CHAPTER I

THE OCCURRENCE OF LIPASE IN THE SKELETAL MUSCLES OF VERTEBRATES AND ITS POSSIBLE SIGNIFICANCE IN SUSTAINED MUSCULAR ACTIVITY

That fat and not glycogen forms the chief fuel in many flying animals in long and sustained flight involving intense muscular activity is gaining increasing support. The high energy value and the property of being easily stored in tissues are certain positive advantages of fat for being used as fuel, particularly when a continuous flow of energy for a long time is needed. Since 1920 considerable amount of work has accumulated demonstrating an appreciable reduction in the muscle fat during prolonged exercise and thereby denoting the possibility of the utilization of fat during such muscular activity. The whole literature on this subject has been reviewed by Weis-Fogh (1952) and George and Jyoti (1955a and '55b) and so it is not necessary to cite them again here. Weis-Fogh showed that in the locust, Schistocerca gregaria, atleast two-thirds of the energy liberated during flight was derived from fat. His estimations also showed that per locust about 60 mg. of fat and 25 mg. of other materials chiefly glycogen were metabolized. George and Jyoti (1955b) found that there is considerable reduction in the lipid content of the breast muscle of good fliers during activity, when the animal is forced to fly for a long time or when the muscle of the pithed animal is continuously stimulated electrically, the extent of reduction varying according to the

duration of the exercise. They (1955a) further observed a reduction in the number of fat globules in the fat loaded narrow fibres of the exercised pectoralis major muscle of the pigeon using proper histological techniques. Recently George and Jyoti (1957) have shown that when a pigeon is subjected to forced flight till it is completely fatigued, about 78% of the energy utilized is derived from fat while only about 22% from glycogen.

If therefore fat is used as fuel in muscular activity the splitting up of fat into fatty acids and glycerol should be an essential step in the process. Fatty acid is then oxidized to carbon dioxide and water through β oxidation and the citric acid cycle. Evidence of such oxidation in mammalian liver cells with the mitochondria as the centres of such action is available from the work of Kennedy and Lehninger (1948). The recent researches of Green and his collaborators (Green, 1954 and 1955) have confirmed beyond doubt that the complex enzyme system in the mitochondria of the cells of the liver, heart, kidney and skeletal muscle can bring about such oxidation. In view of these findings it is reasonable to expect that the oxidation of fatty acids takes place in the mitochondria of muscle cells also. Partial oxidation of fatty acids in the liver leads to the production of ketone bodies which are completely oxidized to carbon dioxide and water in the muscles (Fruton and Simmonds, 1953, Harrow, 1950). It has also been shown that extrahepatic tissues can effect direct oxidation of long chain fatty acids to carbon dioxide and water through

the citric acid cycle without giving rise to ketone bodies (Harrow, 1950, Fruton and Simmonds, 1953).

Having been thus convinced of the possibility of the oxidation of fatty acids in muscle cells in the process of fat utilization during prolonged and intense muscular activity, it was considered worthwhile looking for an enzyme which could hydrolyze fat into fatty acids and glycerol which is an essential step in the utilization of fat.

The presence of lipase in blood, liver and pancreas of vertebrates, in the intestinal tissue of many invertebrates, and in seeds and moulds among plants is well known. But very little however is known regarding its presence in vertebrate tissues other than those mentioned above. The work of Schmidt Nielson and Stene (1939) who noted lipase in the cooked muscle of salmon is the only important work on this subject.

In the present investigation experiments were conducted to see whether there is a true lipase in the pectoralis major muscle of the rock pigeon (Columba livia), and extended this study to the pectoralis major of the bat (Hipposideros speoris) and the domestic fowl (Gallus domesticus) and gastrocnemeus muscles of the fowl and the frog (Rana tigrina).

Detection of Lipase Activity

Materials

Preparation of the Enzyme Material

Portions of the pectoralis major muscle from pigeon which was bled to death by cutting off the head and the

pectoral blood vessels, were cut out and immediately frozen in an ice-acetone bath. The extraction medium used was 70% glycerine which was cooled to 0°C . The muscle was then quickly cut into small pieces, crushed and ground to a fine pulp in a mortar which was also previously cooled to 0°C and kept in an ice bath. Glycerine was slowly added to the pulp, well slaked and allowed to stand for a while. The glycerine soluble fraction was then separated out by squeezing through a cheese cloth. A few drops of toluene were added to the clear filtrate to prevent putrifaction and kept at 0°C in a refrigerator. By this method even though all the enzyme in the muscle might not be extracted, a considerable part of it at least should get dissolved, since glycerine is a good solvent for lipases. This crude glycerine extract was used as the enzyme material in all the experiments.

Substrates

For determining the activity of the extract, four substrates- two triglycerides, tributyrin and triacetin and two vegetable oils, castor oil and olive oil - were used.

Methods

The following methods were employed.

(1) The method of Cherry and Crandall (1932) for determining the lipase activity in blood serum using olive oil as substrate.

(2) Lipase determination with the aid of polyvinyl alcohol (Fiore and Nord, 1949) using all the substrates mentioned above.

(3) Method for lipase (Tributyrinase) determination (Goldstein, Epstein and Roe, 1948) using tributyrin as substrate.

(4) Spectrophotometric method of lipase activity determination (Herr Jr. and Sumner, 1955) using castor oil as substrate.

In all these assays a boiled extract was used as the control with all the other conditions remaining the same. The reaction mixtures were incubated at 40°C and pH maintained at 7 in all the experiments.

Results

In the titrimetric methods 1,2 and 3 an increase in the acidity and in method 4, a decrease in the optical density of the reaction mixture indicated the presence of lipolytic activity.

Comments

In the titrimetric methods the determination of the end point was somewhat difficult owing to precipitation. In order to verify the accuracy of the end point in titration the pH of the titrated material was later determined by electrometric methods. In the case of the polyvinyl alcohol method, the alternate procedure recommended by Fiore and Nord (1949) was found to be quite suitable in judging the end point.

Polyvinyl alcohol, however, was not found to be a very effecient emulsifying medium for certain of the substrates. Tributyrin and castor oil did not yield emulsions at all. But when one or two drops of " Tween 80 " were added, these

substrates formed a fine emulsion in the Waring blendor. It should be mentioned here that although "Tweens" themselves are considered to be substrates for lipase, there is no detectable activity at a low concentration such as two drops in 100 ml. of polyvinyl alcohol solution.

Quantitative Determination of Lipase Activity

Method and Materials

Of all the methods employed in the above experiments the one using polyvinyl alcohol was found to be the most convenient. This method with certain modifications was therefore adopted for all the subsequent studies. Tributyrin was used as the substrate because this yielded the best results in the earlier experiments.

Preparation of the Polyvinyl Alcohol Solution

2.5 gm. of polyvinyl alcohol (Grade 71-30) was shaken in an Erlenmeyer flask with 250 ml. of distilled water. 1.25 ml. of 0.1 N HCl was then added and the mixture heated at 75-85°C for a few minutes, till the solution became clear, when 25 ml. of distilled water was added and the whole heated for some more time. The solution was then cooled and brought to the desired pH by adding 0.1 N NaOH.

Preparation of the Emulsion

To 100 ml. of the polyvinyl alcohol solution were added 2.8 ml. of tributyrin and a single drop of "Tween 80" and emulsified in a Waring blendor for five minutes and was used after some time.

Buffer Solution

A McIlvaine buffer of the same pH as that of the polyvinyl alcohol solution was used.

Preparation of the Enzyme Material

Glycerine extracts of different concentrations were prepared. In each case 100 ml. of 70% glycerine was used for extraction and the quantity of muscle in gm. taken were in multiples of ten. Thus four samples with 10, 20, 30 and 40 gm. of muscle were extracted with 100 ml. of glycerine in each case. In all the studies except that on the effect of concentration of the enzyme, an extract with a concentration of 40 gm. per 100 ml. glycerine was used.

Procedure

Into a 250 ml. Erlenmeyer flask were measured 10 ml. of the emulsion, 5 ml. of the buffer and 5 ml. of the enzyme preparation. The mixture was shaken gently and incubated at 40°C for the desired length of time with constant shaking. For all the studies the incubation period was fixed at 4 hrs. except where it is stated otherwise.

At the end of the incubation period 30 ml. of a 1:1 alcohol- acetone mixture was added to stop further enzymic activity. After 10 minutes it was filtered using a fluted No. 1 Whatman filter paper and 25 ml. of the clear filtrate was titrated against 0.05 N aqueous Na OH using a microburette. 0.3 ml. of a 1% soln. of alcoholic phenolphthalein was used as an indicator. The value thus obtained was doubled and recorded as such.

Control

Two controls were tried. In one the enzyme was boiled for about 10 minutes and used after cooling. In the second, the fresh enzyme preparation was used after the addition of the alcohol- acetone mixture to the substrate, and thereby breaking up the emulsion and rendering the enzyme completely inactive. The readings obtained for these two types of control experiments were found to be identical. The second method of inactivation was employed in the present study because it was found to be more convenient.

Results

The results obtained were as follows:

pH Optimum

Experiments were carried out using reaction mixtures of various pH. McIlvaine buffer of a pH range of 2 to 8 was used. For pH 9 , a 0.2 M soln of Na_2HPO_4 was employed. Lipolytic activity was found to be the highest at pH 8 as can be seen from the graph (Fig. 1).

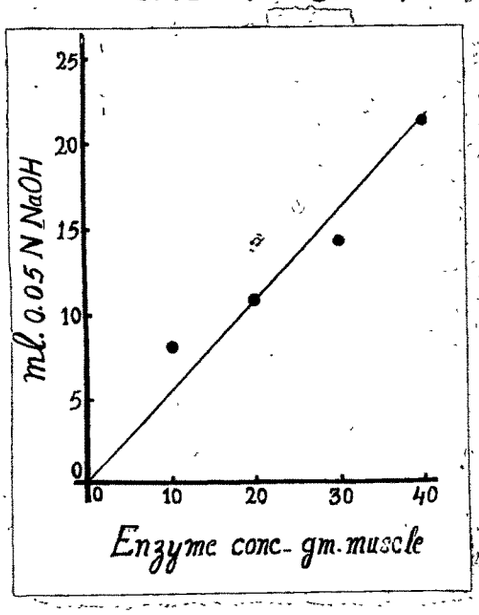

Fig. 1 - pH Curve

Effect of Temperature

The enzymic activity of the extract was studied at different temperatures ranging from 0°C to 60°C, keeping the pH constant at 7.6, which is nearest to the pH of the avian blood. Since time and temperature are interdependent variables it was decided to conduct three sets of experiments with different periods of incubation. It was found that for shorter periods of incubation, the temperature optimum was 50°C whereas the activity is reduced considerably at this temperature when incubated for longer periods, for which the optimum was 40°C. The results obtained in this study are illustrated in Fig.2. It is clearly seen that the enzyme is quite stable at higher temperatures and is less active at lower temperatures.

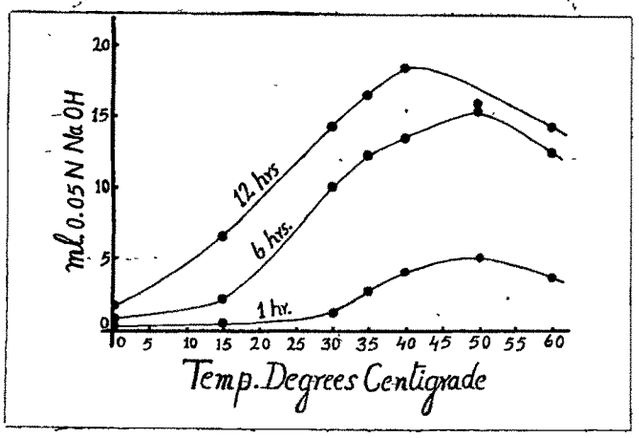

Fig.2 - Time and Temperature Curves

Time Factor in the Enzymic Activity

Several samples were run for varying lengths of time, all other conditions such as pH , temperature and the concentration of the substrate and the enzyme material remaining the same. The pH was adjusted at 7.6. It can be seen from

Fig.3 that the initial velocity of the reaction decreases with time.

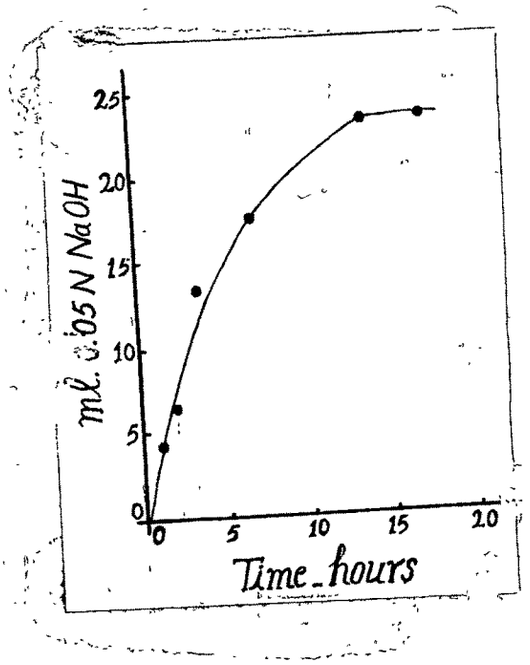

Fig. 3 - Progress Curve

Effect of Concentration of the Enzyme

As mentioned above four different concentrations of the enzyme were prepared for the study. The activity is found tovvary accordingly (Fig.4). The samples were incubated for a period of 14 hrs. and at pH 7.6.

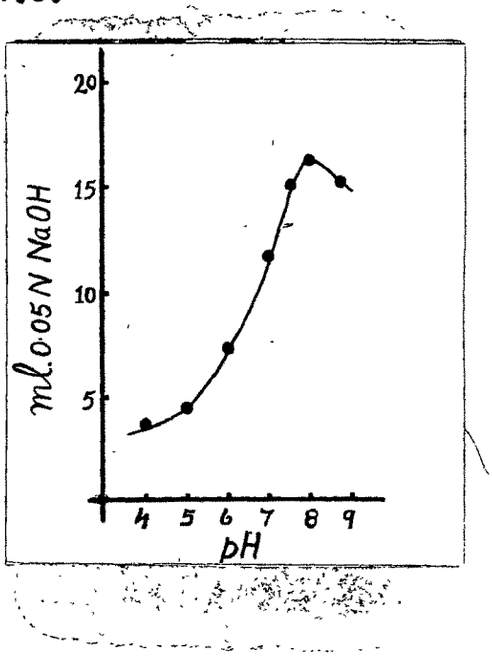

Fig. 4 - Concentration Curve

Action on Different Substrates

Experiments using different substrates were carried out at pH 8 which is the optimum for this enzyme at 40°C. Four substrates viz. tributyrin, triacetin, olive oil and castor oil were tested. The rate of hydrolysis of these substrates come in the following order tributyrin > triacetin > castor oil > olive oil (Table 1).

Table 1

Showing the enzymic action on different substrates

Substrate	Substrate conc. ml./100 ml. PVA*	ml. 0.05N NaOH titrated		
		Control	Sample	Difference
Tributyrin	2.8	7.4	25.2	17.8
Triacetin	1.9	8.1	21.2	13.1
Castor oil	3.0	7.5	10.0	2.5
Olive oil	3.0	7.7	8.8	1.1

* PVA - polyvinyl alcohol solution

Definitions

For the measurement of lipase activity and for the purpose of comparison of the activity in different muscles it was considered necessary to adopt a certain standard. The standard adopted for this study is expressed as lipase units and defined as follows:

Lipase Units

The amount of lipase required to liberate that quantity of butyric acid equivalent to 1 ml. of 0.05 N NaOH, when 10 ml. of the substrate, of the concentration, 2.8 ml. tributyrin in

100 ml. polyvinyl alcohol solution and emulsified with 1 drop of "Tween 80", is allowed to react with 5 ml. of a McIlvaine buffer of pH 8 at 40°C for 4 hrs. the total volume of the reaction mixture being 20 ml.

Lipase Value

The number of lipase units present in 1 gm. of wet muscle.

Calculation

5 ml. of an extract of 40 gm. of the pectoralis major of pigeon in 100 ml. glycerine liberates butyric acid equivalent to 17 ml. of 0.05 N NaOH on the average, under the above conditions. The number of lipase units in 5 ml. of the extract is therefore, 17. Five ml. of the extract contains 2 gm. of muscle with 17 units of lipase in it. So the lipase value of the pectoralis major muscle of pigeon is 8.5. Table 2 shows the lipase value of the different muscles of the four animals examined.

Table 2

Showing the lipase value of different muscles

Name of the animal	Name of the muscle	Lipase units in 5 ml. of enzyme material	Lipase value
Bat	Pectoralis major	22.0	11.0
Pigeon	,,	17.0	8.5
Fowl	,,	2.0	1.0
,,	Gastrocnemeus	2.2	1.1
Frog	,,	1.6	0.8

Discussion

That the active substance present in the muscle extract is an enzyme is evident from the fact that it satisfies the requisite conditions for being considered an enzyme in that it shows a progressive activity at increased periods of incubation, a marked difference in the activity as the concentration is altered, an optimum pH and temperature(Figs. 1-4) It is well known that muscle contains several oxidizing and hydrolysing enzymes. The hydrolysing enzymes are mostly of the nature of esterases. Lipase is essentially an esterase, but is distinguished from other esterases by the fact that it readily hydrolyses lipids, which are esters of organic acids with alcohol, especially triglycerides in preference to any other substrate(Baldwin,1953; Fruton and Simmonds,1953; Sumner and Somers,1953).Tributyrin and triacetin are triglycerides of butyric and acetic acids respectively, and are usually used as substrates in the study of the known lipases, such as of the liver, pancreas, castor bean and fusarium. The hydrolytic activity of the muscle extract on these substrates is considerable and other substrates like olive oil and castor oil are also acted upon though to a lesser extent. The enzyme in the muscle extract therefore is to be regarded as a true lipase.

The possible physiological role of such an enzyme in hydrolysing fat into fatty acids and glycerol during sustained muscular activity has already been pointed out. The amount of lipase present in a muscle should therefore be an index of the extent of possible fat utilization. The utilization of fat

in the muscle could thus be directly correlated with its activity. George and Jyoti ('1955a and '55b) have shown that there is a greater fat store and a greater measure of reduction of fat during activity in the breast muscles of those birds where the narrow fat loaded fibres predominate over the broad glycogen loaded ones. It is therefore natural to expect a greater lipase activity in those muscles where the narrow fibres predominate and my observations also support this view. Thus the pectoralis major muscle of the pigeon, which consists predominantly of narrow fibres has a high lipase value of 8.5 , while that of the fowl consisting of only the large variety is only 1. In contrast to the pigeon breast muscle, the breast muscle of the fowl consisting of the broad glycogen loaded variety of fibres contains very little fat and when exercised electrically, the reduction of fat is extremely small (George and Jyoti,1955b). In the fowl again, the fat content and its reduction during exercise in the more active leg muscle is greater than that of the breast muscle (George and Jyoti, 1955b). The lipase value of 1.1 for the leg muscle of the fowl obtained in my experiments is slightly higher than that for the pectoralis major of the fowl. These observations regarding the fat content and its reduction during activity in the above muscles and their lipase values tend to show a relationship between the lipase content and fat utilization during sustained muscular activity. The lipase value of 11.0 for the pectoralis major muscle of the bat which is higher than that for the pigeon breast muscle may be

attributed to the greater activity, of the animal and its nocturnal habits.

Niemierko in 1929 and Buchwald and Cori in 1931 (as cited by Heilbrunn, 1952) noted a reduction in the fat content in the frog's leg muscle as well after prolonged stimulation. My findings regarding the occurrence of lipase (lipase value 0.8) in the frog leg muscle also, not only provides an explanation for the hydrolysis of fat in the process of fat utilization in this muscle, but they also raise an important question as to whether lipase is present in all vertebrate muscles and perhaps in invertebrate muscles too. Such a possibility has been suggested by Baldwin (1953) when he wrote," It has usually been assumed , though never proved, that fats are hydrolytically split into glycerol and free fatty acids before any oxidation takes place. This is not entirely an unreasonable supposition, for cells of most kinds seem to be furnished with lipolytic enzymes, the action of which is freely reversible. " The results obtained in the present study support this suggestion at least as far as the muscle tissue is concerned and the general conclusion that could be drawn is that the lipase concentration in the muscle would depend upon the extent of fat utilization in the muscle during activity.