A modified perfused incubation system for isolated fat-cells

Investigation of intermediary metabolism by perfusion

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Perfused fat-cells showed similar values for acylglycerol glycerol synthesis from glucose with insulin and for the effects of added palmitate to those in normal incubations and those reported in the literature. Fatty acid synthesis was lower in perfused cells compared with normal incubations, and there was a net release of fatty acids only with the perfused fat-cells. Hence fluxes of metabolites were different in the two incubation systems, and the perfusion system enables the investigation of the flux of metabolites under conditions which may more closely resemble those *in vivo*.

Adipose-tissue metabolism at the cellular level has been investigated by using several techniques. Each technique has certain limitations and disadvantages, some of which are indicated below. The incubation of fat pieces or fat-cells in flasks, which will be referred to as 'normal' incubations, has the disadvantage that it does not represent the environment *in vivo*, since the substrates and products of metabolism are not continually replenished and removed respectively (Rodbell, 1964; Francendese & Digirolamo, 1981). Determinations *in vivo*, perfusion of adipose tissue and perfusion of fat-cells do not allow the sampling of fat-cells to determine the flux of substrates into metabolic products (Robert & Scow, 1963; Allen *et al.*, 1973; Richter & Schwandt, 1981; Bülow & Madsen, 1981; Bülow, 1982). These techniques are usually used for the determination of lipolysis rates. But the net release of lipolytic fatty acids is related to their rate of re-esterification, which can only be determined indirectly by comparing glycerol release with fatty acid release (Bülow, 1982). The references quoted above are only representative of the original or current use of the techniques. The effect of various hormonal and other physiological conditions on blood flow rate in adipose tissue, and the relationship of this to adipose-tissue metabolism, has been reviewed by Rosell & Belfrage (1979).

Most investigations of adipose-tissue metabolism at the cellular level have been carried out in normal incubations. For many determinations, the effect of having a flow of medium, resulting in the setting up of steady states which may more closely

resemble those *in vivo*, has not been investigated. This paper introduces a modified perfusion system with which these effects can be investigated. In the modified system the metabolic products in the perfusate can be determined, fat-cells can be sampled and comparison made with fat-cells incubated normally.

Materials and methods

Chemicals and animals

Bovine serum albumin (fraction V) and pig insulin were obtained from Sigma (London) Chemical Co. (Poole, Dorset, U.K.). Albumin was treated to remove fatty acids as described by Chen (1967). Collagenase was obtained from Boehringer Corp. (London) (Lewes, East Sussex, U.K.). Palmitic acid was obtained from BDH Chemicals (Atherstone, Warwickshire, U.K.), converted into the sodium salt and associated with fatty-acid poor albumin as described by Evans & Mueller (1963). Radiochemicals were obtained from Amersham International (Amersham, Bucks., U.K.). Nucleopore polycarbonate filters (12.0 µm pore size) were a gift from Sterilin (Teddington, Middx., U.K.). All other chemicals were of A.R. grade and were used without further purification.

Male Wistar rats weighing 150–250 g were purchased from Griffin and George (East Preston, West Sussex, U.K.) or were bred in the animal colony at Luton College of Higher Education from these animals. The animals were maintained on cube diet 41B (Bruce & Parkes, 1949) with water supplied *ad libitum*.

Techniques with fat-cells

Preparation. Isolated epididymal fat-cells were prepared by the method of Rodbell (1964) as described by Saggerson & Tomassi (1971).

Perfusion chamber. The filter support was removed from the top of a Sartorius 25mm filter holder, and a hole was cut in the top, leaving a 2mm-wide lip. A polypropylene tube was sealed to the lip with Araldite epoxy resin. The bottom of the filter holder, together with a $12.0\mu\text{m}$ -pore filter to retain the fat-cells, was screwed on to complete the chamber (Fig. 1). Filters with $12.0\mu\text{m}$ pores were used, since this is less than the diameter of fat-cells reported by Zinder *et al.* (1967) and Malgieri *et al.* (1975).

Incubation of fat-cells in shaken perfused chambers. The perfusion chamber was back-perfused to remove air from the tubing and filter holder. Portions of fat-cells were incubated at 37°C in 8 ml (or the indicated volume) of Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932), with additions as indicated in the legend to Table 1. The buffer was previously gassed with O_2/CO_2 (19:1) and filtered through a $12\mu\text{m}$ -pore filter. Perfusion medium of the same composition as that in the chamber, except with Krebs–Ringer bicarbonate buffer with 1% albumin in place of the fat-cells, was warmed to 37°C and pumped in through the seal. The volumes of the perfusion medium and the perfusate were read at frequent intervals, and

adjustments were made to the inflow to maintain the correct volume in the chamber. O_2/CO_2 (19:1) was continuously passed into the air space above the incubation medium.

Flow rate. For dispersed fat-cells the actual flow rate is not a useful value; rather the rate of dilution of the extracellular medium is a better indication of the rate of provision and removal of metabolites. This is given by the relative flow rate (ml/min per ml of incubation medium), which was about 0.1 min^{-1} in these experiments (see Table 1 and the text). The extracellular water in epididymal fat-pads is about 20ml/100g (Denton *et al.*, 1966). From the blood-flow rate *in vivo* of 5ml/min per 100g of tissue determined by Bülow (1982) for dog subcutaneous adipose tissue, the rate of dilution of the extracellular water is 0.25 min^{-1} . These dilution rates are not directly comparable, however, since the plasma component of the extracellular water does not mix freely with the fluid in the interstitial space. Linde & Chisolm (1975) found that the plasma component has a volume about 60% that of the interstitial space, and factors affecting vascular permeability in adipose tissue have been discussed by Rosell & Belfrage (1979).

Incubation of fat-cells in shaken sealed chambers (normal incubations). The incubations were performed in perfusion chambers with a sheet of polythene below the filter to seal the chamber. The buffer was as described above.

Measurement of incorporation of ^{14}C into metabolic products. Samples were taken from the incubation chambers at 20min intervals (or as indicated), and lipids were extracted into hexane (Saggerson & Tomassi, 1971). The incorporation of ^{14}C into acylglycerol glycerol and acylglycerol fatty acids was determined as described by Saggerson & Greenbaum (1970). Small samples were taken from the perfused chamber, ensuring that the rate of dilution was only 8% higher during the final period than during the initial period of incubation.

The incorporation of ^{14}C into perfusate fatty acids was determined by extraction into hexane, then into 50mM- NaHCO_3 in ethanol/water (1:1, v/v); this was acidified and the fatty acids were extracted into hexane for counting of ^{14}C -labelled fatty acids, as described by Sooranna & Saggerson (1975) for lowering the background counts of [^{14}C]-palmitic acid.

Measurement of non-esterified fatty acids. Fatty acids were extracted into hexane and determined as described by Sooranna & Saggerson (1975), or extracted into hexane and determined by the method of Laurell & Tibbling (1967).

Measurement of fat-cell DNA. This was as described by Saggerson (1972).

Measurement of glycerol. This was by the method of Garland & Randle (1962).

Fig. 1. Perfusion chamber

Key: A, filter; B, rubber seal. Not drawn to scale.
Size of tube: 10cm $\times$ 2.5cm.

Calculations and statistics

When more than one sample was taken, rates of incorporation were determined from the slope of the regression line. Incorporations were corrected for any changes in the volume of the medium in the perfused chamber and for dilution by the added palmitate.

The concentration of fatty acids in the perfused incubation chamber at the time of sampling was determined by extrapolation from the initial value and the values for the perfusate. It was assumed that the value for the perfusate represented the mid-point of the time period for the collection of the perfusate.

Each determination was on a separate preparation of fat-cells, and statistical significance of results was determined by Student's *t* test.

Results and discussion

The incorporation of glucose into acylglycerol glycerol in the perfused incubation was the same as in the normal incubation, whereas perfusion decreased fatty acid synthesis by 35% compared with the normal incubation (Table 1a). Synthesis of neither acylglycerol glycerol nor fatty acids was affected by the decrease in fatty acid concentration in the incubation medium which is represented by that in the perfusate (Table 2a). Addition of

0.25 mM-palmitate stimulated acylglycerol glycerol synthesis by 128% and 114%, and fatty acid synthesis by 23% and 45%, in perfused and normal incubations respectively (Table 1b). Similar stimulations by palmitate were found by Saggerson (1972) in normal incubations. With palmitate, perfusion decreased fatty acid synthesis by 38% compared with normal incubations. The results suggest that the decrease in fatty acid synthesis by perfusion is independent of the concentration of exogenous fatty acid. However, a number of determinations over a range of concentrations would be necessary to confirm this suggestion.

By decreasing the incubation volume to 6.0 ml, the dilution rate was increased by 30% to 0.125 min^{-1} . This resulted in a 77% ($P < 0.01$, mean of three determinations) decrease in fatty acid synthesis by perfusion. Lipolysis has been shown to be dependent on the flow of medium in perfused adipose tissue (Scow *et al.*, 1965; Bülow & Madsen, 1981) and *in vivo* (Bülow, 1982). The results presented here show that fatty acid synthesis can also be markedly susceptible to the flow of medium. Whether this occurs under physiological conditions, and the mechanism for the decrease, remain to be determined.

The concentrations of fatty acids in the medium initially (Table 2) are similar to those found by Sooranna & Saggerson (1975) in incubations with fructose and insulin. There was no significant change in the concentration of fatty acids in the normal incubation (Table 2a), as was found by Angel *et al.* (1971) for incubations without glucose or insulin. The conditions reflect a balance between lipolysis and re-esterification. Lipolysis is limited by the activity of hormone-sensitive lipase and re-esterification by the supply of fatty acids, as discussed by Saggerson (1972). The concentration of fatty acids in the perfusate fell after 20 min of incubation (Table 2a). The values represent a net release of fatty acids, and this was calculated from the concentrations in the perfusate and in the incubation chamber (Table 2a; see the Materials and methods section). The values were 3.23, 0.91 and $0.78 \mu\text{mol}/100 \mu\text{g}$ of DNA for 0–20, 20–40 and 40–60 min incubation periods respectively. The pattern of fatty acid release is similar to that found by Scow *et al.* (1965), who used perfused parametrial fat-pad.

The proportion of glucose carbon incorporated into acylglycerol glycerol which could be attributed to the esterification of glucose-derived fatty acids was 8% and 6% for normal and perfused incubations respectively (calculated by the method of Sooranna & Saggerson, 1975). Therefore, assuming the complete esterification of acylglycerol glycerol, the release of fatty acids was not due to differences in the rate of re-esterification of fatty

Table 1. *Effects of perfusion and of palmitate on synthesis of fatty acids and acylglycerol glycerol*

Fat-cells were incubated for 1 h in Krebs–Ringer bicarbonate buffer containing 1% (w/v) albumin, 5 mM-[U- ^{14}C]glucose and insulin (140 nM). (a) No additions; (b) a separate series of determinations in which 0.25 mM-sodium palmitate was added after 20 min. The results are the means $\pm$ S.E.M. of four determinations. Initial relative flow rate was (a) $9.53 (\pm 0.09) \times 10^{-2}$ and (b) $9.65 (\pm 0.06) \times 10^{-2} \text{ min}^{-1}$. Mean fat-cell DNA was (a) 3.5 and (b) $4.8 \mu\text{g}/\text{ml}$. * $P < 0.05$, ** $P < 0.01$ for comparison of incubations with and without palmitate; † $P < 0.05$ for comparison of perfused with normal incubations.

Incubation system	Additions	Incorporation ($\mu\text{g-atoms}/\text{h}$ per $100 \mu\text{g}$ of DNA) into:	
		Acylglycerol glycerol	Acylglycerol fatty acids
(a) Normal	None	6.56 ± 0.68	8.86 ± 1.22
Perfused	None	6.15 ± 1.00	$5.90 \pm 0.53^\dagger$
(b) Normal	None	3.62 ± 0.61	5.73 ± 0.65
	Palmitate	$7.59 \pm 0.97^*$	$8.12 \pm 0.29^*$
Perfused	None	3.96 ± 0.64	3.99 ± 0.59
	Palmitate	$8.72 \pm 0.97^{**}$	$5.03 \pm 0.83^\dagger$

Table 2. *Effect of perfusion on fatty acid concentrations and the release of glucose-derived fatty acids*

The results were determined in the same experiments as described in Table 1. The results are the means $\pm$ S.E.M. of four determinations. * $P < 0.05$, ** $P < 0.05$, *** $P < 0.001$ for comparison with the previous period of incubation; † $P < 0.001$ for comparison of perfusate with the average value over the time period for the normal incubation.

Additions	None-esterified fatty acids (μM)				Perfusate glucose-derived non-esterified fatty acids (ng-atoms/ml per 100 μg of DNA)
	Time (min)	Normal incubation	Time (min)	Perfusate	
(a) None	0	47.0 $\pm$ 2.1	0	47.0 $\pm$ 2.1	
	20	48.5 $\pm$ 7.3	0–20	57.2 $\pm$ 4.3	1.32 $\pm$ 0.39
	40	54.8 $\pm$ 4.3	20–40	22.5 $\pm$ 1.7***, †	3.66 $\pm$ 0.32***
	60	49.5 $\pm$ 2.7	40–60	18.5 $\pm$ 2.4†	4.32 $\pm$ 0.32
(b) None	0	72 $\pm$ 12	0	72 $\pm$ 12	
	20	81 $\pm$ 13	0–20	72 $\pm$ 5	0.88 $\pm$ 0.19
	40	244 $\pm$ 9	20–40	195 $\pm$ 9	3.41 $\pm$ 0.60**
	60	167 $\pm$ 16*	40–60	166 $\pm$ 8	5.83 $\pm$ 1.14
Palmitate					

acids, since this was the same in the two systems. Angel *et al.* (1971) found that the non-esterified fatty acid content of rat epididymal fat-cells from rats of 160–230 g body wt. was about 0.5 $\mu\text{mol/g}$ of lipid. By using a value of 181 μg of DNA/g of lipid (mean of ten determinations, with rats of 150–180 g body wt.), this represents about 0.28 $\mu\text{mol}/100 \mu\text{g}$ of DNA, and indicates that depletion of intracellular non-esterified fatty acids could not contribute significantly to fatty acid release. Hence the net release of fatty acids was due to an increased rate of lipolysis in the perfused compared with the normal incubation, since this is the only source of fatty acid remaining. Under the conditions used, rates of lipolysis in the two systems cannot be compared by glycerol measurements, because the partial hydrolysis of triacylglycerol is significant. Perfusate glycerol concentrations only accounted at most for about 40% of the fatty acids released. This indicates that partial hydrolysis of triacylglycerol was occurring, and this has also been observed in normal incubations under the same conditions (Saggerson, 1972).

The pattern for the release of glucose-derived fatty acids is similar in the presence and absence of palmitate (Tables 2a and 2b). For the fatty acids released by perfusion in the absence of palmitate, the proportion of carbon derived from glucose was 0.09%, 0.50% and 0.66% for the 0–20, 20–40 and 40–60 min periods of incubation respectively (net release of ^{14}C -labelled fatty acids was calculated as for fatty acid release, and assuming the fatty acids have an average chain length of 17 carbon atoms). Zinder *et al.* (1973) showed that a pool containing newly synthesized triacylglycerol was preferentially hydrolysed during lipolysis. The results are consistent with the view that the released fatty acids, both labelled and unlabelled, were mainly derived from a lipolytic pool of fatty acids, as

discussed above, and that these fatty acids were derived from a lipolytic pool of triacylglycerol which contained an increasing proportion of newly synthesized triacylglycerol. The fatty acid pool which is a precursor for esterification of acylglycerol glycerol contained a constant proportion of glucose-derived fatty acids, as discussed above. These results support those of Dole (1961), Vaughan *et al.* (1964) and Winand *et al.* (1971), who found that the lipolytic pool of fatty acids is not in equilibrium with the precursor pool for esterification.

The results in the present paper show that, in the perfused incubation system, the fluxes of glucose and of fatty acids can be significantly different from those in normal incubations. The perfusion system may provide conditions for the determination of the fluxes of metabolites which are more representative of physiological conditions than are normal incubations.

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