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Utilization of Triglyceride by Porcine Muscle

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Animals which are only allowed access to food for a short period during the day, that is meal fed, rather than allowed to feed *ad libitum* adapt to such a regime with an increased capacity for lipogenesis (Leveille and Hanson, 1966). However, between feeds, the fat which has been produced is utilized and a leaner carcass may be obtained from such animals (Friend and Cunningham, 1967). Fat is released from the adipose tissue as free fatty acids which are taken up by peripheral tissues. Some of the fatty acids taken up by the liver are converted to triglycerides which re-enter the circulation as very low density lipoproteins. These triglycerides are degraded by the enzyme clearing factor lipase, in the capillary wall, and the fatty acids enter the tissues. In some species the activity of this enzyme, which is under hormonal control, has been shown to increase in the early stages of fasting and then to decrease after approximately three days (Robinson and Wing, 1970). Clearing factor lipase also degrades chylomicron triglycerides and the ability of tissues of non-ruminants to utilise dietary fat depends upon the activity of this enzyme. Hence it determines the initial distribution of dietary fat in the body.

As part of our studies on factors regulating the storage and utilisation of fat by pigs we have determined the conditions for assaying clearing factor lipase in pig heart muscle and have measured its activity in fed and fasting pigs. Because of the relatively low activity of the enzyme in pig tissues we have devised a new and sensitive radioactive assay.

Methods

Pure bred Large White pigs were obtained commercially and fed *ad libitum* until slaughtered at 150 ± 5 lb live weight. During starvation animals were allowed free access to water. Hearts were obtained at the end of the normal slaughter procedure, approximately fifteen minutes after stunning, and placed in ice. A 5% (w/v) homogenate of left ventricle muscle was prepared in 0.025 M ammonia/ammonium chloride buffer pH 8.1 and 8.0 ml was added to 200 ml of cold acetone and 100 ml of ether at room temperature. The acetone/ether powders were stored *in vacuo* at 4°C overnight.

Assay procedure. Assay medium was prepared by incubating the following mixture in a shaking water bath at 35°C for thirty minutes: triolein ^{14}C emulsion 1.5 ml; 20% dialysed defatted albumin pH 8.1, 6.0 ml; 0.7 M tris-hydroxymethyl aminomethane chloride buffer pH 8.1, 3.0 ml; dialysed pig serum 0.9 ml; 0.9% (w/v) sodium chloride 0.6 ml and 1.5 ml heparin, 14 IU per ml. The triolein emulsion, prepared fresh daily, contained in 5.1 ml of 0.85% sodium chloride, 221 mg pure triolein labelled with 2 μ Ci of glycerol trioleate-1-^{14}C, 11 mg pure lecithin and 25 mg triton x100. The acetone powder of heart muscle was homogenised in 20 ml of 0.025 M ammonia/ammonium chloride buffer pH 8.1 and 1.25 ml of the homogenate was added to 2.25 ml of assay medium in a conical flask. After incubating at 35°C for one hour in a shaking water bath, aliquots were taken for extraction of the fatty acids by the procedure of Dole and Meinertz (1960). The fatty acids were removed from the extract by shaking with alkaline ethylene glycol and the radioactivity of the ethylene glycol phase was determined (Kaplan, 1970). The results obtained with this assay system were comparable to those obtained using Intralipid (A.B. Vitrum, Stockholm) as substrate and titrating the free fatty acids produced. Triton x100 did not affect the activity of the enzyme in assays in which Intralipid was used as substrate. Enzyme activity is expressed as μ moles of fatty acid released per g. wet tissue per hour.

Results

Assay requirements. Clearing factor lipase is only active against the triglycerides of synthetic emulsions if they have been treated with serum. During incubation with serum certain high density lipoproteins are adsorbed on to the emulsion thereby making it a suitable substrate. Table 1 shows the effect of serum concentration on the activity of the enzyme. The volume of the assay was maintained with 0.9% sodium chloride.

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Table 1. Effect of serum concentration on the activity of clearing factor lipase

Serum, ml per assay flask	0	0.05	0.10	0.15	0.20	0.25
Activity, μ moles fatty acid per g wet muscle	8.6	25.8	37.2	47.9	44.7	35.4

Maximum activity was obtained with 0.15 ml of serum and this quantity was used in all assays. Of the assayed activity 80% was dependent upon activation of the substrate with serum.

The effect of emulsion concentration was determined by replacing some of the emulsion with sodium chloride in the assay flask. The activity was maximal at the lowest concentration tested, 2.8 μ moles of triolein per flask, and remained constant up to 12.5 μ moles per flask, which was used in the standard assay. During the assay the triglyceride concentration was virtually unchanged since the most active enzyme released 0.7 μ moles of fatty acid, approximately 2% of the triglyceride fatty acids present.

Heparin activates clearing factor lipase, but the mechanism of this activation has not been established. It is possible that it protects the enzyme against denaturation during the assay. Although heparin was used in the standard assay at a concentration of one international unit per ml we were unable to establish optimal requirements for heparin in this system. Activity increased by 50% between 0.2 and 1.0 IU per ml, but decreased at higher levels. However a similar pattern of increase and decrease was also obtained using tenfold higher levels of heparin.

Protamine inhibits clearing factor lipase activity by binding heparin and removing it from the enzyme. In order to study the significance of heparin in this reaction we added protamine to assays in the presence and absence of added heparin. In both systems 5 mg of protamine sulphate increased the activity by 23%. A similar activation was obtained with 0.25 mg of protamine sulphate per assay.

Under the conditions of the standard assay the enzyme activity was linear for two hours, table 2.

Table 2. The time course of assay of clearing factor lipase.

Time of incubation, minutes	15	30	45	60	90	120
Activity, μ moles fatty acid/g wet muscle	4.7	12.7	21.4	28.9	42.7	53.1

Enzyme activity was also proportional to the quantity of acetone powder added using powder obtained from 0.5-4.0 mg of tissue per ml of assay, table 3.

Table 3. Effect of enzyme concentration on activity.

Wet tissue equivalent per ml assay	0.5	1.0	2.0	3.0	4.0
Fatty acid released, μ moles per hour	0.05	0.09	0.17	0.24	0.32

Sodium chloride inhibits clearing factor lipase, (Robinson, 1963). The effect of increasing concentrations of sodium chloride on pig heart clearing factor lipase are shown in table 4. The lower concentrations of sodium chloride stimulated the enzyme, possibly due to a change in the characteristics of the emulsion, whereas higher concentrations inhibited. 1.0 M sodium chloride inhibited the basal activity by 7.3% and the maximal activity by 84%.

Table 4. Effect of sodium chloride on enzyme activity.

* Sodium chloride conc. M.	0	0.1	0.2	0.3	0.5	1.0
μ moles fatty acid released per g wet muscle.	44.0	72.8	70.5	63.8	37.2	12.1

* This is added sodium chloride above the normal concentration in the assay which was 0.022 M.

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The activity of clearing factor lipase in hearts from fed pigs and from starved pigs is shown in table 5. There was no difference in the activity of gilts and hogs, and each figure includes five hogs and five gilts. There was a slight decrease after 48 hrs starvation.

Table 5. Effect of starvation on enzyme activity.

Length of starvation, hr	0	24	48
* µ moles fatty acid released per g wet muscle	26.7 ± 2.3(10)	23.6 ± 1.7(10)	17.7 ± 2.8(10)

* Results are expressed as means ± S.E.M. with the number of animals in parenthesis.

Discussion

The dependence of the lipolytic activity of pig heart acetone powders on the activation of the substrate with serum characterises the enzyme as clearing factor lipase. Inhibition of the enzyme by 1 M sodium chloride is also characteristic but an activation by lower concentrations of sodium chloride has not been reported. This may be caused by the type of emulsion used for the assay although this assay system has been shown to give quantitatively similar results to other assay methods when compared using acetone powders from mouse heart which are ten times more active than pig heart.

The ill defined effects of heparin on the enzyme activity may also be characteristic of the use of a triton stabilized emulsion, since Schotz et al 1970, found no activation of rat adipose tissue clearing factor lipase by heparin in an assay using triton x100. With these results it was not surprising that protamine failed to inhibit the enzyme, however the observed activation was not expected, and requires further study. The function of heparin is not fully understood. It causes the release of clearing factor lipase into the blood when it is injected into animals, possibly by displacing the enzyme from a similar sulphated polysaccharide in the capillary endothelium, and its presence in the assay is thought to stabilize the enzyme. It is possible however that the tissue enzyme, unlike that released into the blood by heparin, is still attached to a heparin-like molecule against which protamine is inactive.

The effect of starvation on the activity of clearing factor lipase in pig heart differs from that found in rats by Borensztajn and Robinson, 1970 in that there was no increase in the initial stages of fasting, although there was a decrease as found previously by Robinson and Jennings, 1965. It is possible that we failed to observe an increase which took place prior to twenty four hours of starving, however the pig intestine empties slowly (Castle and Castle, 1956), and the lipids of the intestinal lymphatic system are elevated for over ten hours after a meal (Frémont et al, 1970). Furthermore Cunningham and Friend, (1965) showed that the concentration of plasma free fatty acids was still increasing after twenty four hours starvation so that any change in triglyceride levels might be expected to occur later.

These results show that the clearing factor lipase activity is present in pig heart muscle and is similar to that in other species. The changes in the enzyme on starvation indicate that it is probably mainly active in providing the heart with fatty acids from chylomicrons, in the fed state. Using this sensitive assay system, it is proposed to extend these studies to the adipose tissue to determine the way in which this enzyme may control the initial distribution of dietary fat in the body.

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