M. A piece of the pectoralis muscle was quickly excised and blotted free of blood. In every case the bird was brought to the laboratory within 10 minutes after it was shot. The tissue was weighed, washed quickly with ice cold 0.25 M

sucrose solution and homogenized in a chilled mortar with ice cold isotonic sucrose solution. A 10% homogenate was prepared and a portion of this homogenate was preserved at 4°C for the estimation of lipase and SDH activities till the various fractions were isolated from a known volume of the original homogenate. The homogenate made in sucrose solution was used as the starting material for fractionation.

0.25 M sucrose was used throughout eventhough it is reported that sucrose might favour gel formation. In our experience no gel formation was noticed in the sucrose medium and it was also found to be most suitable since it did not affect the activity of these enzymes under investigation.

Particulate fractions of the muscle homogenate were isolated by the conventional differential centrifugation method adopted from the procedures of Hogeboom, Schneider and Palade (1948); Schneider (1948); Schneider and Hogeboom (1956); Chappel and Perry (1953) and Harman and Osborne (1953). Homogenate prepared in isotonic sucrose solution has been found most suitable by these investigators also. All procedures after the muscle was taken from the animal were carried out at temperature between 0 to 4°C. A known aliquot of the original homogenate (10 ml.) was pipeted into centrifuge tubes and centrifuged for 5 minutes at 600 x g. in a 'MSE Super Speed 25' refrigerated centrifuge in order to sediment the myofibrils, nuclei and cell debris. The viscous supernatant phase was poured off and saved for further fractionation. The residue was homogenized

again in a chilled mortar for 2 minutes, resuspended in isotonic sucrose and centrifuged as before for another 5 minutes. The supernatant was mixed with the first supernatant. The residue was again resuspended in a small volume of sucrose and centrifuged as before. The supernatant was mixed with the first and second supernatant and the residue was suspended in a small volume of sucrose and labelled as the myofibrillar fraction and stored at 4°C. The combined supernatant was again centrifuged for 10 minutes at 600 x g. The residue obtained was mixed with the myofibrillar fraction. This myofibrillar fraction consisted mainly of myofibrils and cell debris, but some mitochondria and microsomes might have also been present.

Mitochondria were isolated from the combined supernatant by centrifuging at 7500 x g. for 15 minutes. The translucent supernatant was decanted off and saved for the isolation of microsomes. The residue was resuspended in sucrose with a glass rod and spun again at 7500 x g. for 15 minutes. The sediment was suspended in a small volume of sucrose and labelled as mitochondrial fraction and was stored at 4°C. This fraction was microscopically examined every time to find out the purity of mitochondria free from myofibrils. Cytological examination after staining it with Janus Green B also revealed that this fraction contained only undamaged mitochondria.

The combined supernatant saved for the separation of microsomes was spun at 40,000 x g. for 1 hour. The reddish brown jelly like residue obtained was suspended in a little

volume of sucrose after decanting off the supernatant into a glass stoppered measuring cylinder. This fraction was labelled as the soluble fraction and the jelly like residue was labelled as the microsomal fraction. The microsomal fraction was also examined microscopically to ensure that it was free from mitochondria.

All the different fractions obtained were diluted with ice cold sucrose solution in such a way as to bring all the dilutions equal except that of the supernatant. The contents were then thoroughly mixed by shaking. The lipase and SDH activities of the original homogenate as well as the various fractions were determined simultaneously.

The lipolytic activity was determined manometrically by the method of Martin and Peers (1953) as adapted by George, Vallyathan and Scaria (1958) using a bicarbonate carbon dioxide buffer system of pH 7.4 at 37^{o}C. 4% tributyrin (v/v) emulsified in 0.0148 M bicarbonate with a drop of 'Tween 80' was used as the substrate. The rest of the experimental procedures were same as described in Chapter 1. Lipase activity is expressed as µl CO_2 evolved per mg. protein per hour at 37^{o}C.

Succinic dehydrogenase activity was assayed according to the method of Kun and Abood (1949) using triphenyl tetrazolium chloride (TTC) as the hydrogen acceptor. The experimental procedure was same as described in Chapter 2. The enzyme activity is expressed as µg formazan formed per mg. protein per hour at 37^{o}C under aerobic conditions. Protein was estimated in

duplicate samples according to the micro-Kjeldahl steam distillation method (Hawk et al, 1954).

Results

The per centage recovery and the specific activities of lipase and SDH respectively of the original homogenate as well as the various fractions of the muscle in the post-migratory and pre-migratory periods are given in Tables 1 to 4. In the post-migratory period (December) the per centage recovery of lipase was found to be more than that of SDH whereas in the pre-migratory period (April) the per centage recovery of lipase was lower than that of SDH while SDH recovery was the same as it was in the post-migratory period. A distinct difference in the activity of both enzymes in all the fractions was observed in the two periods studied. The soluble fraction contained the highest concentration of lipase in the post-migratory period whereas in the pre-migratory period the microsomal fraction contained the highest concentration of lipase. In the post-migratory period lipase activity of the mitochondrial fraction was higher than that in the pre-migratory period. The myofibrillar fraction also showed a slight difference in lipase activity, it being lower in the pre-migratory period.

There was no marked difference in the recovery of SDH activity in the two periods. In both cases the recovery of this enzyme was found to be 80 to 90%. However, a considerable difference in SDH activity was observed in the mitochondrial and microsomal fractions. In both periods mitochondrial fraction

showed considerably higher SDH activity. In the pre-migratory period there was higher level of SDH activity in the microsomal fraction. The mitochondrial fraction also showed an increase in the enzyme concentration in the pre-migratory period. The muscle homogenate too showed a remarkable increase in SDH activity prior to migration.

Figures 1 and 2 gives a graphic representation of the diurnal changes in the lipase and SDH activities of the various subcellular fractions during the post- and pre-migratory periods. Significant differences in the activities of the two enzymes in all the fractions as well as the whole homogenate of the muscle, were also noticed between the results obtained in experiments done early morning and late evening. During the post-migratory period lipase and SDH activity of the homogenate in the evening was higher than that in the morning, whereas in the pre-migratory period it was more in the morning. In both periods enzyme activity of the myofibrillar fraction was high in the evening except for lipase in the pre-migratory period. In the case of mitochondrial fraction higher enzyme level was obtained in the evening only in the post-migratory phase. The microsomal lipase activity was very high in the evening in the post-migratory period, but during the pre-migratory period it was low in the evening and high in the morning. The SDH activity of the microsomes was high in the evening for the post-migratory period and low in the evening for the pre-migratory period. Most striking differences were seen in the

lipase activity of the soluble fraction. In both the periods it was high in the evenings and a considerable decrease was noted in the pre-migratory period both in the evening as well as morning.

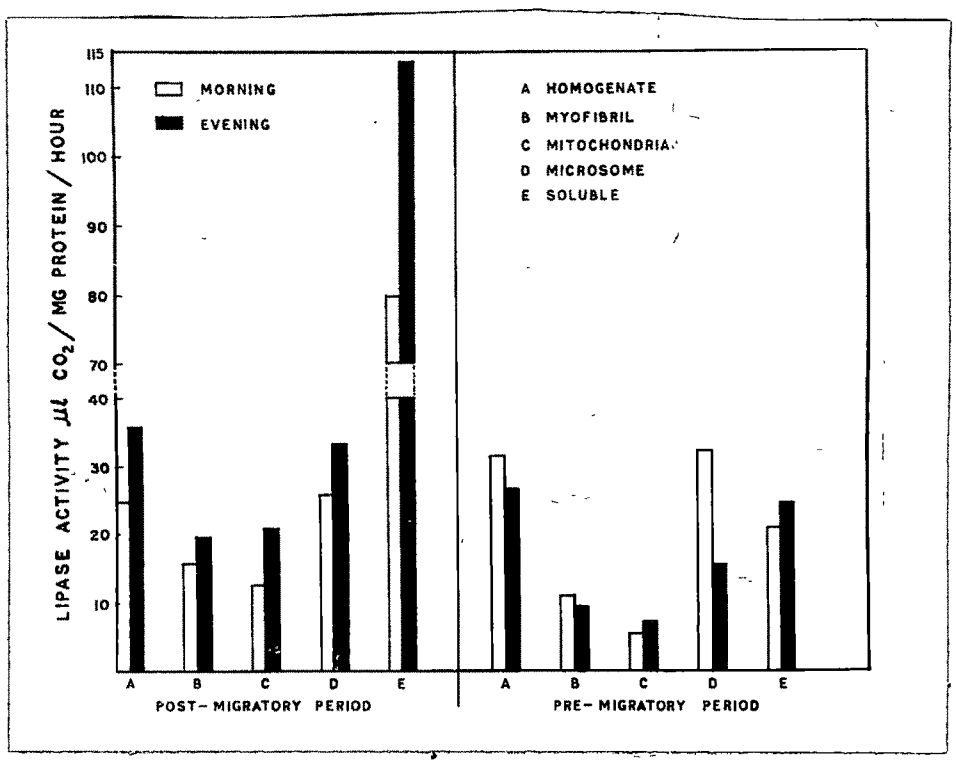

Figure 1. Showing the graphic representation of the seasonal and diurnal changes in the lipase activity of the various subcellular particulates of the breast muscle homogenate of the Rosy pasbor during the post-migratpry and pre-migratory periods.

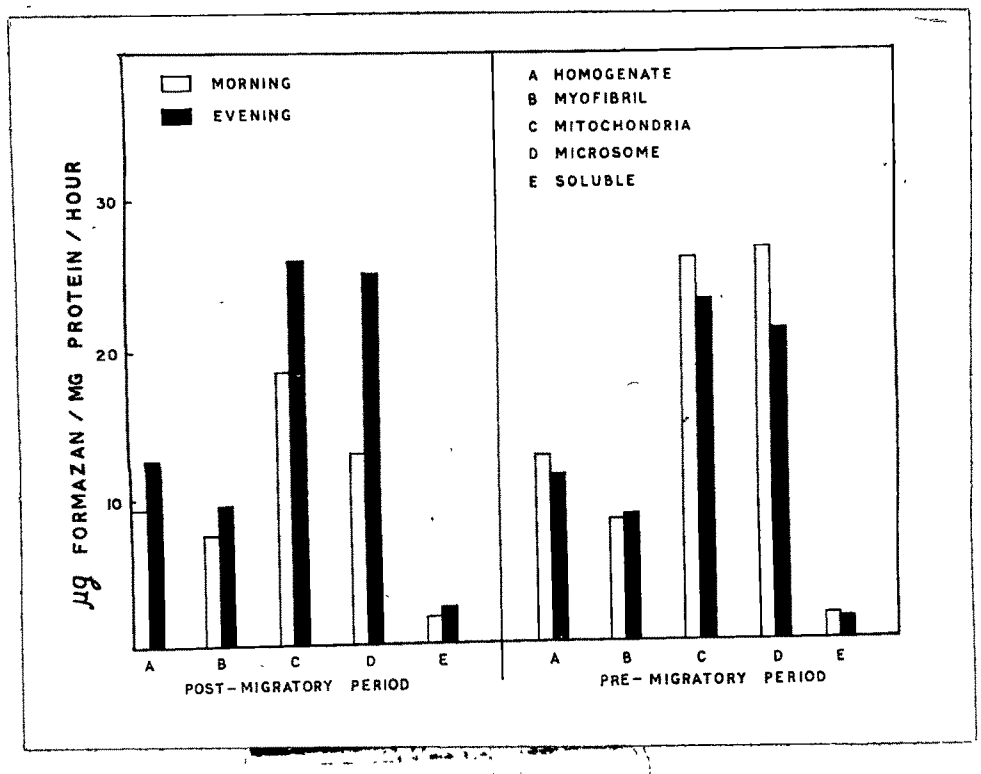

Figure 2. Showing the graphic representation of the seasonal and diurnal changes in the succinic dehydrogenase activity of the various subcellular particulates of the breast muscle homogenate of the Rosy pastor during the post- and pre-migratory periods.

Enzyme activity of the myofibrillar fraction also showed some fluctuations which might be due to the presence of some mitochondria and few microsomes adhering without being isolated. A pure myofibrillar fraction was not attempted in

Table 1

The per centage recovery and the specific activities of lipase and SDH activity in the original homogenate and the various particulate fractions of the breast muscle of Rosy pastor in the morning of the post-migratory period.

Period & Time	Fraction	Lipase activity µl CO_2/ mg. protein/ hour. Mean	S. D.	Recovery %	SDH activity µg formazan/ mg. protein/hour. Mean	S. D.	Recovery %
Post-migratory period (December) Morning	Homogenate	24.96	± 2.328	94.69	9.175	± 0.7795	90.53
	Myofibrils (600 x g.)	16.01	± 3.075		7.520	± 1.6877	
	Mitochondria (7,500 x g.)	12.61	± 1.781		18.322	± 5.5199	
	Microsomes (40,000 x g.)	25.81	± 4.472		12.848	± 3.5355	
	Soluble (40,000 x g.)	79.66	± 8.465		1.645	± 0.6531	

The figures indicate the average values of 5 sets of experiments.

Table 2

The per centage recovery and the specific activities of lipase and SDH in the original homogenate and the various particulate fractions of the breast muscle of Rosy pastor in the evening of the post-migratory period.

Period & Time	Fraction	Lipase activity µl CO_2/ mg.protein/ hour. Mean	S. D.	Recovery %	SDH activity µg. formazan/ mg. protein/ hour. Mean	S. D.	Recovery %
Post-migratory period (December) Evening	Homogenate	35.912	± 5.036	82.32	12.471	± 1.550	75.50
	Myofibrils (600 x g.)	20.170	± 0.393		9.450	± 1.582	
	Mitochondria (7,500 x g.)	20.897	± 5.351		25.930	± 6.264	
	Microsomes (40,000 x g.)	30.750	± 7.013		24.754	± 4.382	
	Soluble (40.000 x g.)	113.783	± 11.32		2.391	± 1.380	

Figures indicate the average values of 5 sets of experiments.

Table 3

The per centage recovery and the specific activities of lipase and SDH in the original homogenate and the various particulate fractions of the breast muscle of Rosy pastor in the morning of the pre-migratory period.

Period & Time	Fraction	Lipase activity µl CO_2/ mg. protein/ hour		Recovery %	SDH activity µg. formazan/ mg. protein/ hour		Recovery %
		Mean	S. D.		Mean	S. D.	
Pre-migratory period (April) Morning	Homogenate	31.494	± 6.272	56.34	12.510	± 1.470	81.530
	Myofibrils (600 x g.)	11.195	± 3.819		8.160	± 1.943	
	Mitochondria (7,500 x g.)	5.675	± 1.988		25.605	± 1.337	
	Microsomes (40,000 x g.)	32.156	± 8.954		26.398	± 4.143	
	Soluble (40,000 x g.)	20.900	± 6.745		1.574	± 0.444	

Figures indicate the average values of 5 sets of experiments.

Table 4

The per centage recovery and the specific activities of lipase and SDH in the original homogenate and the various particulate fractions of the breast muscle of Rosy pastor in the evening of the pre-migratory period.

Period & Time	Fraction	Lipase activity µl CO_2/ mg. protein/ hour.	Recovery %	SDH activity µg. formazan/mg. protein/ hour.	Recovery %
Pre-migratory period (April) Evening	Homogenate	26.702 ± 2.674	50.58	11.249 ± 2.449	79.89
	Myofibrils (600 x g.)	9.463 ± 3.031		8.493 ± 1.431	
	Mitochondria (7,500 x g.)	7.178 ± 1.685		23.010 ± 2.808	
	Microsomes (40,000 x g.)	15.662 ± 1.834		20.810 ± 1.997	
	Soluble (40,000 x g.)	24.184 ± 4.133		1.679 ± 0.403	

The figures indicate the average of 5 sets of experiments.

these studies.

Increase in body weight during the pre-migratory period with a corresponding increase in the fat content of the breast muscle and adipose tissue has been noted. More than 3/4 of the population of Rosy pastors migrated by the third week of April and the rest in the last week of April. A striking increase in the size and weight of the gonads prior to migration was also observed.

Discussion

From the results obtained it is clear that there are significant differences in the lipase activity of the various subcellular fractions of the muscle homogenate between the two periods under investigation viz. post-migratory and pre-migratory. In the post-migratory period the lipase activity was higher in the soluble as well as microsomal fractions whereas in the pre-migratory period the enzyme activity of the mitochondrial and soluble fractions respectively was comparatively low. Mitochondria are known to contain all the cyclophorase system of enzymes (Green, 1951) and are also shown to be the important sites of fatty acid oxidation (Kennedy and Lehninger, 1949). Muscle fibres possessing numerous mitochondria have been shown to possess high lipase as well as oxidative enzyme activities (George and Talesara, 1961). The role of lipase in the muscle for the breakdown of fat into fatty acids as the initial step necessary in the utilization of fat, has also been shown by George and Talesara (1962a). In the light of these findings,

the lower figures obtained for lipase activity in mitochondria in the pre-migratory period denote supression of lipolytic activity and thereby also of active fatty acid oxidation. The enzyme activity of the soluble fraction which was very high in the post-migratory period was distinctly low in the pre-migratory period. It should, however, be mentioned here that the recovery of the enzyme in this period was only 56 and 50% (morning and evening respectively) in contrast to 95 and 82% (morning and evening respectively) in the post-migratory period. But such a phenomenal drop in the enzyme activity was not seen in the homogenate. This indicates that in the pre-migratory period, there is a control system active in the muscle, which lowers the activity of the enzyme. The action of this system, which might be hormonal in nature, becomes all the more pronounced when the cellular organization is disrupted.

The microsomal fraction on the other hand showed more or less equal activity of the enzyme in both the periods though there was a distinct diurnal difference in the enzyme activity. The fact that microsomes are the sites of lipid synthesis in the cell (Green, 1960) denotes a special significance for the high concentration of lipase present in the microsomes in bringing about the esterification of the fatty acids synthesized. In this respect it is of importance to cite the work of Borgstrom (1953) and Jedeikin and Weinhouse (1954) who have shown that the biosynthesis of neutral fats or triglycerides from free fatty acids is by the reversal of

lipase action. The synthesis of neutral fats from labelled palmitate has also been reported by Stein et al, (1957). They have also suggested that the microsomes may bring about the initial steps while the mitochondria catalyze the final step in the conversion of fatty acids to triglycerides. Furthermore, the role of microsomes in fatty acid synthesis has been reported by Lachance et al, (1958), Abraham et al, (1960) and Matthes et al, (1960). In support may be mentioned the work of Matthes et al, (1960) showing a 5 to 10 fold increase in lipogenesis by the addition of microsomes to the supernatant fraction.

In the post-migratory period lipase activity in the soluble fraction was highest compared to that in the pre-migratory period. The enzyme activity was also found to be more in the evenings in both the periods. The occurrence of a high concentration of the enzyme in the soluble fraction may be due to the fact that this is a water soluble enzyme and may well have been derived from the mitochondria and microsomes during the successive washing procedures employed in the isolation of the different particulates. Recent studies on the lipase activity of the particulate fractions of the pigeon breast muscle (George and Talesara, 1962a) and that of the duodenal homogenate (Dinella and Meng, 1960) have shown the highest recovery of the enzyme from the soluble fraction. However, in the present studies the soluble fraction during the pre-migratory period was found to have very little enzyme activity compared to that in the post-migratory period.

The myofibrillar fraction also showed fluctuations in its enzyme (lipase and SDH) activity. This may be attributed to the mitochondria and microsomes still adhering to the myofibrils. In the pre-migratory period lipase activity in the myofibrillar fraction was low. It is difficult to believe that this is due to the total removal of the mitochondria and microsomes since all the experimental operations were similar in all cases.

In the post-migratory period, SDH activity in the mitochondria was found to be more in the evening than in the morning. In the pre-migratory period, on the other hand, it was high in the mitochondria in both the pre- and post-migratory periods, which denotes a high potentiality for oxidative metabolism. Microsomes also showed high concentration of this enzyme particularly more in the mornings of the pre-migratory period. The enzymes taking part in the oxidation of fatty acids are also known to catalyze the reversible reactions leading to the synthesis of fatty acids. However, it has not been possible to demonstrate the synthesis of fatty acids by the isolated mitochondria alone which indicate the possibility that synthesis and utilization of fat do not take place at the same site in the cell (Mahler, 1957; Kennedy, 1957; Langdon, 1957). If that is the case the higher concentration of lipase and SDH in the microsomal fraction may be considered as for the synthesis of fat before migration. Furthermore, microsomes during the pre-migratory period showed a higher activity of both the enzymes in the early mornings, suggesting that synthesis takes place

in the night during rest. Recent studies reported by George and Iype (1963a) on the lipase activity of the pigeon heart muscle particulate fractions also showed a higher enzyme level in the microsomes. They attributed the high lipase activity in this cell fraction to the role of this enzyme in the esterification of fatty acids that are synthesized. The observation on the increased activity of both the enzymes in the microsomal fractions during the pre-migratory period suggests that the microsomes are the sites of lipogenesis in the muscle and that there is an increased synthesis of fat during the pre-migratory period. Quantitative data on the seasonal changes in the fat content of the muscle in these birds also show that there is augmented increase in fat content (Chapter 8). Incidentally, lipase level in the mitochondrial fraction during the pre-migratory period was found to be low. It is now established that lipase is necessary for the breakdown of fat into fatty acids which is the initial step in the utilization of fat. It is realized from these observations that in the pre-migratory period the muscle is more adapted for the synthesis and mobilization of fat, rather than utilization. This mechanism is probably controlled by some physiological factors. Evidence for a lower capacity for fat utilization in the pre-migratory period is available from the investigation on the *in vitro* oxidation of butyrate by muscle (Chapter 10). It should be mentioned here that an increase in the SDH activity of the muscle in the pre-migratory period observed by George and Talesara (1961a) was also confirmed in these studies. This

increase in SDH activity during the pre-migratory period while the capacity for fatty acid oxidation was diminished, is to be regarded as building up the potentiality for oxidative metabolism which would become effective when the physiological trigger for migration is pulled.

During the pre-migratory period there is a clear fall in the lipase activity of the soluble fraction. These changes in lipase activity of the soluble fraction may be due to the higher concentration of fat present in the muscle separated by homogenization and further centrifugation. However, even after the removal of the fat from the supernatant fluid there was no change in the enzyme activity. The per centage recovery of lipase was found to be too low in the pre-migratory period when compared to the post-migratory period. It was found that there was no considerable change in the enzyme activity of the whole homogenate other than a slight decrease. An attempt was also made to find out if there is any distinct change in the different fractions by using activators like glutathione and ATP, since these are known to activate the pigeon breast muscle lipase (George and Scaria, 1959). It was observed that the slight activation noted in the different fractions of the muscle was similar. Since lipase is known to be a stable enzyme this loss in its activity and that too particularly more in the soluble fraction during the fractionation procedures could not be accounted for at present.

Recently Farner et al, (1961) observed in the white-

crowned sparrows subjected to long day photoperiodic treatment increased deposition of fat with a corresponding reduction in glycogen in the breast muscle and liver. They have suggested a diurnal cycle of fat synthesis from glycogen during the night. The changes observed in the enzyme activity of the different subcellular fractions in the present study during the evening and morning hours support their hypothesis that synthesis of fat takes place during the night. The high microsomal enzyme activities of SDH and lipase in the early morning experiments is possibly due to the fact that active synthesis takes place in the night and during the day time fat may be utilized to a limited extent.

Considering these significant enzymic changes in the different particulate fractions of the muscle in the Rosy pastor during the post- and pre-migratory periods and during the day and night it could be assumed that there is a favourable mechanism existing in these birds for the biosynthesis of lipids during the pre-migratory period. It is also logical to assume from the present studies that there is a slow-down mechanism of fat utilization by the mitochondria. The low lipase activity in the mitochondria and the low capacity for fatty acid oxidation by the breast muscle homogenate in the pre-migratory period also support this hypothesis.

It was also observed that the muscle glycogen and phosphorylase activity in the muscle of Rosy pastor increase significantly before migration (Chapter 9).This could be

accounted for on the possibility of lipid synthesis from the carbohydrates which form the bulk of food of these birds during the pre-migratory period. Merkel (1958) noted that in the White Throat (Sylvia communis) and the Robin (Erithacus rubecula) the respiratory quotient reaches its peak in the spring and that in the autumn it is also very high during pre-migratory period. He obtained high quotients, over one, which indicate that fat is being formed from starchy foods. It could be assumed that fatty acids are synthesized from carbohydrates and are esterified with glycerol to form triglycerides. It is well known that the addition of glucose augments lipogenesis and oxidation of acetate to CO_2 (Lyon et al, 1952; Masri et al, 1952; Meades et al, 1952). In this context, similarity in the utilization of acetate by fasted and diabetic liver is striking, since in both conditions fatty acid synthesis is depressed whereas formation of acetoacetate and cholesterol and acetate oxidation, are not. The most likely explanation is that, lipogenesis depends upon an active glycolysis (Lyon et al, 1952). Observations made on changes in blood glucose level and changes in endocrine activity in the same species also give support to the hypothesis of an increased biosynthesis of fat during the pre-migratory period (George and Naik, Unpublished). In addition to all these enzymatic mechanisms, the nutritional status and hormonal mechanisms are also known to play an important role in the net synthesis and breakdown of fats. Other than these factors the intracellular localization of enzyme systems and the potential physiological urge may also be in operation.