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Clearing-Factor Lipase in Obese Hyperglycaemic Mice (*ob*/*ob*)

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1. Clearing-factor lipase was assayed in acetone–ether-dried powders of heart and epididymal fat-pads of lean and genetically obese mice (*ob*/*ob*). In both tissues the enzyme activity in the adult was higher in the obese mice. 2. In heart the enzyme activity was unchanged from 8 to 48 weeks of age in lean mice, but in obese mice it increased between 8 and 12 weeks of age and remained elevated. 3. Starvation produced changes in the heart clearing-factor lipase activity in obese, but not lean, mice. 4. The clearing-factor lipase activity of epididymal fat-pads decreased rapidly during 24h starvation in both lean and obese mice, but the activity in the obese mice remained higher than that in lean mice. 5. Plasma triglyceride and cholesterol concentrations were determined in both lean and obese mice. Triglyceride concentrations were not greatly different, but the obese mice were hypercholesterolaemic. Plasma cholesterol concentrations were not correlated with changes in clearing-factor lipase activity.

Mice which are homozygous for the obese gene (*ob*) are characterized by the excessive deposition of body fat. Although many metabolic abnormalities have been described in these animals (Mayer, 1960), the primary cause of the obesity has not been ascertained. There are extensive reports of a defect in fatty acid mobilization by adipose tissue from obese animals, but the ability of tissues to take up lipid from the plasma has not been investigated. Such changes might be expected since total plasma lipids are reported to be increased in obese mice (Mayer & Silides, 1958) and plasma cholesterol is increased (Mayer & Jones, 1953; Christophe & Mayer, 1959).

The removal of triglycerides from plasma is believed to be controlled by the enzyme clearing-factor lipase (Robinson, 1970). The activity of this enzyme varies under different physiological conditions. In the fed animal the activity is high in adipose tissue and low in heart muscle, whereas during starvation the activity in adipose tissue declines and that in the heart may increase. Such changes are believed to be under hormonal control, and in diabetes the activity of the adipose-tissue enzyme is lowered, whereas that in the heart is increased. The mechanism by which clearing-factor lipase activity is controlled *in vivo* is not known; however, *in vitro* the activity of the enzyme in rat adipose tissue is increased by insulin (Salaman & Robinson, 1966) and inhibited by compounds which increase the concentration of cyclic AMP (adenosine 3′:5′-cyclic monophosphate), such as catecholamines and caffeine (Wing *et al.*, 1966; Wing & Robinson, 1968). The hyperinsulinaemia of the *ob*/*ob* strain of obese mice is well established (Stauffacher *et al.*, 1967) and this, together with the low activity of adenylate cyclase in the adipose tissue of obese mice (Enser, 1970), might be expected to produce changes in clearing-factor lipase activity, if either is involved in the regulation of this enzyme *in vivo*.

In the present study the clearing-factor lipase activity in heart and adipose tissue from obese mice has been measured by a new assay procedure. In addition the plasma concentration of cholesterol and triglycerides, both of which may induce changes in the activity of clearing-factor lipase (Fielding, 1970; Huang & Kako, 1970; Shafrir & Biale, 1970), have been measured in an attempt to determine what modifiers of clearing-factor lipase are responsible for the changes in activity observed under different physiological conditions.

Materials and Methods

Animals

Mice of the C57 Bl/6J strain carrying the obese (*ob*) gene, originally obtained from the Jackson Laboratory, Bar Harbor, Maine, U.S.A., were bred in our own colony. All animals were obtained from the mating of siblings. The lean controls were taken from litters that did not contain obese mice and whose parents were shown not to carry the *ob* gene by backcrossing experiments. They were not separated from the obese line by more than two generations and were of the same sex and age, ±2 days, as the obese mice. Female mice were used to study the clearing-factor lipase of heart and male mice to study that of adipose

tissue. All mice were fed on a commercial diet (Oxoid breeding diet). They were killed between 11.00h and 12.00h and starvation was started by removal of food at 11.00h.

Tissue preparations

Mice were anaesthetized with diethyl ether and then decapitated. The heart ventricles or epididymal fat-pads were rapidly removed, rinsed in 0.9% NaCl, blotted and weighed. Acetone–ether-dried powders were then prepared by the method of Borensztajn *et al.* (1970) with a tissue homogenate prepared in 0.025M-NH_3–NH_4Cl buffer (pH8.1). The powders were stored overnight at 4°C *in vacuo* before assay.

For determination of plasma lipids, mice were anaesthetized with ether and blood samples were taken from the orbital sinus (Stone, 1954) into heparinized tubes. The tubes were capped and centrifuged at approx. 2000*g* for 15min at room temperature. The plasma was removed and immediately added to the appropriate extraction medium. When serum was prepared instead of plasma, the assays gave the same results, but plasma was the preferred preparation because of greater yield and less frequent haemolysis. Serum for clearing-factor lipase assays was prepared from recalcified citrated plasma (Salaman & Robinson, 1966) by using mouse blood obtained from the orbital sinus. Rat serum was not used since it gave only 75% of the activity obtained with mouse serum.

Clearing-factor lipase assay

A radioactive assay based on the method of Schotz *et al.* (1970) was used to determine clearing-factor lipase.

A triolein emulsion was prepared as follows. A 5.0ml volume of cold (4°C) 0.85% NaCl was placed in a 10ml Vortex beaker. To this was added, in ether solution, 221.5mg of triolein containing 2μCi of glycerol [1-^{14}C]trioleate (0.9ml) and 11mg of phosphatidylcholine. The mixture was homogenized at full speed with an MSE homogenizer for 10min. The jacket of the container was then filled with water at 60°C and the mixture was homogenized for a further 5min to remove the ether. Triton X-100 (25mg) in 0.1ml of water was then added and the mixture was homogenized for another 10min. This emulsion was stable for 24h and, although the fat floated after 72h, it could readily be dispersed by swirling. However fresh emulsions were prepared daily.

The assay medium was based on that of Borensztajn *et al.* (1970), except that the ^{14}C-labelled triolein emulsion replaced the chyle or Intralipid. The medium consisted of [^{14}C]triolein emulsion (1vol.); 20% (w/v) defatted bovine albumin adjusted to pH8.1 with NaOH (4vol.); 0.7M-tris–HCl buffer, pH8.1 (2vol.); mouse serum, dialysed against 0.9% NaCl (0.8vol.); 0.9% NaCl (0.2vol.) and heparin, 14i.u./ml (1vol.). It was incubated at 35°C for 30min to activate the triolein emulsion and 2.25ml portions were then placed in 10ml conical flasks. A homogenate of the acetone–ether-dried powder in 0.025M-NH_4Cl, pH8.1 (1.25ml), was added and the mixture incubated in a shaking water bath at 35°C for 1h. The homogenate was made up to contain acetone–ether-dried powder equivalent to 3mg of heart muscle, or 100–250mg of fat-pad, per 1.25ml. The activity of the enzyme was linear with time for 90min when studied with acetone–ether-dried powders from the hearts of lean and obese mice. The activity of the enzyme from heart was constant at concentrations between 0.5 and 9.0mg/ml. At the end of the incubation, a 2.0ml sample was taken for extraction of lipids by the method of Dole & Meinertz (1960). Fatty acids were removed from the extract by shaking a 5.0ml portion with 2.0ml of alkaline ethylene glycol (Kaplan, 1970) (0.1ml of 10M-NaOH diluted to 10.0ml with ethylene glycol), and the radioactivity was determined. This procedure only extracted 0.09% of the triglyceride radioactivity. All radioactive samples were counted to 2000 total counts. The efficiency of counting in Kaplan's (1970) scintillation mixture was 75% under the conditions used as determined with internal standards. Tissue from each mouse was assayed in duplicate, and samples from lean and obese mice of the same age, sex and treatment were assayed together.

Determination of cholesterol

Total plasma cholesterol was determined by the method of Zak (1957). Free cholesterol was determined by the method of Brown *et al.* (1954) by using aluminium chloride to gather the digitonin precipitate. The extraction and sampling procedure was scaled down so that 0.2ml of plasma could be assayed. Because of the poor recovery of cholesterol below 20μg, the plasma was extracted in 2.0ml of ethanol–acetone (1:1, v/v) containing 15μg of free cholesterol/ml and the plasma cholesterol was therefore determined as an increment.

Determination of triglyceride

Lipid was extracted from 0.2ml of plasma with 1.0ml of Dole's extraction mixture (Dole & Meinertz, 1960). A portion (1.6ml) of the final heptane phase was added to 100mg of silicic acid, followed immediately by 1.0ml of chloroform. After mixing and standing for 10min, 1.0ml samples of the solution were taken for triglyceride glycerol determination by the method of Van Handel & Zilversmit (1957). This procedure removed phospholipids (Ho, 1970) and gave a quantitative recovery of triglyceride.

Chemicals

Where possible chemicals were of analytical grade and all solvents were redistilled. Triolein was purified on columns of Florisil by using the solvent system described by Carroll (1961) and the fractions containing pure triglyceride, as determined by t.l.c. on silica-gel G plates developed with hexane–diethyl ether–acetic acid–methanol (100:20:2:1.8, by vol.), were pooled. Egg phosphatidylcholine was purified by elution from a column of neutral alumina with ethanol, and gave a single spot on t.l.c. Albumin (fraction V from bovine plasma, Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.) was defatted by the method of Chen (1967). Triolein, Florisil, egg phosphatidylcholine and heparin were purchased from British Drug Houses Ltd., Poole, Dorset, U.K. Triton X-100 was from Sigma (London) Chemical Co. London S.W.6, U.K. Glycerol [1-^{14}C]trioleate was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Intralipid (A. B. Vitrum, Stockholm, Sweden) was obtained from Vestric Ltd., Bristol, U.K.

Presentation of results

Clearing-factor lipase activity is expressed as μmol of fatty acid released/h per g of fresh tissue. Because it was not possible to carry out all the determinations in each experiment at one time, all the treatments were studied at random and the results shown are the means of a minimum of two assays carried out at least 1 week apart.

Results

Assay requirements

The radioactive clearing-factor lipase assay was devised to allow determination of clearing-factor lipase activity in tissues with low activity. To confirm that Triton X-100 did not change the activity of the enzyme, quantities of Triton X-100 were added to assays with Intralipid as substrate. No inhibition was observed below 0.6mg/ml when acetone–ether-dried powders from mouse heart ventricles were used and 0.35mg of Triton X-100/ml was used in the standard assay. When assays with the triolein emulsion were compared with assays in the same medium in which the triolein emulsion was replaced by Intralipid, the quantity of fatty acid released, determined by radioactivity, was the same as that found by titration in the Intralipid assays. This was additional evidence that Triton X-100 did not affect the enzyme.

Clearing-factor lipase is characterized by its requirement for a lipoprotein substrate (Korn, 1955), which can be prepared by incubating triglyceride emulsions with serum. Fig. 1 shows the serum dependence of the triolein emulsion assay. In the absence of serum only 20% of the maximum lipolytic activity was present. Although the clearing-factor lipase activity of plasma is low, the mouse serum contained a lipase that is active under these assay conditions, and suitable control assays were done. Although this activity varied with different batches of serum, it never amounted to more than 16% of the total activity.

High concentrations of NaCl inhibit clearing-factor lipase activity (Korn, 1955) and in the present study the activity was decreased to 23% at 0.5M and to 17% at 1.0M (100% = 350μmol/h per g wet wt. of tissue).

Heart clearing-factor lipase activity during growth

Changes in the clearing-factor lipase activity of the hearts of lean and obese female mice during growth are shown in Fig. 2. At 8 weeks the activity was the same in both types of animal, although the weight of the obese mice was considerably higher at this time. Between 8 and 12 weeks of age there was a rapid increase in the clearing-factor lipase activity of the hearts from obese mice until it was approx. 50% higher than in lean mice. The activity remained at this elevated value in the obese mice up to 48 weeks of age. The activity in the hearts from lean mice changed little during growth.

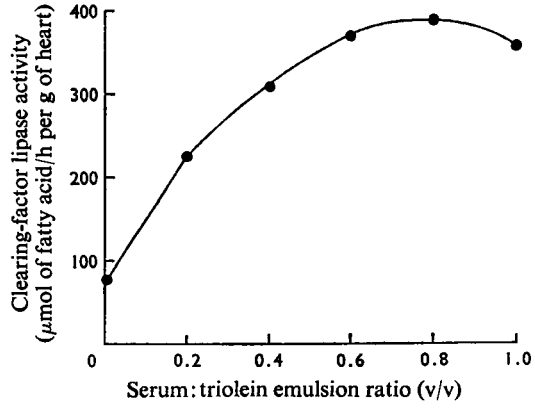

Fig. 1. *Requirement of serum for clearing-factor lipase activity*

Enzyme was from hearts of two 12-week-old female mice. The quantity of triolein emulsion was kept constant and the amount of serum used during the activation of the emulsion was varied. The volume was kept constant with 0.9% NaCl.

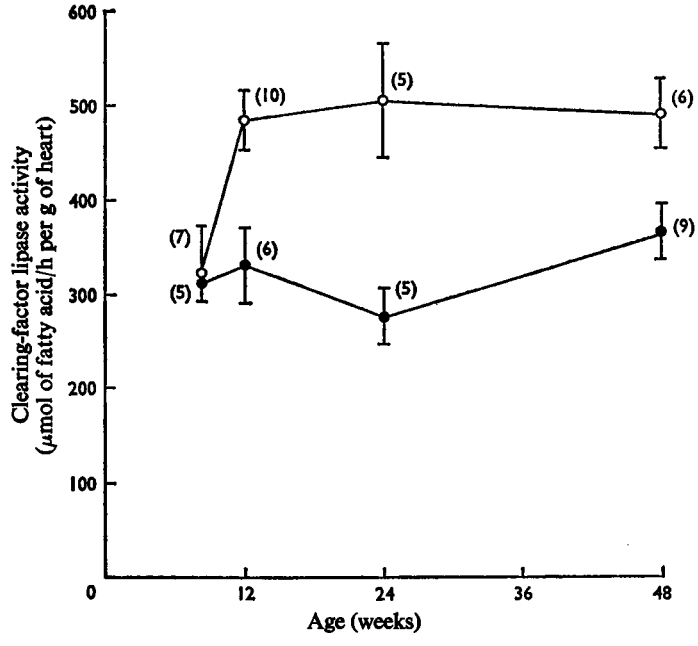

Fig. 2. *Effect of age on the clearing-factor lipase activity of heart muscle from lean and obese mice*

Acetone–ether-dried powders of ventricles from individual mice were assayed in duplicate. ●, Lean mice; o, obese mice. Results are means and the bars represent the S.E.M. with the number of animals in parentheses. The weights of the mice were: 8-week lean 19.0±0.6g (6); 8-week obese 33.2±1.4g (6); 12-week lean 22.62±1.32g (6); 12-week obese 44.12±0.66g (10); 24-week lean 24.17±0.73g (5); 24-week obese 56.19±1.25g (5); 48-week lean 26.57±0.83g (9); 48-week obese 70.05±2.35g (6).

Table 1. *Effect of starvation on the clearing-factor lipase activity of heart muscle from lean and obese mice*

For experimental details see the text. Results are means±S.E.M. with the number of animals in parentheses.

Type of mouse	Period of starvation (h) ... Age (weeks)	Clearing-factor lipase activity (μmol of fatty acid released/h per g of fresh tissue) 0	24	48	72
Lean	12	330±40 (6)	318±49 (7)	310±16 (6)	320±39 (7)
Obese	12	484±30 (10)	432±32 (6)	428±23 (5)	267±40 (8)
Lean	24	279±29 (5)	294± 9 (11)		307±18 (6)
Obese	24	508±59 (5)	374±14 (12)		356±13 (7)

	Period of starvation (h) ... Age (weeks)	Weight (g)	Weight loss after starvation (g) 24	48	72
Lean	12	21.51±1.01	2.27±0.12	3.57±0.09	4.35±0.10
Obese	12	43.01±0.50	3.30±0.16	5.09±0.40	6.04±0.23
Lean	24	25.24±0.79	2.33±0.28		4.25±0.59
Obese	24	58.91±0.94	3.52±0.22		5.99±0.51

Effect of starvation on the clearing-factor lipase activity of the heart

Starvation for periods of up to 72h did not affect clearing-factor lipase activity of hearts from lean, 12-week-old mice. However, there was a slight initial decrease in the activity of hearts from obese mice of the same age on starvation for 24h, and after 72h of starvation the activity in the hearts from obese animals was decreased below that of the lean animals (Table 1). With 24-week-old mice, in the static phase of obesity, the pattern was somewhat different in that the activity in hearts from obese mice fell more sharply during the first 24h without food and then remained essentially unchanged.

Epididymal fat-pad clearing-factor lipase activity

The clearing-factor lipase activity in epididymal fat-pads from obese mice was 87% higher than that in fat-pads from lean mice (31.0 compared with 16.6 μmol/h per g of tissue) (Fig. 3). Since the quantity of dry matter recovered as acetone–ether-dried powder from the fat-pads of the obese mice was lower than that from the fat-pads of the lean mice (23.4mg/g compared with 28.4mg/g), the activity in the fat-pads from obese mice was even greater on a fat-free basis. The mean weight of the pair of fat-pads from the obese mice was 1.75±0.08g (18), compared with 0.88±0.05g (12) for the lean mice. The total activity in the pair of fat-pads of the obese mouse was therefore 54.3 μmol/h, compared with 14.6 μmol/h for the fat-pads from lean mice, an increase of 3.7-fold.

Effect of starvation on the clearing-factor lipase activity of epididymal fat-pads

Starvation decreased the clearing-factor lipase activity in fat-pads from both lean and obese mice (Fig. 3). In the first 24h the activity/g of tissue decreased by 63% in the tissue from lean mice and by 42% in that from obese mice. Little change in activity occurred in either group between 24 and 48h of starvation, but between 48 and 72h the activity in the fat-pads from obese mice declined slightly, whereas the activity in those from the lean mice increased by 84%.

There was no significant change in the weight of the fat-pads from obese mice during 72h of starvation, so the mean final clearing-factor lipase activity/pair of fat-pads in the obese mouse was 26.0 μmol/h. The weight of the fat-pads from lean mice decreased from 0.88 to 0.50g and the enzyme activity in the lean mice after 72h of starving was therefore 4.16 μmol/h per pair of fat-pads: that is, the fat-pads of the obese mice finally had 6.25 times the activity of those of the lean mice.

Plasma concentrations of glyceride and cholesterol

At 8 and 12 weeks of age, plasma glyceride concentrations were 36 and 30% higher respectively in lean mice than in obese mice (Table 2). At 24 weeks of age the triglyceride concentrations were very similar and, at 48 weeks, the plasma triglyceride concentration in the obese mice exceeded that of the lean mice by 36%.

The total plasma cholesterol concentration in lean mice changed very little with age (Fig. 4), from 83mg/100ml at 8 weeks to 91mg/100ml at 48 weeks of age. In the obese mice, however, it was 60% higher than in the lean mice at 8 weeks of age and double that in the lean mice at 48 weeks. The unesterified cholesterol concentration was also higher in the obese mice, 34.6±2.4mg/100ml (8) compared with 20.6±1.7mg/100ml (7) at 8 weeks and 38.5±1.8mg/100ml (6) compared with 21.6±1.0mg/100ml (6) at 12 weeks.

Starvation for 72h increased total plasma cholesterol concentrations in both lean and obese mice. The greatest changes were observed in 12-week-old female

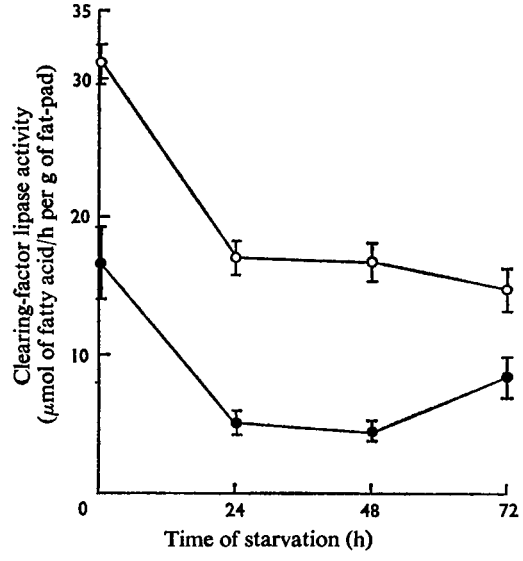

Fig. 3. *Effect of starvation on the clearing-factor lipase activity of epididymal fat-pads from lean and obese mice*

The activity was assayed in duplicate in acetone–ether-dried powders of fat-pads from individual mice. ●, Lean mice; ○, obese mice. Six obese and six lean mice were used at each stage. Bars represent the S.E.M. The lean mice weighed 31.29±0.59g and the weight decreased by 3.02±0.81g, 4.34±0.33g and 5.65±0.28g after starvation for 24, 48 and 72h respectively. The obese mice weighed 57.45±1.25g and the weight decreased by 3.45±0.36g, 4.79±0.38g and 6.25±0.58g after starvation for 24, 48 and 72h respectively. The mice were 24 weeks old.

Table 2. *Effect of age on concentration of plasma glycerides*

For experimental details see the text. Female mice were used. Results are means±S.E.M. with the number of animals in parentheses.

Type of mouse	Age (weeks) ...	Concn. of plasma glycerides (μmol/ml)			
		8	12	24	48
Lean		1.08±0.16 (9)	0.83±0.09 (9)	1.09±0.11 (6)	0.93±0.25 (6)
Obese		0.79±0.09 (8)	0.64±0.13 (9)	0.95±0.24 (5)	1.45±0.11 (5)

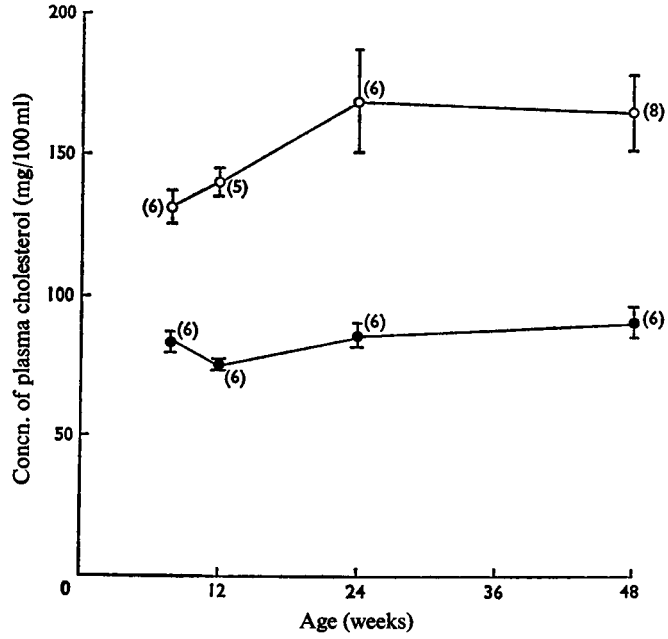

Fig. 4. *Effect of age on total plasma cholesterol concentrations in lean and obese mice*

Cholesterol was determined on plasma prepared from heparinized blood of fed mice. ●, Lean mice; ○, obese mice. Numbers of animals are given in parentheses and the bars represent the S.E.M.

mice, where the concentration in the lean mice increased by 32% and that in the obese mice by 40%. In 24-week-old male mice the increases were 11% (lean mice) and 33% (obese mice).

Discussion

The increases in tissue clearing-factor lipase activities described in the present paper add to the large number of metabolic variations described in obese hyperglycaemic mice (*ob/ob*). If the increased epididymal fat-pad activities are characteristic of those of adipose tissue as a whole in the obese animal, this would suggest an increased capacity of this tissue for triglyceride uptake and this could clearly be a factor contributing to the obesity. Again, if the activity in cardiac muscle is representative of that of the whole muscle mass and the increased activity of this organ is of the functional type, then triglyceride utilization by muscle may also be increased, at least in the adult animal.

Clearing-factor lipase is distributed both intracellularly and extracellularly and there is evidence that the extracellular enzyme is the active and functional form (Enser *et al.*, 1967; Cunningham & Robinson, 1969). Starvation is believed to result in a loss of the active enzyme in adipose tissue. However, the obese mice, although they contained much more clearing-factor lipase activity/g of epididymal fat-pad in the fed state, lost proportionately much less activity

than the lean mice during starvation for 24h. This suggests that part of the increased enzyme may be of the stable, intracellular, inactive type. The changes in the fat-pad clearing-factor lipase activity during starvation (Fig. 3) are the result of a decrease in the clearing-factor lipase activity and a loss of fat. When the fat-pad weight decreases more rapidly than the clearing-factor lipase activity there will be an increase in this activity/g of tissue. Such a change is seen in lean mice between 48 and 72h of starvation, but not in the obese mice, because the weight of their fat-pads did not decrease during starvation.

The mechanism by which clearing-factor lipase activity is regulated is not known. Wing *et al.* (1966) demonstrated that insulin promoted an increase in the activity of the enzyme in rat epididymal fat-pads, and a requirement for insulin for activity *in vivo* has been demonstrated in alloxan-diabetic rats (Meng & Goldfarb, 1959; Schnatz & Williams, 1963). Since hyperinsulinaemia is characteristic of obese mice of this strain (Christophe *et al.*, 1959; Stauffacher *et al.*, 1967), this could be one factor contributing to the increased clearing-factor lipase activity. Insulin presumably acts by inhibiting adenylate cyclase (Butcher *et al.*, 1968), thus decreasing the concentration of cyclic AMP in the fat-cell and so favouring an increase in clearing-factor lipase activity (Wing & Robinson, 1968). I have previously found a decreased activity of adenylate cyclase in epididymal fat-pads of obese mice (Enser, 1970), but it is not known if insulin is responsible for this effect.

Hollenberg (1960) and Borensztajn *et al.* (1970) observed an increase in the total clearing-factor lipase activity of the rat heart during starvation. We did not find this in the mouse, although it is possible that such an increase might be over after 24h in the smaller animal.

Alloxan diabetes has also been reported to increase the clearing-factor lipase activity of rat heart (Kessler, 1963), and the finding of a higher activity in the obese mice with their hyperinsulinaemia was therefore unexpected. However, the musculature of obese mice is known to be highly resistant to the effects of insulin (Stauffacher *et al.*, 1967; Genuth *et al.*, 1971). Moreover the increase in the heart clearing-factor lipase activity occurs in the obese mice between 8 and 12 weeks of age and much later, therefore, than the development of obesity, hyperinsulinaemia and muscle resistance to insulin (Genuth *et al.*, 1971).

The concentrations of total plasma cholesterol found in the present study agree well with those of Mayer & Jones (1953), but unesterified cholesterol has not previously been determined in obese mice. In the obese mice hypercholesterolaemia preceded the increase in heart clearing-factor lipase activity, but since, during starvation, the activity of clearing-factor lipase decreased while plasma cholesterol concentrations increased there is no correlation between plasma cholesterol concentrations and clearing-factor lipase activity. Nor does the present study indicate any correlation between plasma triglyceride concentrations and tissue clearing-factor lipase activity.

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