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Clearing-Factor Lipase in Adipose Tissue

A POSSIBLE ROLE OF ADENOSINE 3′,5′-(CYCLIC)-MONOPHOSPHATE IN THE REGULATION OF ITS ACTIVITY

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1. The rise in clearing-factor lipase activity that occurs when epididymal fat bodies from starved rats are incubated in appropriate media *in vitro* is inhibited in the presence of 6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP (1mM). 2. Inhibition occurs at a concentration of glucose in the incubation medium of 1·3mg./ml. or less, but not at a glucose concentration of 2·4mg./ml., unless caffeine (1mM), an inhibitor of 3′,5′-(cyclic)-nucleotide phosphodiesterase, is also present. Caffeine (5mM) alone inhibits the rise in clearing-factor lipase activity at a glucose concentration of 2·4mg./ml. of medium. 3. The concentration of free fatty acids in the epididymal fat bodies normally falls during incubations *in vitro* as the rise in clearing-factor lipase activity occurs. In the presence of 1mM-6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP, however, either the tissue free fatty acid concentration is increased or it does not fall to the same extent. The concentration of glucose in the incubation medium is important in determining the direction and extent of the changes in tissue free fatty acid concentration that occur in the presence of 6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP. 4. Free fatty acid concentrations in epididymal fat bodies *in vivo* rise as the clearing-factor lipase activity of the tissue falls during starvation. 5. The possibility that the concentration of 3′,5′-(cyclic)-AMP in adipose tissue may regulate clearing-factor lipase activity, and that the regulation may occur through effects of the nucleotide on tissue free fatty acid concentrations, is discussed.

The enzyme clearing-factor lipase or lipoprotein lipase is believed to regulate the uptake of triglyceride fatty acids by the extrahepatic tissues. It is thought to act at, or close to, the surface of the capillary endothelial cells of particular tissues on triglycerides in the circulating blood to produce FFA.* These, according to the physiological state of the animal and the tissue concerned, are either oxidized immediately or re-esterified and stored as triglycerides (see Robinson, 1963, 1968).

The uptake of circulating triglyceride fatty acids by adipose tissue and their storage therein as triglycerides is of particular significance in relation to the present work. As with the uptake of glucose and its storage as glycogen by the liver and skeletal muscle, the process is of primary importance when the energy stores of the body are being built up: for instance, it occurs in the fed state but not in the starved one (Bragdon & Gordon, 1958). Consistent with a role of clearing-factor lipase in regulating this uptake is the finding that the activity of the enzyme in adipose tissue is high in fed but low in starved animals (Hollenberg, 1959; Cherkes & Gordon, 1959; Páv & Wenkeová, 1960; Robinson, 1960).

In recent work to study how the variations in adipose-tissue clearing-factor lipase activity might be brought about, intact epididymal fat bodies from starved rats were incubated *in vitro* in a medium, designated CRM, that contained dialysed serum, glucose and heparin (Salaman & Robinson, 1966; Wing, Salaman & Robinson, 1966). A progressive increase in the clearing-factor lipase activity towards that characteristic of tissue from fed animals occurred. The extent of the increase was markedly influenced by the hormonal composition of the incubation medium and, notably, was enhanced by insulin and inhibited by catecholamines.

Insulin, on the one hand, and catecholamines, on the other, have marked and opposing actions on both the synthesis and the breakdown of the

*Abbreviations: FFA, free fatty acid(s); CRM, complete reconstituted medium.

glycogen stores of the body; these actions are exerted through appropriate inhibiting or activating effects on the enzymes glycogen phosphorylase (EC 2.4.1.1) and UDP-glucose–glycogen glucosyltransferase (glycogen synthetase, EC 2.4.1.11) (Caputto, Barra & Cumar, 1967). Mobilization as FFA of triglycerides stored in adipose tissue is also under hormonal control, being increased by catecholamines and inhibited by insulin, through the activation and inhibition respectively of a lipase in adipose tissue that is distinct from the clearing-factor lipase (Jungas & Ball, 1963; Rizack, 1964; Butcher, Ho, Meng & Sutherland, 1965). All these hormonal effects are now thought to be mediated through changes in the concentration of 3′,5′-(cyclic)-AMP in the tissues concerned (see Butcher & Sutherland, 1967).

In view of the hormonal effects on clearing-factor lipase activity already observed and the proposed role of the enzyme in regulating the uptake of triglyceride fatty acids from the circulation for replenishment of the adipose-tissue triglyceride stores, it seemed that 3′,5′-(cyclic)-AMP might also be concerned in determining the activity of this enzyme (Robinson, 1968). The present work provides evidence consistent with this hypothesis.

MATERIALS AND METHODS

Animals. Epididymal fat bodies were taken from male albino rats of the Wistar strain as described by Salaman & Robinson (1966). The animals had been either given their normal laboratory diet (Oxoid pasteurized breeding diet) until they were killed, or starved for periods of up to 48hr. They weighed between 120 and 145g. before starvation and between 100 and 130g. after starvation for 48hr. Fat bodies from fed animals weighed between 85 and 155mg., with a mean weight (±S.D.) for 40 fat bodies of 127 (±23) mg. For animals starved for 48hr., the range was between 50 and 145mg. with a mean (±S.D.) for 75 fat bodies of 90 (±23) mg.

Technique of incubation of fat bodies. Most of the experiments that are reported involved the incubation of groups of epididymal fat bodies in particular media. The methods used, including that of pairing fat bodies, have been described previously (Salaman & Robinson, 1966; Wing *et al.* 1966; Wing & Robinson, 1968). The media are described as modifications of that (CRM) used by Salaman & Robinson (1966), differing from it only with respect to glucose concentration or in the presence of additional components. Details are given with the individual experiments.

Assay of clearing-factor lipase. Clearing-factor lipase activity was measured in epididymal fat bodies immediately after their removal from the animal and also in systems consisting of epididymal fat bodies and an incubation medium combined. In the latter case measurements were made after the systems had been incubated for 3hr. at 37°. Heparin was a component of the incubation medium and, consequently, at the end of the incubation enzyme activity was distributed between the fat bodies and the medium (Salaman & Robinson, 1966; Wing *et al.* 1966). The enzyme was therefore assayed in the fat bodies and medium combined.

The method of assay has been described in detail previously (Salaman & Robinson, 1966; Wing & Robinson, 1968). Briefly, it involved the making of acetone–ether-dried preparations and the determination of the ability of the enzyme in homogenates of such preparations to hydrolyse chylomicron triglycerides to FFA, which could be measured by titration. When the acetone–ether-dried preparations were made from fat bodies immediately after their removal from the rats, a slight modification of the technique described by Wing & Robinson (1968) was used. The fat bodies were first homogenized in serum containing heparin (0·5unit/ml.), 2ml. of serum being used for each fat body. The homogenizer was rinsed with 2ml. of 0·9% NaCl solution and the rinse was added to the homogenate. The whole was poured into 200ml. of cold acetone and the procedure described by Salaman & Robinson (1966) was then followed. A similar modification was used when preparations were made after the fat bodies had been incubated for 3hr. In this case the fat bodies and the incubation medium were first homogenized together and then, with a 2ml. rinse of 0·9% NaCl solution, poured into the cold acetone.

The medium used to assay the enzyme in homogenates of acetone–ether-dried preparations was slightly modified from that described by Salaman & Robinson (1966) and had the following composition: 2vol. of 20% (w/v) albumin in water, pH8·1; 1vol. of a chyle–serum mixture (1:1, v/v); 1vol. of 0·7M-tris–HCl buffer, pH8·1 at room temperature; 0·5vol. of heparin (14 units/ml.). Usually 2·5ml. of the homogenate was added to 4·5ml. of this assay medium at 37° and incubation was carried out in a shaking water bath for either 1·5 or 2hr. FFA were estimated before and after the incubation as described previously (Salaman & Robinson, 1966).

Enzyme activities are expressed as μmoles of FFA released from the chylomicron triglyceride substrate/fat body/hr. of assay (Salaman & Robinson, 1966). The fat bodies were weighed in all the experiments reported, but expression of the results in terms of fat-body weight does not affect the conclusions.

In the present experiments the mean (±S.D.) initial activity of the enzyme in five fat bodies removed from five animals that had been starved for 48hr. was 1·8 (±0·2) μmoles of FFA released/fat body/hr. of assay.

Measurement of FFA concentration in tissue and medium. This was carried out by the modification of the method of Dole (1956) described by Trout, Estes & Friedberg (1960). The modification eliminates acidic phospholipids and lactic acid from the extracts containing FFA by introducing an additional washing stage. Titration of the FFA was by the method described by Salaman & Robinson (1961).

Normally, after their removal from the animal, the epididymal fat bodies were briefly rinsed in 0·9% NaCl solution, blotted dry and weighed. They were then minced coarsely and homogenized in the extraction mixture described by Dole (1956), except that 2N-H_2SO_4 instead of N-H_2SO_4 was used (Dole & Meinertz, 1960). A volume of 5ml. of extraction mixture was used for each fat body. When tissue FFA concentrations were determined after incubation, a similar procedure was adopted.

Some experiments were carried out in which the fat bodies were frozen by immediately clamping them between

aluminium plates cooled in liquid N_2, the technique being a modification of that described by Wollenberger, Ristau & Schoffa (1960). The fat bodies were then ground finely in a mortar in the frozen state before being added to the extraction mixture. The FFA concentration in tissue extracted in this way did not differ significantly from that of tissue extracted without prior freezing.

It was found necessary to adhere to the times described by Dole & Meinertz (1960) for tissue extraction and subsequent separation of the extraction mixture into two phases. When these times were greatly exceeded, hydrolysis of some of the tissue triglycerides occurred and the tissue FFA content was significantly increased. That this increase was due to triglyceride hydrolysis, rather than to more complete FFA extraction, was shown in control experiments with purified triglycerides and ^{14}C-labelled FFA.

Tissue FFA concentrations are expressed as μmoles of FFA/fat body, each value being based on the mean of at least two titrations. Expression of the results in terms of fat-body weight does not affect the conclusions. FFA concentrations in the medium (μmoles of FFA/ml.) are based on the results of duplicate or triplicate titrations of 1 ml. samples of the incubation media.

Glucose concentration in incubation media. This was determined, in duplicate, at the beginning and end of the incubations, by the method of Huggett & Nixon (1957).

Statistical analysis. Where the significance of the differences between means was calculated, the Behrens test was used (Fisher & Yates, 1957). A probability value, P, of $< 0{\cdot}05$ was considered significant.

Chemicals. Sources of most of the constituents of the incubation media have been given by Salaman & Robinson (1966), Wing *et al.* (1966) and Wing & Robinson (1968). 6-*N*-2′-*O*-Dibutyryl-3′,5′-(cyclic)-AMP was purchased from Boehringer Corp. (London) Ltd., London, W. 5, and caffeine and theophylline were from British Drug Houses Ltd., Poole, Dorset.

The experiments to determine whether hydrolysis of triglycerides occurred during the extraction of FFA from adipose tissue were carried out with triolein and tripalmitin mixtures, from which traces of FFA had been removed by treatment with Florisil (Carroll, 1961; Enser, Kunz, Borensztajn, Opie & Robinson, 1967), and [1-^{14}C]oleic acid and [1-^{14}C]palmitic acid purchased from The Radiochemical Centre, Amersham, Bucks. The triglycerides were obtained from British Drug Houses Ltd.

RESULTS

The experimental portion of this study falls into two parts. In the first, it is shown that a derivative of 3′,5′-(cyclic)-AMP inhibits the rise in clearing-factor lipase activity that occurs when epididymal fat bodies from starved rats are incubated in an appropriate medium *in vitro*. Changes that occur in the FFA concentrations in tissue and medium during such incubations are altered in the presence of the derivative. In the second portion of the work, therefore, the possibility that changes in FFA concentration could themselves affect the clearing-factor lipase activity of adipose tissue is examined.

Effects of 6-N-2′-O-*dibutyryl*-3′,5′-(*cyclic*)-*AMP on the rise in clearing-factor lipase activity that occurs when epididymal fat bodies from starved rats are incubated* in vitro

3′,5′-(Cyclic)-AMP does not penetrate into cells freely (Sutherland, Øye & Butcher, 1965), and where the action of this nucleotide on intact tissues is studied it has been usual to employ the derivative 6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP. Use of this compound has the further advantage that it is less readily broken down by 3′,5′-(cyclic)-nucleotide phosphodiesterase than is 3′,5′-(cyclic)-AMP itself (Posternak, Sutherland & Henion, 1962).

In CRM. Preliminary experiments were carried out in which epididymal fat bodies from starved rats were incubated for 3 hr. in the complex medium, CRM, that had been used in our earlier work (Salaman & Robinson, 1966; Wing *et al.* 1966). The rise in clearing-factor lipase activity that occurred was not inhibited in the presence of 1 mM-6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP.

Butcher *et al.* (1965) reported that, when epididymal fat bodies from starved rats were incubated in an albumin solution, the amount of FFA appearing in the medium was markedly increased in the presence of 6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP at a concentration of approx. 1 mM. They interpreted this finding as indicating that the lipase concerned with mobilization of the adipose-tissue triglycerides had been activated. However, with CRM, no evidence for activation of this lipase was obtained, insofar as the concentration of FFA in the medium in systems containing the nucleotide derivative was the same as that in systems without it.

In media containing glucose at concentrations of 0·2–1·3 mg./ml. The experiments of Butcher *et al.* (1965) were carried out with glucose-free media, whereas CRM contains glucose at a concentration of 2·4 mg./ml. The increase in clearing-factor lipase activity that occurs when fat bodies from starved rats are incubated in CRM is dependent on the presence of glucose in the medium (Wing *et al.* 1966). The effect of 6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP on this increase therefore cannot be studied in glucose-free media. However, the concentration of glucose required is relatively low. Maximal increases in activity occur at concentrations as low as 0·25 mg./ml., and even at 0·02 mg./ml. some increase is demonstrable.

The results in Table 1 show that 1 mM-6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP has a marked inhibitory effect on the increase in clearing-factor lipase activity at both 0·25 and 0·02 mg. of glucose/ml. of medium.

In the present experiments, the mean glucose concentration at 9 a.m. in the plasma of a group of

Table 1. *Effect of* 6-N-2′-O-*dibutyryl*-3′,5′-(*cyclic*)-*AMP on the clearing-factor lipase activity and FFA concentration of epididymal fat bodies incubated* in vitro

In each test, two fat bodies, each from a starved rat, were incubated at 37° in 5ml. of a medium having the composition of CRM except that glucose was present at the concentration shown. The paired fat bodies were incubated in 5ml. of the same medium containing, in addition, 6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP (1mM). After 3hr. either clearing-factor lipase was measured in acetone–ether-dried preparations made from the fat bodies and the medium combined or FFA were determined in the incubation medium and in the fat bodies. Mean values are expressed ±S.D. with the numbers of tests in parentheses. *P* values relating tests made in the presence of 6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP to those made in its absence are shown; N.S., not significant.

Concn. of glucose in medium (mg./ml.)	6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP ...	Final clearing-factor lipase activity (μmoles of FFA/fat body/hr.)		Increase in FFA concn. in medium (μmole/ml.)		Final tissue FFA concn. (μmoles/fat body)	
		Absent	Present	Absent	Present	Absent	Present
0·02		5·0±1·5 (12)	2·2±0·6 (12)	0·22±0·03 (4)	0·76±0·16 (4)	0·22±0·06 (4)	2·07±0·82 (4)
		$P<0·001$		$P<0·001$		$P<0·01$	
0·25		10·6±2·6 (12)	5·3±1·5 (12)	0·22±0·06 (7)	0·40±0·14 (8)	0·24±0·08 (7)	0·71±0·61 (8)
		$P<0·001$		$P<0·01$		N.S.	
0·75		11·0±1·6 (7)	7·3±0·9 (7)	0·28±0·05 (8)	0·40±0·08 (8)	0·23±0·05 (8)	0·35±0·22 (8)
		$P<0·001$		$P<0·01$		N.S.	
1·3		11·5±2·7 (8)	7·9±2·5 (8)	0·31±0·05 (6)	0·45±0·06 (6)	0·23±0·07 (6)	0·31±0·12 (6)
		$P<0·02$		$P<0·01$		N.S.	

six rats on their normal diet was 1·3mg./ml. The concentration in rats that have been starved for 48hr. is about 0·75mg./ml. (Wing *et al.* 1966). It was decided to study the effects of the 3′,5′-(cyclic)-AMP derivative at these physiological concentrations of plasma glucose. The results in Table 1 show that 1mM-6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP has an inhibitory effect on the increase in clearing-factor lipase activity that occurs in media containing glucose at each of these concentrations. The degree of inhibition, though less than at a glucose concentration of 0·25mg./ml., is similar at each concentration and significant.

The possibility that the utilization of glucose in the medium might be altered in the presence of 6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP was investigated in these experiments. At an initial concentration of glucose of 0·25mg./ml. of medium, the concentration after incubation for 3hr. fell to between 0·15 and 0·19mg./ml. However, there were no significant differences in the values found in the absence and presence of the 3′,5′-(cyclic)-AMP derivative.

The changes in these experiments in FFA concentration in medium and tissue are considered below.

In CRM containing caffeine. Caffeine is a potent inhibitor of the phosphodiesterase that normally breaks down 3′,5′-(cyclic)-AMP in adipose tissue (Rizack, 1964). As such it was used by Butcher *et al.* (1965), at a concentration of 1mM, to potentiate the actions of those hormones that, by raising the adipose-tissue 3′,5′-(cyclic)-AMP concentration, activate the lipase concerned with mobilization of the stored triglycerides. The results in Table 2 show that when caffeine (1mM) as well as 6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP (1mM) is present in CRM, the rise in clearing-factor lipase activity is markedly inhibited, even at an initial concentration of glucose in the medium of 2·4mg./ml. Moreover, under these conditions, the changes in FFA concentration in the tissue and medium indicate that activation of the lipase concerned with mobilization of the adipose-tissue triglycerides occurs in the presence of the nucleotide derivative.

Changes in FFA concentration during the incubation in vitro *of epididymal fat bodies from starved rats*

In the absence of 6-N-2′-O-*dibutyryl*-3′,5′-(*cyclic*)-*AMP.* In the experiments described in Table 1, the mean (±S.D.) initial tissue concentration of FFA measured in eight fat bodies taken from eight starved rats was 0·60 (±0·15) μmole/fat body. The initial FFA concentration in the medium was 0·20μmole/ml. During the incubations in the absence of the 3′,5′-(cyclic)-AMP derivative, therefore, the FFA concentration in the medium rises and the tissue FFA concentration falls as the

Table 2. *Effect of* 6-N-2′-O-*dibutyryl*-3′,5′-(*cyclic*)-*AMP and caffeine on the clearing-factor lipase activity and FFA concentration of epididymal fat bodies incubated* in vitro

In each test, two fat bodies, each from a starved rat, were incubated at 37° in 5ml. of CRM (glucose concn., 2·4mg./ml.). The paired fat bodies were incubated in 5ml. of the same medium containing, in addition, 6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP (1mM) and caffeine (1mM). After 3hr. either clearing-factor lipase was measured in acetone-ether-dried preparations made from the fat bodies and medium combined or FFA were determined in the incubation medium and in the fat bodies. Mean values are expressed ±S.D. with the numbers of tests in parentheses. *P* values relating tests made in the presence of 6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP and caffeine to those made in their absence are given.

Caffeine and 6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP	Final clearing-factor lipase activity (μmoles of FFA/fat body/hr.)	Increase in FFA concn. in medium (μmoles/ml.)	Final tissue FFA concn. (μmoles/fat body)
Absent	10·7±3·2 (5)	0·11±0·03 (4)	0·35±0·06 (4)
Present	3·1±1·1 (5)	1·01±0·02 (4)	2·56±0·33 (4)
	$P<0·001$	$P<0·001$	$P<0·001$

Table 3. *Changes in FFA concentration when epididymal fat bodies from starved rats are incubated* in vitro

In each test, a fat body from a starved rat was incubated at 37° in 2·5ml. of a medium having the composition of CRM except that glucose was present at a concentration of 0·25mg./ml. instead of 2·4mg./ml. After various times, FFA were estimated in the medium and in the fat body. The results at each time are expressed as means ±S.D. with the numbers of tests in parentheses.

Incubation time (hr.)	Total FFA in medium (μmoles/2·5ml.)	Total tissue FFA (μmole/fat body)
0	0·38	0·62±0·16 (6)
1·5	0·75±0·03 (4)	0·17±0·04 (4)
3	1·25±0·27 (4)	0·18±0·08 (4)
6	1·65±0·09 (4)	0·23±0·07 (4)

clearing-factor lipase activity of the system increases.

These findings indicate that FFA move from the tissue into the medium during the incubation (see Jungas & Ball, 1963). However, the rise in the amount of FFA in the total medium volume of 2·5ml./fat body is greater than the decline in the amount in the tissue. Moreover, in further experiments at a glucose concentration of 0·25mg./ml. of medium, it was shown that, whereas the tissue concentration of FFA falls to its lowest level after 1·5hr., the concentration in the medium continues to rise for at least 6hr. (Table 3). These experiments indicate that an additional source is contributing FFA to the medium and it seems probable that tissue or medium triglycerides are being hydrolysed during the incubation period.

In the presence of 6-N-2′-O-*dibutyryl*-3′,5′-(*cyclic*)-*AMP*. The rise in FFA concentration in the medium that occurs when fat bodies are incubated in media containing either 0·02 or 0·25mg. of glucose/ml. is always greater in the presence of the 3′,5′-(cyclic)-AMP derivative than in its absence (Table 1). At a glucose concentration of 0·02mg./ml., the tissue FFA concentration rises markedly above the initial value of 0·60μmole/fat body, instead of falling as in the absence of the derivative. At the higher glucose concentration of 0·25mg./ml., the changes in tissue FFA concentration are more variable, as shown by the high S.D. value. Though the mean final tissue concentration is slightly greater than the mean initial, in some individual experiments it was as low as in the absence of the 3′,5′-(cyclic)-AMP derivative, whereas in others marked increases in concentration occurred.

At higher glucose concentrations of 0·75 and 1·3mg./ml., the rise in FFA concentration in the medium in the presence of the 3′,5′-(cyclic)-AMP derivative is still significantly greater ($P<0·01$) than that which occurs in its absence (Table 1). However, in contrast with the situation at lower glucose concentrations, the tissue FFA concentration falls both in the absence and in the presence of the derivative; though the final mean values are somewhat higher in the presence of 6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP than in its absence, the differences between the means are not significant.

At an initial glucose concentration of 2·4mg./ml., but in the presence of both caffeine and 6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP, FFA concentrations rise markedly above the initial values both in the medium and in the tissue (Table 2).

These changes in FFA concentration presumably reflect primarily the extent of activation of the lipase concerned with hydrolysis of the adipose-tissue triglycerides (Butcher *et al.* 1965). Additional factors that may be operating are considered below.

FFA concentration and adipose-tissue clearing-factor lipase activity

In the experiments just described the inhibitory effect of 6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP on

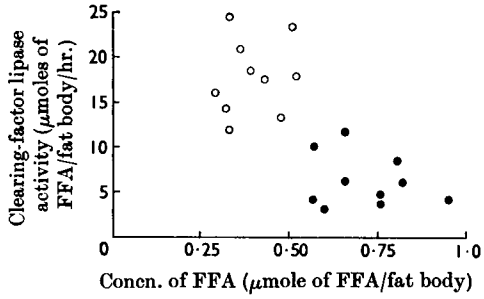

Fig. 1. Clearing-factor lipase activity and FFA concentration in rat epididymal fat bodies after a short period of starvation. Each point represents a test on the fat bodies of two rats. In each test two fat bodies were taken for measurement of clearing-factor lipase activity and the two paired fat bodies were analysed for FFA. Tests were carried out on fat bodies removed either between 9 a.m. and 9.30 a.m. (○) or between 3 p.m. and 3.30 p.m. in rats that had been starved from 9 a.m. (●).

clearing-factor lipase activity could have been exerted through alterations in FFA concentration brought about by the nucleotide derivative. Further experiments were therefore carried out to see whether, in other situations, changes in the activity of the enzyme were accompanied by changes in FFA concentration.

Changes in adipose-tissue clearing-factor lipase activity and FFA concentration during starvation. The initial rate of decline in the clearing-factor lipase activity of epididymal fat bodies during starvation in the rat is rapid. In animals starved from 8 a.m., for example, the activity falls by at least 50% in 6hr. (Wing & Robinson, 1968). The concentration of FFA in rat adipose tissue is known to rise during starvation as the adipose-tissue triglycerides are mobilized (Buckle, 1963; Butcher *et al.* 1965; Stoner & Matthews, 1966), but the effect of such a short period of starvation as 6hr. is not known, so far as we are aware. The results in Fig. 1 show that, in fact, the fall in adipose-tissue clearing-factor lipase activity in rats starved from 9 a.m. to 3 p.m. is accompanied by a rise in the tissue FFA concentration.

Effect of caffeine on adipose-tissue clearing-factor lipase activity and FFA concentration. Control studies carried out with the experiments described in Table 2 showed that a concentration of 1mM-caffeine in the medium did not by itself inhibit the rise in clearing-factor lipase activity that occurred when fat bodies from starved rats were incubated in CRM. However, with caffeine alone at 5mM, inhibition of the rise in clearing-factor lipase activity was observed. This inhibition occurred without any effect on the concentration of FFA in the tissue or medium (Table 4). Inhibition by 5mM-caffeine of the rise in clearing-factor lipase activity has already been reported briefly (Robinson, 1968).

Table 4. *Effect of caffeine on the clearing-factor lipase activity and FFA concentration of epididymal fat bodies incubated* in vitro

In each test, two fat bodies, each from a starved rat, were incubated at 37° in 5ml. of CRM. The paired fat bodies were incubated in 5ml. of the same medium containing caffeine (5mM) in addition. After 3hr. either clearing-factor lipase was measured in acetone–ether-dried preparations made from the fat bodies and the medium combined or FFA were determined in the incubation medium and in the fat bodies. Mean values are expressed ±S.D. with the numbers of tests in parentheses. *P* values relating tests made in the presence of caffeine to those made in its absence are shown; N.S., not significant.

Caffeine	Final clearing-factor lipase activity (μmoles of FFA/fat body/hr.)	Increase in FFA concn. in medium (μmole/ml.)	Final tissue FFA concn. (μmole/fat body)
Absent	12·4±2·1 (7)	0·16±0·07 (9)	0·22±0·04 (9)
Present	7·0±0·9 (7)	0·14±0·05 (9)	0·21±0·07 (9)
	$P<0·001$	N.S.	N.S.

Similar inhibitory effects on the rise in clearing-factor lipase activity have been obtained with 5mM-theophylline, another inhibitor of the phosphodiesterase that breaks down 3′,5′-(cyclic)-AMP (Sutherland *et al.* 1965). FFA concentrations were not measured in these studies.

DISCUSSION

3′,5′-(*Cyclic*)-*AMP and the regulation of adipose-tissue clearing-factor lipase activity*

The observations made with 6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP and with caffeine in the present study suggest that 3′,5′-(cyclic)-AMP could function as an inhibitor of clearing-factor lipase in adipose tissue. They are consistent with our earlier findings on the inhibitory effects of catecholamines (Wing *et al.* 1966), since the latter have been shown to raise the concentration of 3′,5′-(cyclic)-AMP in adipose tissue under appropriate conditions (Butcher *et al.* 1965).

The proposal that 3′,5′-(cyclic)-AMP may regulate the activity of the lipase concerned with mobilization of the adipose-tissue triglycerides is

based on the findings that the nucleotide is an activator of the lipase and that changes in its tissue concentration that are brought about by hormones, by caffeine and by 6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP *in vitro* can be correlated with changes in lipase activity (Rizack, 1964; Butcher *et al.* 1965; Butcher, Sneyd, Park & Sutherland, 1966). This, of course, does not imply that the tissue nucleotide concentration is the only determinant of the activity of this lipase. Its activity is higher in adipose tissue from starved animals than in tissue from fed animals, for example; but, to our knowledge, it has not been shown that the concentration of the nucleotide in adipose tissue is increased on starvation. The possibility has been raised that a rise in the amount of the lipase in the tissue, possibly as a result of growth hormone and corticosteroid action, may occur on starvation (Fain, Kovacev & Scow, 1965).

Similarly, though 3′,5′-(cyclic)-AMP may be one regulator of clearing-factor lipase activity, changes in its tissue concentration need not be responsible for the fall in the activity of the enzyme that occurs in adipose tissue on starvation. Nevertheless, the evidence that the activities of both clearing-factor lipase and the lipase concerned with triglyceride mobilization in adipose tissue are affected by 3′,5′-(cyclic)-AMP does raise the possibility that the concentration of the nucleotide may, in appropriate situations, control the balance between the storage and breakdown of triglycerides. It could therefore be a factor concerned in determining the extent of both triglyceride and glycogen (Caputto *et al.* 1967) storage in the tissues of the body.

Possible role of FFA in the regulation of adipose-tissue clearing-factor lipase activity

If 3′,5′-(cyclic)-AMP in adipose tissue is a regulator of the activity of clearing-factor lipase, its action need not be a direct one. Thus the activation by 3′,5′-(cyclic)-AMP of the lipase concerned with triglyceride mobilization could lead to alterations in FFA concentration that might themselves be responsible for the effects observed. Ho & Jeanrenaud (1967) have emphasized that a rise in the concentration of FFA in adipose tissue may have important secondary metabolic effects. Nikkilä & Pykälistö (1968) have shown that, after the injection of nicotinic acid, the activity of clearing-factor lipase in adipose tissue rises when FFA release is decreased, as a result of inhibition of the mobilization of triglycerides from the tissue.

In the tissue. In the present study, at low concentrations of glucose in the incubation medium, inhibition of the rise in activity of clearing-factor lipase in adipose tissue by 6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP *in vitro* was associated with a raised tissue FFA concentration at the end of the incubation period (Table 1). The tissue FFA concentration *in vivo* rose as the adipose-tissue clearing-factor lipase activity fell during starvation (Fig. 1).

These findings are certainly consistent with a mediating function of the tissue FFA concentration. However, in the experiments *in vivo*, paired groups of fat bodies were used for the measurement of FFA concentration and enzyme activity. It is clear from Fig. 1 that the clearing-factor lipase activity at a particular FFA concentration varied over a wide range. Conversely, similar enzyme activities were found at very different FFA concentrations. Thus it does not appear from these results that the tissue FFA concentration was an important regulator of the clearing-factor lipase activity. In support of this are the results of experiments *in vitro* with caffeine alone (Table 4), albeit at a concentration of 5mM, which is greater than that employed in other studies (Butcher *et al.* 1965), in which inhibition of the rise in clearing-factor lipase activity occurred in the absence of any effects on tissue FFA concentrations. Finally, detailed consideration of the experiments with 6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP shows that: (*a*) in the presence of 0·25mg. of glucose/ml. of medium, the tissue FFA concentrations at the end of the incubations were sometimes lower and sometimes higher than the initial ones and yet the rise in clearing-factor lipase activity was always diminished (Table 1); (*b*) in the presence of 0·75 or 1·3mg. of glucose/ml. of medium, significant inhibition of the increase in clearing-factor lipase activity was observed when the final mean tissue FFA concentrations were only slightly and not significantly greater than in the absence of the 3′,5′-(cyclic)-AMP derivative (Table 1).

These findings are difficult to reconcile with an important role of the tissue FFA, unless the pattern of change in tissue FFA concentration with time is markedly altered in the presence of 6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP or unless the FFA concentration in a particular cell compartment, rather than that in the whole tissue, is significant in this context.

In the incubation medium. Inhibition of the rise in clearing-factor lipase activity by 6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP is associated also with a rise in the FFA concentration in the incubation medium that is greater than that which occurs in its absence (Tables 1 and 2). However, Wing *et al.* (1966) have already shown that the FFA concentration in the medium can be raised to 1·4 μmoles/ml., by the prior addition of sodium oleate or palmitate, without significantly affecting the increase in clearing-factor lipase activity that occurs on incubation. The experiments were carried out at a

concentration of glucose in the medium of 2·4mg./ml., but they have been confirmed at a concentration of 0·25mg./ml. (D. R. Wing & D. S. Robinson, unpublished work). The final FFA concentration in the medium was considerably higher than that reached in the presence of 6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP in the experiments of the present study. It should be emphasized that the tissue FFA concentration at the end of the incubation is not significantly affected by the addition of FFA to a concentration of 1·4 μmoles/ml. of medium.

Inhibition of the increase in clearing-factor lipase activity was observed when sodium oleate was added to the incubation medium to an initial concentration of 2·8 μmoles/ml. (D. R. Wing & D. S. Robinson, unpublished work). However, such FFA concentrations are considerably in excess of those normally found in plasma *in vivo* (Carlson, Boberg & Högstedt, 1965) and, *in vitro*, exceed the concentration that can readily be bound by the plasma albumin in the incubation medium (Goodman, 1958). The significance of this finding is therefore uncertain.

Glucose, FFA and clearing-factor lipase activity in adipose tissue

In the experiments reported, 6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP did not increase the concentration of FFA in the medium when fat bodies were incubated in CRM containing glucose at a concentration of 2·4mg./ml. Moreover, at lower concentrations of glucose in the medium, the effectiveness of the nucleotide derivative in raising the FFA concentrations in the tissue and medium above those observed in its absence became less as the glucose concentration was increased (Table 1). The most reasonable interpretation of these findings is that a proportion of the FFA released are normally available for re-esterification and that the extent to which this process occurs increases as the glucose concentration rises (Jungas & Ball, 1963; Leboeuf, 1965; Vaughan & Steinberg, 1965). However, a direct inhibitory effect of glucose, or a metabolite thereof, on the activation of the lipase concerned with mobilization of the adipose-tissue triglycerides cannot be excluded on present evidence.

Glucose availability may also modify any direct action of 3′,5′-(cyclic)-AMP on clearing-factor lipase activity. Thus 1mM-6-*N*-2′-*O*-dibutyryl-3′,5′-(cyclic)-AMP alone was an effective inhibitor at a concentration of 1·3mg. or less of glucose/ml. of medium, but not at the higher glucose concentration of 2·4mg./ml. The inhibitory effects of the 3′,5′-(cyclic)-AMP derivative did not differ significantly at 0·8mg. and 1·3mg. of glucose/ml. of medium (Table 1), concentrations that are characteristic respectively of plasma from starved and fed rats. However, the possibility that the plasma glucose concentration, in conjunction with the tissue 3′,5′-(cyclic)-AMP concentration, might play a physiological role in the regulation of clearing-factor lipase activity in adipose tissue seems to require further consideration (cf. Wing *et al.* 1966).

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