Studies on the

Triglyceride - Fatty acid Cycle

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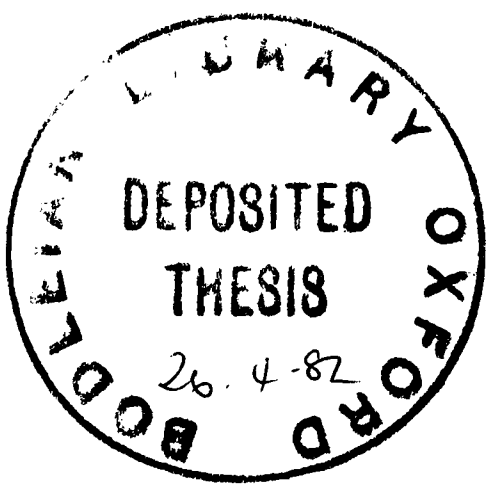

Trinity term
1981

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To

my wife

and

my parents

- * -

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Abstract

The triglyceride-free fatty acid (TG-FFA) cycle was studied in white adipose tissue. The major aims of the study were 1) to see if the rate of TG-FFA cycling (i.e. FFA reesterification) and the sensitivity properties (see Newsholme & Crabtree, 1976, Biochem. Soc. Symp. 41, 61-109) were affected by various treatments, and 2) to measure the rate of cycling *in vivo* and assess its contribution to the metabolic rate of an animal.

There are two ways of estimating the rate of TG-FFA cycling; the first is based on the release of glycerol and FFA from the tissue, and the second on the synthesis of the glyceryl and FFA moieties of triglyceride. Experimental agreement between the two methods is very good. It is shown that the rate of TG-glycerol synthesis can be estimated by measuring the incorporation of tritium from tritiated water into the TG-glycerol moiety; this method is used to study the TG-FFA cycle *in vivo*.

Experimental results indicated that the rate of TG-FFA cycling in white adipose tissue *in vitro* and *in vivo* is affected by various short- and long-term treatments. However, the reesterification of FFA in adipose tissue can only account for perhaps 1% of the basal metabolic rate of a mouse, and perhaps 4% of the increase in osygen consumption observed in fenoterol-treated mice.

The equations of Newsholme & Crabtree (1976) describing the sensitivity properties of substrate cycles are extended and used to show that the TG-FFA cycle increases the sensitivity of control of FFA release from adipose tissue. The degree of sensitivity attainable is variable depending on the treatment used.

The use of tritiated water for estimating TG-FFA cycling is tentatively extended to brown adipose tissue. It is suggested that the rate of cycling could be used as an indicator of sympathetic activity in brown and white adipose tissue.

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Chapter 2 Materials and methods

Chapter 3 Methods of measuring the rate of TG-FFA cycling

CHAPTER ONE

Introduction to substrate cycles

1.1 What is a substrate cycle?

In his book, "The integrative action of the nervous system", Sir Charles Sherrington (1947) made the following statement:

"Where two muscles would antagonise each other's action the reflex arc, instead of merely activating one of two, when it activates the one, causes depression of the other. The latter is an inhibitory effect".

These lines summarise Sherrington's idea that the integrative action of the nervous system ensured reciprocal inhibition of antagonistic muscles, the benefits being obvious. This principle is thought to have a parallel in the control mechanisms which regulate the flux through the various biochemical pathways present in the cell (see Tepperman and Tepperman, 1970). In this chapter, it is hoped to elucidate the possible advantages to be gained in the control of the flux through a metabolic pathway if the Sherrington metaphor is not followed to the full.

A metabolic pathway can be defined as "a series of reactions initiated by a flux-generating step and terminated by a product that is either a pathway-substrate for another flux-generating reaction or one that is transferred to the environment or to a metabolic 'sink', eg structural or storage material", (see Newsholme, 1979). The reactions in a pathway can be classified into two types; those which are close to equilibrium, and those that are far from equilibrium - non-equilibrium reactions. Changing environmental conditions may require that the direction of flux through a metabolic pathway is reversed. In an equilibrium reaction, the

the reversal of the direction of flux is achieved simply by small changes in the concentration of reactants and/or products (see Newsholme and Crabtree, 1976). The mechanism of the backwards reaction is a reversal of that of the forward reaction. However, altering the direction of flux in a non-equilibrium reaction by simply reversing the mechanism of the forward reaction would require very large changes in the concentrations of substrates and/or products. For several reasons, this method of reversing a non-equilibrium reaction is not possible. For example, large changes in the concentrations of metabolites would cause osmotic, solubility and ionic problems to the cell, and would also be liable to cause side-reactions. Also, large changes in concentration would require a long time to be completed, meaning the control of the pathway would be 'sluggish'. However, large changes in the concentrations of these metabolites can be avoided by using an enzyme different from that used in the forward reaction. This enzyme catalyses the reverse reaction using a chemical mechanism which is different from that of the forward reaction. Hence the possibility exists that two opposing non-equilibrium reactions, catalysed by different enzymes and using different reaction mechanisms, may by simultaneously active. The fact that the two reactions are opposing and non-equilibrium means that they require a source of energy, and this is commonly paid for by linking one of the reactions to the hydrolysis of ATP.

At first sight the loss of energy by having two opposing reactions appears wasteful and thus detrimental to the cell. This led to the name 'futile cycle' for any such combination of reactions. However, there are definite metabolic advantages to be gained by having such opposing non-equilibrium reactions simultaneously

active (see section 1.3), and hence the name 'substrate cycle' is considered more appropriate.

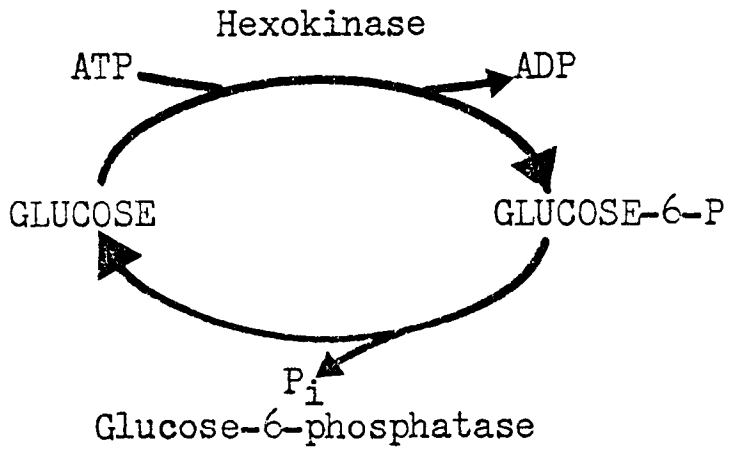
1.2 Examples of Substrate Cycles

If two sets of opposing reactions are found in the same compartment of the cell, the problem arises as to how the enzymes are controlled so that when the flux is in one direction, the reverse enzyme is totally inhibited (and vice versa). As Newsholme and Crabtree (1976) point out, such a situation is very unlikely if not impossible; the efficiency of metabolic control cannot be such as to totally inhibit the activity of an enzyme. It would require the enzyme protein to be destroyed and its synthesis prevented. Consequently, when two such opposing enzymes exist in the same compartment they will both be simultaneously active and catalyse a substrate cycle.

Using the criterion of two opposing non-equilibrium reactions together with the present knowledge of metabolic pathways, potential sites of substrate cycling can be predicted. The classical examples of substrate cycles in a metabolic pathway are the ones which are possible in the glycolytic and gluconeogenic pathways. The conversions between glucose and glucose-6-phosphate, between fructose-6-phosphate and fructose-1,6-diphosphate, and between phosphoenolpyruvate, pyruvate and oxaloacetate are all possible substrate cycles. The breakdown and synthesis of triglyceride is another cycle which is possible in a metabolic pathway. Obviously, there are many examples of potential substrate cycles, and they are not peculiar to metabolic pathways. The control of the activity of an enzyme by a phosphorylation/dephosphorylation mechanism is similar to a substrate cycle. In this cycle the two opposing reactions are catalysed by the protein kinase and phosphatase, and the phosphorylated

Fig 1.1 Examples of possible substrate cycles (see section 1.2)

1.



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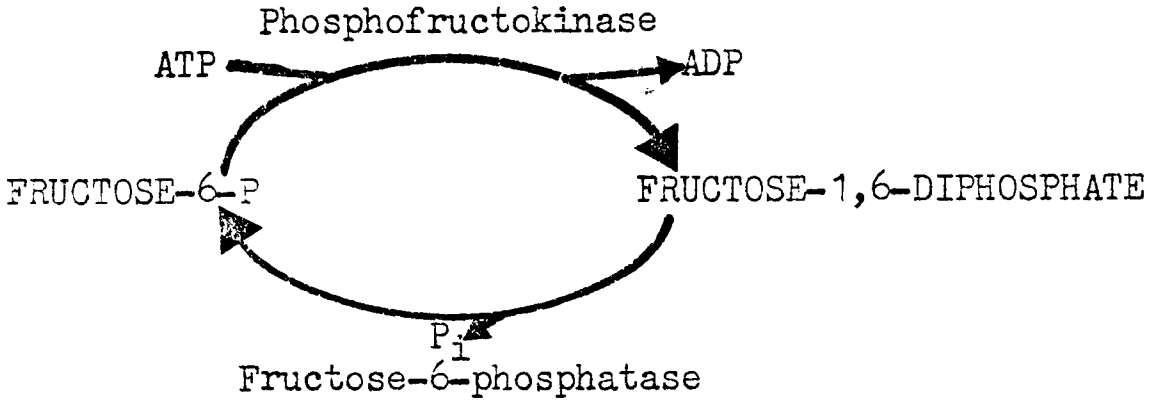

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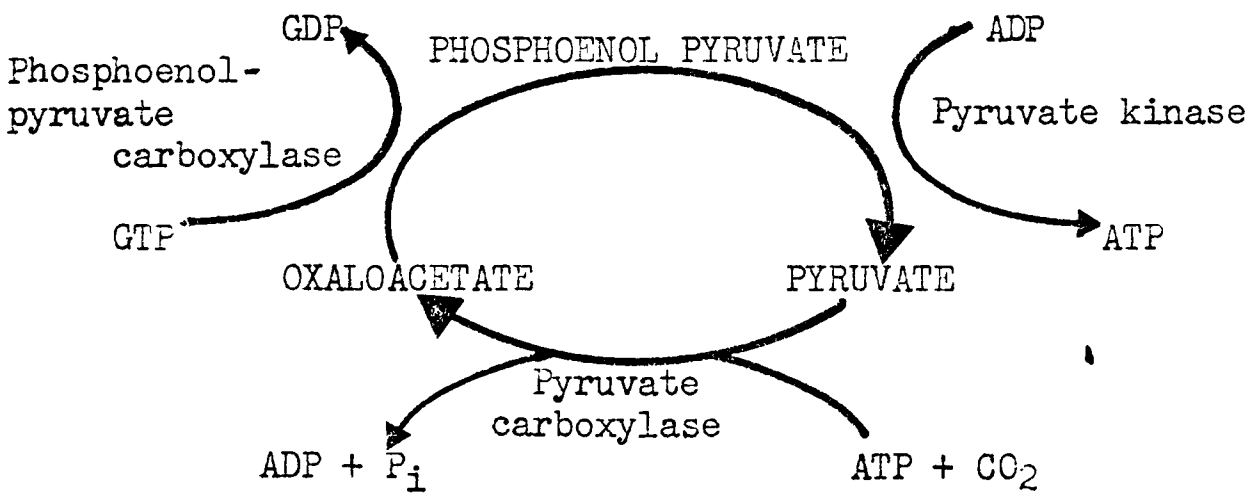

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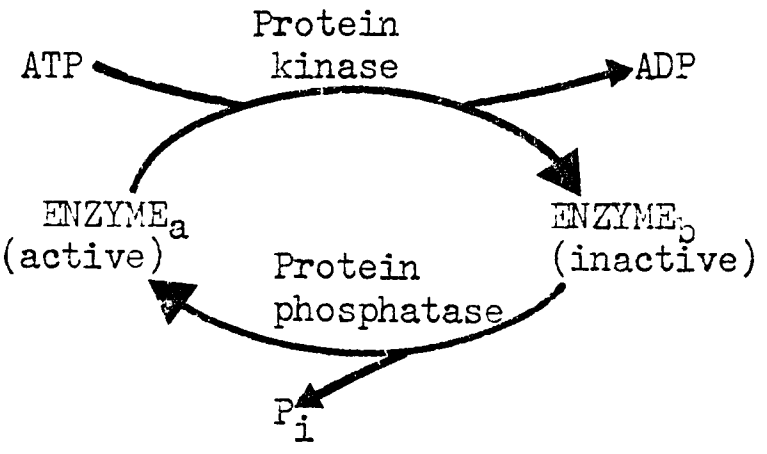


and non-phosphorylated forms of the enzyme are the substrates and products of the cycle. (This is not strictly a substrate cycle, since the flux through the cycle is only transitory because the amount of protein is almost constant. There is a flux through the cycle of enzyme protein being synthesised and broken down, but the direction of this flux is not reversible by alterations in the activities of the protein kinase and phosphatase. Hence the term 'interconversion cycle' is used to describe the interconversion of two forms of an enzyme - see Newsholme & Crabtree, 1976). Some examples of substrate cycles are illustrated in fig 1.1.

It has been known for many years that the body's storage and structural components are continuously being turned over. By following the incorporation of heavy water into body constituents, it was shown that there is a continuous synthesis and degradation of lipid, carbohydrate and protein occurring in the animal (see Schoenheimer & Rittenberg, 1936). If the synthetic processes are not completely switched off whilst the degradative processes are active (and vice versa), then this turnover must represent a rate of substrate cycling. The maintenance of this dynamic state within the animal must require large amounts of energy, and hence teleologically it is reasonable to suppose that if this continual turnover of body components is found, it must have some advantages to the animal. Katz & Rognstad, (1975) have pointed out that "the possibility that some cycles may have no specific function but are simply due to the imperfections in control of metabolism cannot be altogether dismissed". However, substrate cycles do have a variety of properties which are very useful in the control of a metabolic pathway. Katz & Rognstad also say, "recycling may not be as wasteful as it appears to be on first sight. Control, in any system, implies a decrease in entropy, and this requires expenditure of energy". The advantages

in the control of metabolism that the ability to cycle substrate will give to an animal are discussed in the next section.

1.3 Properties of Substrate Cycles

A hypothetical metabolic pathway is shown in fig 1.2. The starting substrate for the pathway is S, and P is the product. The reactions catalysed by E_1 and E_2 are non-equilibrium reactions, and if they are simultaneously active within the same cellular compartment then they catalyse a substrate cycle.

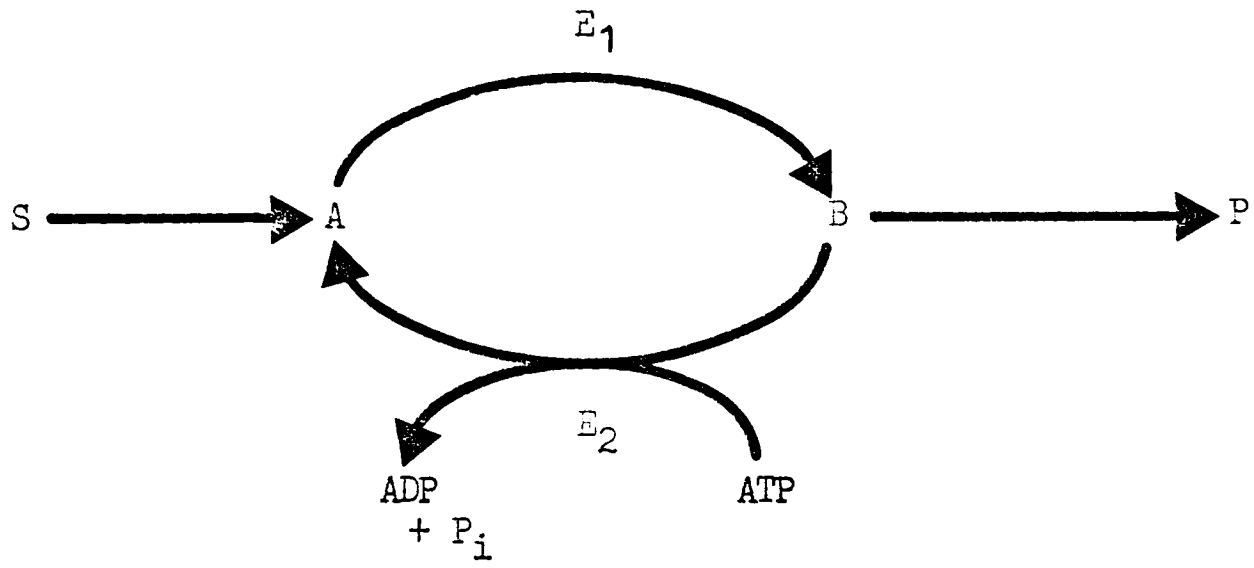


Fig 1.2 A hypothetical metabolic pathway

A substrate cycle consists of two fluxes, a flux through the pathway (given by the difference in the activities of E_1 and E_2) and a flux around the cycle (given by the rate of conversion of B back to A, i.e. the activity of E_2). In this example the flux around the substrate cycle is driven by the hydrolysis of ATP. There are three main advantages that this substrate cycle will give to the control of the flux through the pathway (see Newsholme & Crabtree, 1976, for full discussion on these properties of substrate cycles). These are:

i) they provide a means of reversing the flux through the pathway

ii) they provide a feedback link within the pathway, and

iii) they provide a mechanism for increasing the sensitivity of the control of flux through the pathway.

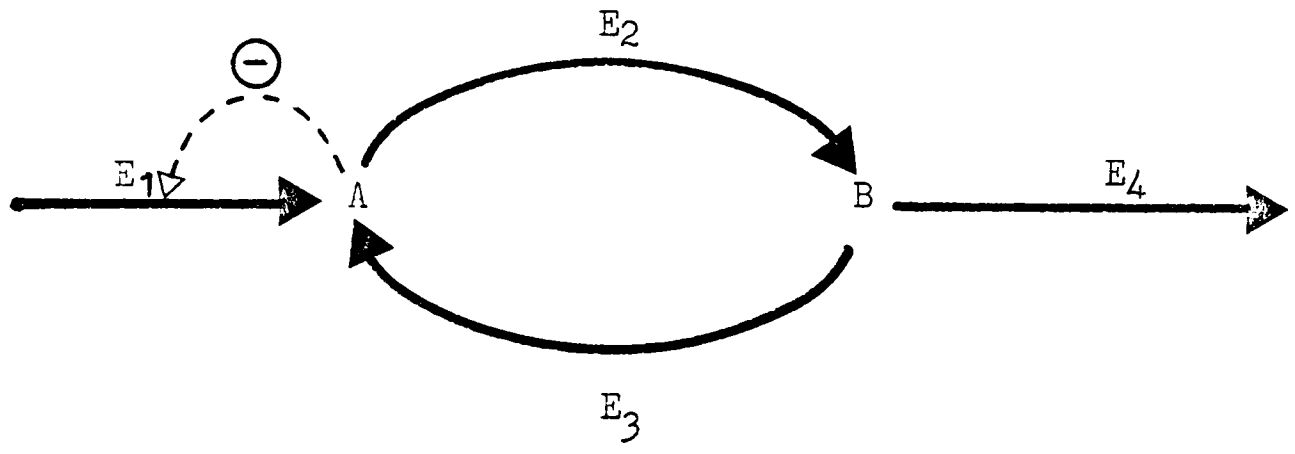
1.3.1 Reversibility of the pathway

The direction of flux through the above pathway will be determined by the relative activities of E_1 and E_2. If E_1 is more active then E_2, then there will be formation of P from S. However, if the concentrations of A and/or B, or the concentrations of any allosteric regulators of E_1 or E_2, change such that E_2 is more active than E_1, then the flux through the pathway will be reversed in direction and S will be formed from P. If E_1 was totally inhibited whilst E_2 was active, in order to reverse the flux a much greater change in the concentration of regulator would be required then is needed to change the direction of flux when a substrate cycle is operating.

Substrate cycles differ from equilibrium reactions in that the direction of flux may be changed by an allosteric regulator, but they are alike in that both of them may be regulated by mass-action effects of their substrates and/or products. (See Newsholme & Crabtree, 1976, for full discussion of the properties of equilibrium and non-equilibrium reactions and substrate cycles.)

1.3.2 Provision of a feedback link

A substrate cycle can provide a feedback link between two non-equilibrium reactions that are separated within a metabolic pathway by the cycle. Thus in the hypothetical system:



A change in the activity of E_4 can be communicated to E_1 via the change in the concentration of A which is influenced by the mass-action effect of B on the reaction catalysed by E_3.

1.3.3 Sensitivity of the control of pathway flux

Sensitivity may be defined as a function that measures the magnitude of a response to a given stimulus. Applying this to the control of enzyme activity, sensitivity is the ratio of the % change in flux through the reaction in response to a given % change in the concentration of regulator molecule, i.e.

$$\text{Sensitivity} = \frac{\%\text{ change in enzyme activity}}{\%\text{ change in regulator concentration}}$$

$$= \frac{(\Delta E/E)}{(\Delta R/R)}$$

where R = concentration of regulator molecule

E = enzyme activity at concentration of regulator molecule R

ΔR = change in concentration of regulator molecule

ΔE = change in enzyme activity in response to ΔR

The presence of a substrate cycle in a metabolic pathway gives a mechanism for increasing the sensitivity of the control of flux through that pathway. Before this property of substrate cycles is

discussed, the sensitivity of other possible mechanisms for controlling pathway flux will be considered.

The flux through a metabolic pathway is controlled by the activity of the so-called flux-generating step. When the activity of the enzyme catalysing the flux-generating step is increased, then the flux through all the other enzymes in the pathway will also be increased. The flux-generating step is a non-equilibrium reaction that is saturated with the pathway substrate (see Newsholme, 1980). The activity of the flux-generating enzyme (or indeed any non-equilibrium enzyme) may be controlled by changes in the concentration of one or more regulator molecules. Hence the sensitivity of the flux-generating step to the changes in concentration of regulator molecule is important to the control of the flux through the pathway.

Broadly speaking, there are two ways in which the activity of an enzyme may respond to changes in the concentration of a regulator molecule; it may exhibit a hyperbolic response to changes in regulator, or it may exhibit a sigmoidal response (positive cooperativity). These two mechanisms differ from each other in the sensitivity of response to the regulator molecule, and each mechanism will now be discussed. It should be noted that these discussions refer to the actions of a regulator on a non-equilibrium reaction. The regulator molecule could be either the substrate molecule for the enzyme or it could be an allosteric affector for the enzyme which can either increase or decrease the enzyme's activity (see Newsholme & Crabtree, 1978, for a more detailed discussion of the sensitivity of these reactions).

1.3.3.1 Sensitivity of a hyperbolic-response enzyme

For an enzyme which exhibits a hyperbolic response to increases in substrate/allosteric effector concentrations, the sensitivity to

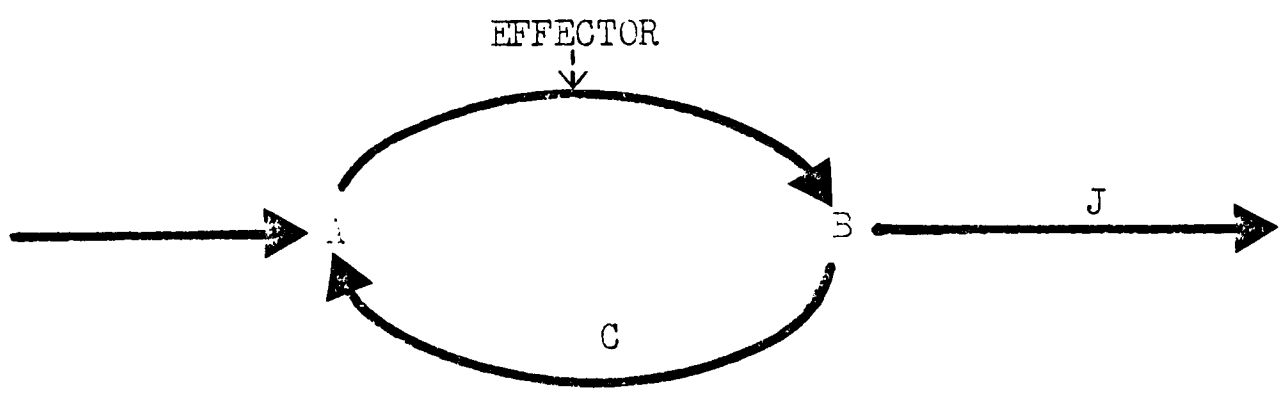
changes in the concentration of regulator will approach unity if the regulator concentration is below the K_m/K_i of the enzyme. In other words, at these concentrations of regulator the enzyme activity is proportional to the regulator concentration. However, at concentrations of the regulator which are greater than K_m/K_i, the sensitivity will approach zero because an increase in the concentration of regulator will not lead to any change in flux.

1.3.3.2 Sensitivity of a positively-cooperative enzyme

If an enzyme responds in a sigmoid manner to changes in the concentration of regulator, the sensitivity of that enzyme to changes in the concentration of regulator will have a value of up to four. The actual value of the sensitivity will depend upon which portion of the response curve is considered. Factors that limit the degree of sigmoidicity in the response of an enzyme have been discussed in detail by Newsholme & Crabtree, (1973).

1.3.3.3 Sensitivity and substrate cycles

Newsholme & Crabtree, (1976) showed that when two opposing non-equilibrium reactions formed a substrate cycle, they provided a mechanism of variable sensitivity for the control of the flux through a non-equilibrium reaction. If the non-equilibrium reaction is also a flux-generating step for a pathway, then the substrate cycle will increase the senstivity of control of the flux through the pathway. In the following hypothetical pathway:



the pathway flux is J and the cycling flux is C. The effector is an allosteric regulator of the enzyme converting A to B. If the concentrations of A and B remain constant, then the rate of the forward reaction of the substrate cycle is (J + C). The sensitivity that this substrate cycle will give in response to changes in the concentration of a regulator of the forward reaction of the cycle (i.e. either the effector for the forward enzyme or the substrate for the reaction) is given by:

$$\text{Sensitivity} = 1 + \frac{\text{Cycling Rate}}{\text{Pathway Flux}} = 1 + C/J$$

The derivation of this equation assumes:

i) that the concentrations of A and B are in a steady state

ii) that the rate of the reverse reaction of the substrate cycle remains constant, and

iii) that the response of the forward reaction of the cycle is linearly related to the change in regulator concentration.

Note that this latter assumption implies that the enzyme catalysing the forward reaction of the cycle is, for example, a hyperbolic-response enzyme acting at concentrations of substrate/effector below the K_m/K_i concentration. If the enzyme was one that exhibited positive cooperativity (and thus a sensitivity of up to four) then the equation above would not be valid. Appendix I shows that if the sensitivity of the forward reaction of the substrate cycle to changes in concentrations of regulator is denoted by 'n', then the sensitivity of the change in pathway flux is given by:

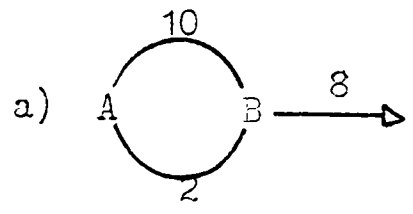
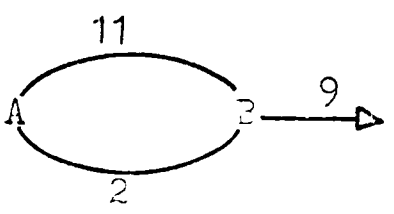
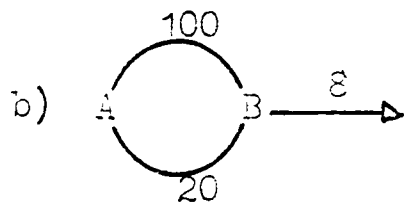
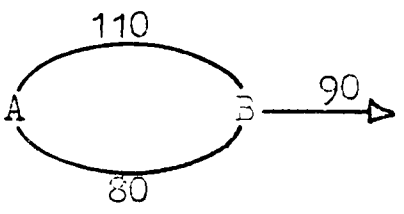
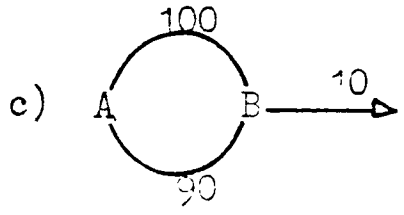
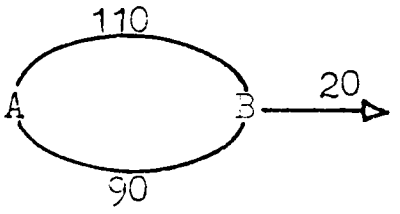
$$(n + \frac{nC}{J}) = n(1 + \frac{C}{J})$$

For any given value of n, therefore, a substrate cycle will give a (1 + C/J)-fold increase in the sensitivity of control of pathway flux.

Thus a substrate cycle will give a mechanism for the control of flux through a metabolic pathway and the sensitivity of this mechanism is dependent on the ratio of the rate of cycling to the pathway flux. Substrate cycles therefore have an advantage over the two control mechanisms previously described in that their sensitivity is variable and it can (theoretically at least) have very high values. The concept of variable sensitivity is best illustrated by numerical examples - see table 1.1. This table shows an ordinary hyperbolic-response enzyme, an enzyme which exhibits positive cooperativity, and a substrate cycle under three different conditions. The first column gives the starting enzyme activity, and the next column gives the enzyme activity after the regulator has changed in concentration and stimulated the enzyme by 10%. The final column gives the sensitivity of the response to the change in regulator molecule.

The hyperbolic response and positively-cooperative enzymes can have a maximum sensitivity of up to 1 and greater than 1 respectively. The substrate cycle in case (a) shows that when the ratio of cycling: flux is 2:8, the percentage response in pathway flux is 1.3 times the percentage change in regulator molecule concentration. Case (b) shows that even though the rate of cycling is ten times that in the case (a), the sensitivity is unchanged. This therefore illustrates that it is the ratio of cycling: flux that is important in determining the sensitivity of the cycle; this ratio is the same in both (a) and (b). In case (c), where the ratio cycling: flux is 9:1, a much greater sensitivity is exhibited than is seen in (a) and (b).

Table 1.1 Numerical examples to show how substrate cycles impart variable sensitivity to the control of pathway flux

Starting Enzyme Activity	Activity after 10% Change in Regulator Concentration	Sensitivity = $\frac{\%\text{ change in flux}}{\%\text{ change in regulator}}$
Hyperbolic response Enzyme A —10→ B	A —11→ B	$\frac{10\%}{10\%} = 1$
Sigmoid response Enzyme A —10→ B	A —14→ B	$\frac{40\%}{10\%} = 4$
Substrate Cycle		
a) 		$\frac{13\%}{10\%} = 1.3$
b) 		$\frac{13\%}{10\%} = 1.3$
c) 		$\frac{100\%}{10\%} = 10$

The hyperbolic- and sigmoid-response enzymes have been assumed to be acting at their most sensitive. In the substrate cycle the enzyme catalysing the forward reaction is assumed to have a hyperbolic response to regulator with a sensitivity of unity.

The equations given above for the sensitivity of a substrate cycle all assume that when the activity of the forward reaction is stimulated there is no concomitant change in the back reaction of the cycle. If, at the same time as it stimulates the forward reaction, the regulator inhibits the back reaction of the cycle, then there will be a greater change in pathway flux for a given change in the concentration of regulator than if the forward reaction alone were stimulated. However, note that the potential gain in flux through the pathway by inhibiting the activity of the back-reaction is limited (the flux through the back-reaction can only be reduced to a minimum of about zero), whereas the forward reaction has the possibility of being activated by much more than 100%. The maximum increase in pathway flux that is obtainable by the inhibition of the back reaction is therefore limited.

The increased sensitivity obtained by substrate cycling is not without cost to the cell. The simultaneous action of two opposing non-equilibrium reactions requires an input of energy, and substrate cycling will therefore produce heat. The greater the rate of substrate cycling, the greater the rate of heat production - but also for a given pathway flux, the greater the sensitivity of the substrate cycle. The ability of a substrate cycle to vary in sensitivity led to the idea that the rate of cycling (and thus sensitivity of control and heat loss) may be increased only during times when extra sensitivity in metabolic control is required. Such times might typically be in storing food after a meal, during the early stages of exercise, or during the anticipation of exercise - for example, a sprinter on the blocks waiting to start a race. It is perhaps constructive to consider this sprinter in more detail. In section 1.1, the Sherrington metaphor was introduced; this states that when one muscle is activated, the muscle whose action is

antagonistic to it is inhibited. At first sight, it appears that the Sherrington metaphor is stating that a 'muscular substrate cycle', i.e. two opposing muscles simultaneously active, does not occur. However, further consideration shows that the Sherrington metaphor in fact requires that a 'muscular substrate cycle' exists; there must be some tone in the antagonistic muscle in order for it to be inhibited at the same time as the opposing muscle is activated. In the case of the sprinter on the blocks, in order to gain the sensitivity required for a rapid departure from the blocks, he increases his 'rate of muscular substrate cycling' by increasing the tension of the opposing muscles. He does not just relax on on the blocks and then activate one set of muscles to move off when the starting gun goes. Thus he has the muscles which are going to propel him forward already tensed, and the antagonistic muscles to these are also tensed (in order to stop him leaving the blocks before the gun). At the start of the race, one set of muscles is activated further and the antagonistic muscles are inhibited (or perhaps deactivated) thus giving a large increase in force very quickly. In this 'muscular substrate cycle', the cycle is "primed" by the brain simultaneously activating the antagonistic muscles, so that there is a greater sensitivity of response to the stimulus produced when the gun actually goes off. It has been suggested that substrate cycles in metabolic pathways might similarly be "primed" on anticipation of, for example, exercise. The cycles could be stimulated by the action of one regulator, meaning that the sensitivity to another regulator (for example the substrate for the forward reaction) would be increased. In this way the substrate cycle is stimulated to provide increased sensitivity only during times of need, and thus the problem of wasting energy by

the substrate cycle is minimised.

1.3.3.4 Interconvertible forms of enzymes

In section 1.3.3.3, the sensitivity of the control of the flux through a pathway was achieved by substrate cycling of intermediates within the pathway. In order to significantly improve the sensitivity of control at a regulatory point in the pathway, the rate of cycling really needs to be of the same order (and/or greater) as the net flux through the pathway. This cycling of metabolites could therefore account for the wastage of much energy, leading to the suggestion given above that the cycling of metabolites might only be increased during certain times when increased sensitivity is required. However, one way of avoiding a large amount of the energy usage involved in the substrate cycling of metabolites is to have a substrate cycle using the enzyme protein itself as the substrate for the cycle. Thus the enzyme is interconverted between two forms by the action of two other enzymes. Often a phosphorylation - dephosphorylation is involved. Thus an interconversion cycle, catalysed by the kinase and the phosphatase, is established and the relative activity of these two enzymes determines the concentration of the active form of the enzyme. This in effect represents a 'coarse control' of enzyme activity, since the concentration of the enzyme is being changed (though not by the more usual method of protein synthesis and degradation). The sensitivity of this method of controlling the activity of an enzyme has not been determined, but it is believed that it will be greater than unity (Newsholme & Crabtree, 1978). The interconversion cycle in this example is different from the substrate cycles considered so far, in that the cycle is being used to set the concentration of the substrates for the cycle (i.e. the enzymes themselves), rather than setting the flux through a pathway

(as is the case with the metabolite substrate cycles considered above).

1.3.4 Substrate cycles, thermogenesis and weight control

Because of their ability to produce heat, it has been suggested that the presence of substrate cycles could be important in thermogenesis. Substrate cycles could explain the specific dynamic action of food (i.e. the increased consumption of O_2 and production of heat that is seen after a meal). The increase in substrate cycling after a meal would be in order to provide the sensitivity required for food storage. It has also been suggested that substrate cycles could be the mechanism with which an animal is able to regulate its body weight to prevent obesity during times of over-feeding (see Newsholme & Crabtree, 1976, for a detailed discussion of these roles of substrate cycles).

1.4 Experimental evidence for substrate cycling

The experiments which support the existence of substrate cycles have been reviewed in detail by Katz & Rognstad (1975) and Newsholme & Crabtree (1976). Therefore the experiments done in various tissues which support the existence of substrate cycles will only be summarised.

Studies on the mechanisms of the reactions in the glycolytic and gluconeogenic pathways have shown that specific hydrogen atoms on glucose are exchanged with water in certain of the reations in these pathways. Hence by using glucose labelled with tritium on specific carbon atoms, the possible existence of substrate cycles in the glycolytic pathway has been investigated. The basis of this method is to use tritium/ carbon-14 dual-labelled glucose with the tritium present on a specific carbon atom, and follow the loss of the tritium label at various stages of glycolysis. When the tritium-

labelled glucose passes through certain of the glycolytic reactions, the tritium label will be lost. Thus, if the ratio of $^{3}H/^{14}C$ on the various glycolytic intermediates falls during an incubation, this implies that the tritium atom originally present on the sugar has been lost. The fall in this ratio may therefore indicate the cycling of a substrate. By using glucose labelled with tritium on different carbon atoms, the existence of different substrate cycles can be investigated, (see Katz & Rognstad, 1975, for full details of this method).

Using this technique of dual labelling, evidence has been gathered which suggests that in liver the F6P-FDP cycle, the glucose-G6P cycle and the phosphoenol pyruvate-pyruvate-oxaloacetate cycles are active (Clark et al., 1973; Katz & Rognstad, 1975). Apparently these cycles are also active in kidney cortex; during active gluconeogenesis in this tissue, carbon from ^{14}C-labelled glucose present in the medium appeared in CO_2, lactate and amino acids i.e. glycolytic products (Rognstad et al., 1970). This approach of using isotopic reversibility to show glucose utilisation during gluconeogenesis has given similar results in liver (Rognstad et al., 1973). Further evidence for the F6P-FDP cycle in liver has been obtained by the technique of measuring the randomisation of carbon-14 from [1-^{14}C]- galactose (Van Schaftingen et al., 1980). However, all of these experiments have simply indicated that a particular substrate cycle exists. The actual rate of cycling has been determined for only three substrate cycles; the triglyceride-fatty acid cycle in adipose tissue (see section 1.5), the glucose-glucose-6-P cycle in liver, and the F6P-FDP cycle in liver.

Clark et al., 1974, determined the rate of the F6P-FDP cycle in isolated hepatocytes. They found that the rate of this cycle

varied in response to several different substrates and the presence of glucagon. The ratio of the rate of cycling to the pathway flux (see section 1.3.3) was found to be variable and under some conditions reached quite high values, implying that the cycle provided a mechanism of variable sensitivity for the control of glycolysis and gluconeogenesis. The substrate cycle was active during both gluconeogenesis and glycolysis, the direction of the pathway flux depending on the relative activities of phosphofructokinase (PFK) and fructose diphosphatase (FDPase).

The F6P-FDP cycle has also been studied in the flight muscle of bumble-bees (see Newsholme & Crabtree, 1978). In a survey of the activities of phosphofructokinase and fructose diphosphatase in the muscle of a wide selection of animals, it was found that the activity of FDPase was unusually high in the flight muscle of bumble-bees (Newsholme et al., 1972). Furthermore, unlike fructose diphosphatase from every other muscle investigated, the enzyme from bumble-bees flight muscle was not inhibited by AMP. This led to the speculation that the F6P-FDP cycle may function in bumble-bees as a thermogenic mechanism to maintain muscle temperature at about 30°C which is necessary for flight. Clark et al.,(1973) injected dual-labelled glucose into bumble-bees and found that the F6P-FDP cycle did not operate at a measurable rate whilst the bee was flying. However, when the bee was resting there was considerable cycling, providing the air temperature was low. The results support the hypothesis that in bumble-bees this substrate cycle is a mechanism for the maintenance of body temperature.

The glucose-glucose-6-P cycle has been studied in hepatocytes (Katz & Rognstad, 1975). The rate of cycling was found to depend greatly on the glucose concentrations and on the dietary status of

the animals. The activity of this cycle is highest in animals fed on high carbohydrate diets (100 μmoles ATP hydrolysed/ g liver/ hour). This rate of cycling is halved or quartered in hepatocytes from rats fed a normal diet or fasted. The rate of glucose-glucose-6-P cycling in these hepatocytes could amount for perhaps 5% of the oxygen consumption of the hepatocytes. If the F6P-FDP and pyruvate-oxaloacetate-phosphoenol pyruvate cycles were also taken into account, the substrate cycles present in the glycolytic pathway could account for 10% of the oxygen consumption of isolated hepatocytes (Newsholme & Gevers, 1967; Katz & Rognstad, 1975).

1.5 The triglyceride-free fatty acid (TG-FFA)substrate cycle

1.5.1 Introduction to the TG-FFA cycle

This cycle has historic importance since it was the first metabolic substrate cycle for which direct evidence was obtained. In 1959 Leboef et al. noticed that the rate of glucose incorporation into triglyceride-glycerol was increased in the presence of adrenaline without any increase in fatty acid synthesis being observed. To explain this, they postulated that some of the FFA released in lipolysis were reesterified. This idea was confirmed by experiments in which the rate of release of free fatty acid (FFA) from incubated adipose tissue was found to be less than that expected on the basis of the amount of glycerol released (complete hydrolysis of triglyceride should yield 3 FFA for every glycerol released). The discrepancy was interpreted as being due to the reesterification of the FFA (see Steinberg, 1963).

The triglyceride-fatty acid cycle is shown in fig 1.3. It consists of the reactions of the lipolytic and esterification pathways (these pathways are covered in more detail in chapters 3 and 4 - see figs 3.1 and 4.1. Note that this substrate cycle therefore

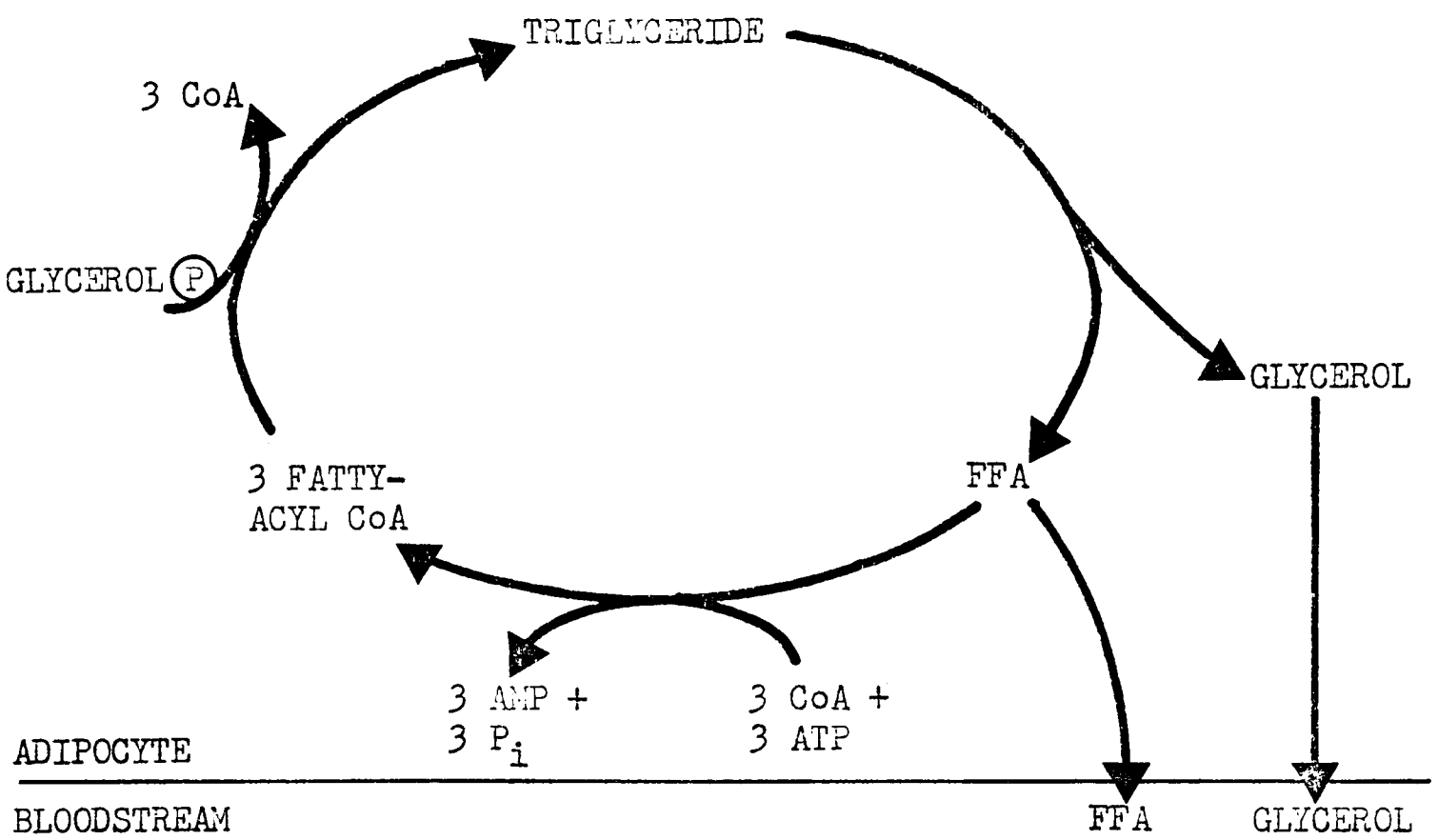


Fig 1.3 The triglyceride-free fatty acid cycle

has more than one reaction in each of the two directions of the cycle. In a cycle of this sort, the sets of reactions which make up each side of the cycle can contain equilibrium reactions, but overall each set must be non-equilibrium, and the two sets of reactions must not share common intermediates. Diglyceride is in fact an intermediate which is common to both pathways, and thus there is the possibility of the triglyceride-diglyceride cycle - this is discussed further in section 3.6.

The last reaction in the synthesis and the first in the breakdown of triglyceride have to be non-equilibrium since they are involved in the metabolism of a fuel that is used for storage. Non-equilibrium reactions have the advantage that they can be controlled by allosteric regulators (Newsholme & Crabtree, 1976). If an equilibrium reaction was to be used to store a fuel, then the concentration of fuel would always be in equilibrium with its precursors. In order to store more fuel, the concentration of precursors would therefore have to

be permenantly raised, and this could lead to osmotic, ionic, solubility and side-reaction problems.

1.5.2 Possible roles for the TG-FFA cycle in adipose tissue

Five roles have been suggested for this cycle. These are as follows and are discussed in detail below.

1) It links the rate of FFA supply to changes in blood glucose concentration
2) it increases the sensitivity of the control of FFA flux
3) it provides feedback regulation of fatty acid mobilisation
4) it clears the build-up in ATP that would otherwise occur when FFA are synthesised from glucose, and
5) it has a role in thermogenesis and weight control.

1.5.2.1 Linking FFA supply to blood glucose concentrations

As far as is known, glucose is the primary fuel for respiration in the fed state in almost all animals, and triglyceride is the reserve fuel. When the animal's supply of glucose (liver glycogen) becomes low, it has to have a mechanism for switching to its reserve fuel of triglyceride. The TG-FFA cycle could provide such a mechanism for linking the breakdown of triglyceride to the concentration of glucose in the blood.

For the TG-FFA cycle to operate, it must have a supply of glycerol phosphate with which FFA are reesterified. Since the activity of glycerol kinase in white adipose tissue is low (see chapter 3), the glycerol phosphate is not obtained by the phosphorylation of the glycerol released in lipolysis, but instead is derived from blood glucose via the glycolytic pathway. Alterations in the concentration of glucose in the blood will lead to changes in the rate of glucose entry into the cell. If the rate of input of glucose into the cell decreases to an extent such that the concentration of glycerol

phosphate becomes rate-limiting for the fatty acyl CoA synthetase reaction, then the rate of FFA esterification will be decreased. Hence, assuming that lipolysis stays constant, there will be a greater release of FFA from the cell thus increasing the blood concentration of FFA. The increased levels of FFA in the blood will increase their use as oxidative substrates and thus have a sparing effect on the metabolism of glucose.

Why should white adipose tissue have a low glycerol kinase activity? One reason is that in a starving man, the glycerol released by the hydrolysis of triglyceride provides the substrate for 50% of the glucose that is synthesised; if adipose tissue had a high glycerol kinase activity, then the glycerol might not be available for gluconeogenesis in the liver. It is tempting to speculate that another reason for the low activity of glycerol kinase in white adipose tissue is so that the TG-FFA cycle mechanism for linking the release of FFA from adipose tissue to the lowered availability of glucose may operate. If there was a large glycerol kinase activity present in white adipose tissue, a supply of glycerol phosphate would be continuously available for FFA reesterification. Thus variations in the supply of glucose to the adipocyte would not affect the availability of glycerol phosphate and would therefore have no effect on the release of FFA from the cell. It is interesting that brown adipose tissue (Knight & Myant, 1970; Portet et al., 1974) liver (Hems, 1975; Kondrup, 1979) and red muscle (Eaton & Steinberg, 1961; Kreisberg, 1966; Crass, 1977; Di Mauro et al., 1980), all tissues which store and break down triglyceride and therefore have the ability to TG-FFA cycle, all have glycerol kinase activity (Knight & Myant, 1970; Robinson & Newsholme, 1969; Newsholme & Taylor, 1969). None of these tissues are thought to release significant quantities

of FFA into the blood (Newsholme & Start, 1973) and it seems possible that the presence of glycerol kinase is a means for these tissues to maintain the ability to TG-FFA cycle, and thus to obtain the advantages of substrate cycling, without being dependent on a supply of glucose from the blood.

The control of FFA release by the availability of glucose requires that the concentration of glycerol phosphate drops to a level such that it becomes limiting for FFA esterification. However, there is some doubt about whether the concentration of glycerol phosphate in adipose tissue ever gets this low (see discussion in chapter 4) which means that this mechanism for the control of FFA release may not occur *in vivo*. On the other hand, as suggested by Newsholme & Start (1973) the mechanism whereby a decreased glucose availability increases the release of FFA due to a lowered rate of reesterification could have been very important in the early stages of the evolution of metabolism (see chapter 7).

1.5.2.2 Sensitivity of control of FFA flux

The control of the hydrolysis of triglyceride is thought to be mainly via the action of hormones on the activity of the hormone-sensitive triglyceride lipase. This enzyme is regulated by a phosphorylation/dephosphorylation mechanism (see chapter 4). Since triglyceride lipase is the flux-generating step for triglyceride mobilisation (see chapter 3), operation of the TG-FFA cycle will increase the sensitivity of the release of FFA in response to regulators of the triglyceride-lipase or esterification reactions. Similarly, even though the esterification reactions are not flux-generating for the storage of FFA as triglyceride (Robinson, 1970), the esterification reaction is overall a non-equilibrium reaction (see above) and the TG-FFA cycle will therefore increase the sensitivity

of the esterification reactions to regulators.

1.5.2.3 Feedback regulation of FFA release

In view of the role of FFA as a fuel for oxidation in other tissues (particularly muscle), the rate of release of FFA from adipose tissue must be precisely controlled. If mobilisation of FFA is too slow, the muscle will not receive sufficient fatty acid for its needs; if the release is too fast, then the concentrations of FFA in the blood could reach toxic levels and lead to tissue damage (Rodbell, 1966; Spector & Fletcher, 1978).

Adipose tissue is a very dispersed tissue; it is not present as a discrete entity such as is the case with liver. Thus a precise control of the overall rates of mobilisation of fatty acids from all the fat depots in the animal might be physiologically difficult. Lipolysis in adipose tissue is known to be stimulated both by nervous and humoral means, (see Hales et al., 1978). The list of agents that stimulate lipolysis in rat adipose tissue has been described by Steinberg (1963) as 'almost embarrassingly long'. Many of these lipolytic agents can be considered as non-physiological, but nevertheless teleologically it seems likely that there should be some sort of feedback regulation which governs the level of FFA in the blood. It has been suggested by Newsholme & Crabtree (1976) that FFA themselves can provide such a mechanism due to their effects on the TG-FFA cycle.

The FFA released from adipose tissue are made available to other tissues by being transported in the blood. Increased utilisation of FFA would lower blood FFA and thus also the concentration of FFA in the adipocyte. If the concentration of FFA in the fat cell falls such that the rate of esterification is reduced, then a greater proportion of FFA released in lipolysis will leave the cell rather

than being reesterified. Similarly, if the rate of mobilisation is too high, the blood fatty acid concentration will be increased and esterification will be stimulated, thus lowering the rate of FFA release into the blood.

1.5.2.4 Clearing ATP build-up during fat synthesis

In a theoretical analysis of the reactions involved in the synthesis of triglyceride, Flatt (1970) showed that if glucose was the substrate for triglyceride synthesis, a net synthesis of ATP within the cell must accompany the synthesis of triglyceride. This increase in ATP could theoretically limit the rate of triglyceride synthesis. However, Evans & Garratt (1977) showed that the activity of the TG-FFA cycle was such that it could prevent any increase in cellular ATP. Thus the synthesis of triglyceride from glucose is prevented from being self-limiting by the activity of the TG-FFA cycle (see chapter 7).

1.5.2.5 TG-FFA cycle, thermogenesis and weight control

Two of the roles often suggested for adipose tissue are as mechanical (for example, around the kidney) and thermal insulators. However, in view of the heat-producing properties of substrate cycles, it has been suggested that the TG-FFA cycle may have a role as a thermogenic mechanism (see Ball, 1965; Baldwin, 1970; Sestoft, 1980). To quote Ball & Jungas (1961);

"George Cahill has made the interesting suggestion that perhaps subcutaneous fat should be thought of not just as an insulating blanket but as an electric blanket."

Other suggestions for the role of the TG-FFA cycle are as an explanation for the thermic effect of food and as a means of controlling body weight (Newsholme & Crabtree, 1976).

1.6 Aims of this thesis

Perhaps because of the methodological problems involved, the study of substrate cycles has been largely concerned with simply determining whether such cycles occur in the living cell. The existence of several substrate cycles has been qualitatively demonstrated in various tissues (see reviews by Katz & Rognstad, 1975; Newsholme & Crabtree, 1976); however, quantitative data on the rate of cycling is less common. The theoretical properties of substrate cycles, in particular their role as a mechanism for importing variable sensitivity to the control of the flux through a reaction, have been well described (Newsholme & Crabtree, 1976; Newsholme, 1979; 1980). In spite of this, there has been little if any experimental work done on the sensitivity properties of substrate cycles. One of the best known substrate cycles is the TG-FFA cycle in white adipose tissue; this cycle has the advantage that it is easily measured in a tissue that is simple to study *in vitro*. Despite these advantages, experimental work on the TG-FFA cycle has consisted of little more than the demonstration that FFA are reesterified within adipose tissue, together with the estimation of the contribution of the cycle to the metabolic rate of the animal. There has been no systematic study on the response of the cycle to various *in vitro* or *in vivo* treatments. In this thesis, therefore, the effects on the TG-FFA cycle of a variety of acute and chronic treatments are investigated, with the aim of establishing the importance of this substrate cycle in the response of the animal to a variety of situations.

CHAPTER TWO

Materials and Methods

2.1 Chemicals

Bovine serum albumin (fraction V), activated charcoal, L-noradrenaline, ACTH, PG-E_1, TSH glucagon, triiodothyronine, cortisol-21-Na-succinate, propylthiouracil, metyrapone, phenylephrine and triton WR-1339 were obtained from Sigma (London) Chemical Co., Poole, Dorset, BH17 7NH.

ATP, PEP, NADH, triethanolamine hydrochloride and 3-hydroxybutyrate were bought from Boehringer Corporation (London), Lewes, E. Sussex.

Tris, D-glucose and toluene (low in sulphur) were bought from Fisons Scientific Apparatus, Loughborough, Leicestershire.

Butyl PBP, PPO and POPOP were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks.

Propranolol, fenoterol and phentolamine were a gift from Dr J.R.S. Arch, Beechams Pharmaceuticals Biosciences Research Division, Yew Tree Bottom Road, Great Burgh, Epsom, Surrey.

Insulin was a gift from the Welcome Research Laboratories, Langley Court, Beckenham, Kent, BR3 3BS.

'Siliclad' was purchased from Clay Adams Ltd. (U.K. agents; Arnold R. Horwell Ltd., 2 Grange Way, Kilburn High Road, London, NW6 2BP).

'Repelcote' was from Hopkin & Williams Ltd., Chadwell Heath, Essex.

^{3}H-water, U-^{14}C-glucose, U-^{14}C-glycerol, ^{3}H and ^{14}C-hexadecane were obtained from The Radiochemical Centre, Amersham, Bucks, HP7 9LL.

All other chemicals were from either BDH or Fisons, and were of the purest grade available.

2.2 Enzymes

Collagenase (lyophilised, from Clostridium histolyticum, no. 1517124); Adenosine deaminase (calf intestime, 200 units/ mg, 10 mg/ ml); Lactate dehydrogenase (bovine heart, 50 mg/ ml); Glycerol kinase (from Candida mycoderma, 5 mg/ ml); and Pyruvate kinase (rabbit muscle, 10 mg/ ml) were obtained from Boehringer.

2.3 Animals

Male Sprague-Dawley and Wistar rats were obtained from OLAC (1976) Ltd., Blackthorn, Bicester, Oxon., OX6 OTP.

Female P.O. mice were from Dept. of Pathology, South Parks Road, Oxford.

Female CFLP, CD1, C57Bl, ob/ob and ob/+ mice were obtained from the MRC Laboratory Animals Centre, Carshalton, Surrey, and were purchased by Beecham Pharmaceuticals and donated to the author by Dr J.R.S. Arch.

2.4 Equipment

Scintillation counting was done on either a Beckman LS 233, an Intertechnique SL 4000 or a Beckman 8100 scintillation counter.

Spectrophotometric work was done using a Gilford Stasar III.

Centrifuging was done using either a Beckman model TJ6 refrigerated bench centrifuge or a MSE bench centrifuge.

2.5 Preparation of defatted albumin

In view of the expense of defatted albumin, Sigma Fraction V bovine serum albumin (BSA) was bought and de-fatted essentially according to the method of Chen (1967).

Bovine serum albumin (20g) was poured on top of distilled water (150 ml) and left to dissolve (approx 4 hours). Activated charcoal (10g) was washed with distilled water by stirring and then allowing

the charcoal to settle, when the supernatant was aspirated using a vacuum line. In this way the fatty contamination, seen as a layer floating on top of the water, could be removed (as opposed to drawing the fatty contamination through the charcoal if a filter funnel was used to to drain the charcoal). After the second wash the charcoal was separated using a Buchner funnel and Whatman No. 1 paper. The charcoal was then mixed in with the BSA, and the pH adjusted to 3.0 using conc. HCl. The mixture was stood in ice on a magnetic stirrer and stirred for an hour. At the end of this period the charcoal was removed, firstly by filtration on a Buchner funnel with Whatman No. 1 paper, and then by filtration using a 0.22μm Millipore filter. The solution was then neutralised with NaOH and the volume measured. Knowing the starting volume, the fraction of solution that was lost in the preparation could be calculated, and the solution was then made up to a final concentration of BSA of 10% w/v using distilled water.

The BSA solution was then dialysed at 4°C against three changes of 50 volumes of 0.9% NaCl solution saturated with O_2:CO_2 (95%:5%). This ensures three things. Firstly, it removes any low molecular weight molecules present. Secondly, it brings the solution to a defined osmotic composition, and finally it gases the albumin whilst avoiding frothing. After dialysis, the 10% albumin solution was stored frozen at -20°C in plastic scintillation vials.

2.6 Incubation Buffers

Incubation of fat cells and fat pads was in Krebs-Ringer Bicarbonate buffer, containing half the recommended amount of calcium and 4% w/v BSA. The buffer components were stored separately in bottles at 4°C (except the BSA which was deep frozen) and on the day of the experiment the incubation buffer was made up as follows:-

10% BSA in 0.9% NaCl	51.4	vols
0.9% NaCl	48.6	"
1.15% KCl	4	"
0.11 M $CaCl_2$	1.5	"
2.11% KH_2PO_4	1	"
3.82% $MgSO_4.7H_2O$	1	"
1.3% $NaHCO_3$	21	"
TOTAL	128.5	vols

On the day of the experiment, all of the buffer components except the albumin were mixed together and gassed with O_2:CO_2 (95%:5%) - this avoids frothing the albumin. The albumin was then added and because it had been gassed during the dialysis stages (see section 2.5) its addition did not affect the degree of saturation of the final buffer. The pH of the buffer was adjusted to 7.4 using either NaOH or HCl.

2.7 Preparation and storage of hormones and enzymes

(i) Adenosine deaminase

The enzyme was bought from Boehringer as an ammonium sulphate suspension. Prior to its use in fat-cell incubations, the ammonium sulphate was removed. This was done by diluting the enzyme solution in Krebs Ringer Bicarbonate buffer to 130 µg/ ml. The enzyme solution was dialysed twice against 200 volumes of O_2:CO_2 (95%:5%) - gassed Krebs Ringer buffer at 4°C. The resulting solution was frozen at -20°C in 1 ml aliquots.

(ii) Insulin

Insulin solutions were kept in plastic tubes, and diluted using Krebs Ringer buffer with 4% albumin. Use of albumin and plastic tubes lessens the problem of insulin absorption to the tube (see Cecil & Robinson, 1975).

Insulin was dissolved in 3 mM HCl at a concentration of 40 µg/ ml and frozen at -20°C in 20 µl aliquots. These were diluted for use

on the day of the experiment.

(iii) Catecholamines and adrenergic drugs

Noradrenaline and related compounds are fairly quickly oxidised in solution. Hence noradrenaline, and the drugs fenoterol, propranolol, phenylephrine and phentolamine were all made up in incubation buffer immediately before use.

(iv) Glucagon

Crystalline glucagon (extracted from a mixture of bovine and porcine pancreas, packaged under nitrogen, Sigma, lot no. 48C-0418) was dissolved in 3 mM HCl at 2 mg/ ml and frozen at -20°C in 60 µl aliquots.

(v) Adrenocorticotrophic hormone (ACTH)

Porcine adrenocorticotrophic hormone (from Sigma, Grade II, lot no. 85C-0337) was dissolved in water at a concentration of 10 mg/ ml and frozen at -20°C in 40 µl aliquots.

(vi) Prostaglandin ($PG-E_1$)

Prostaglandin E_1 (from Sigma, batch no. 18C-0333) was dissolved in ethanol at a concentration of 2 mg/ ml and stored at -20°C. Aliquots of this stock solution were used for addition to incubation media.

(vii) Thyroid Stimulating Hormone (TSH)

Thyroid stimulating hormone from Sigma (supplied as a sterile powder, batch no. 76C-0152) was dissolved in water at a concentration of 10 U/ ml and frozen at -20°C in 50 µl aliquots.

(viii) Triiodothyronine (T-3)

L-triiodothyronine for injection was dissolved in saline at a

concentration of around 80 µg/ ml (depending on size of animal) at pH of 11. For a given series of injections triiodothyronine was made up fresh and frozen in plastic tubes at -20°C and stored in the dark in aliquots as it is light sensitive and absorbs to glass.

(ix) Propyl thiouracil (PTU)

Propyl thiouracil was made up fresh for a given series of injections. It was dissolved in saline at a concentration of around 5 mg/ ml (depending on size of animal) at pH 11 and frozen at -20°C in aliquots.

(x) Cortisol

Cortisol for injection was used either as cortisol acetate (which was bought as a sterile suspension ready for injection) or as cortisol-21-Na succinate. The latter was easily dissolved at 60 mg/ ml in saline for injection, and was made up fresh for a given experiment. During the period of dosing for an experiment the solutions were stored at 4°C.

(xi) Metyrapone

Metyrapone was dissolved at a concentration of about 80 mg/ ml in saline. It was made up fresh for a series of experiments and during the experiments kept at 4°C.

(xii) Triton WR-1339

Triton WR-1339 was usually dissolved at a concentration of about 150 mg/ ml by standing overnight with water at room temperature. (It can be made up by warming under a tap and swirling, but this tends to froth the solution, which is undesirable from a handling point of view when dealing with tritiated water.)

2.8 Assay for glycerol

The concentration of glycerol in neutralised H_2SO_4/TCA extracts of adipocyte and fat-cell incubation media were performed using a modification of the method of Garland & Randle (1962). The following stock buffer solution was prepared:-

0.1 M triethanolamine HCl, pH 7.6

4 mM $MgSO_4$

This was kept at 4°C. A stock solution of ATP (100 mM, pH 7.6) was stored in 1 ml aliquots at -20°C. On the day of assay, the assay-mixture was prepared as follows:-

Stock buffer solution	60 ml
NADH (disodium salt)	15 mg
PEP (tricyclohexylammonium salt)	15 mg
ATP (100 mM, pH 7.6)	1 ml
Lactate dehydrogenase (5 mg/ ml suspension)	150 µl
Pyruvate kinase (10 mg/ ml suspension)	60 µl

The buffer was divided into two, and to one half was added glycerol kinase (suspension of 5 mg/ ml) at a concentration of 100 µl suspension per 30 ml assay mix. Two 50-150 ul lots of the neutralised TCA extract of the incubation medium were pipetted into separate tubes. To one of them the assay mix with glycerol kinase was added, and to the other the glycerol kinase-free assay mix was added. After mixing, they were allowed to stand for an hour, after which time the optical density at 340 nm was measured using the through-flow Gilford Stasar spectrophotometer. The concentration of glycerol was estimated from the difference in optical density between the two tubes. For each assay, standard solutions of glycerol were run through the whole procedure and used to plot a calibration graph.

2.9 Assay for free fatty acids

FFA were assayed by a modification of the method of Novak (1965). FFA are extracted into an organic phase using a modified Dole's mixture (Dole & Meinertz, 1960). This method does not suffer from interference by bilirubin and other substances. The composition of the organic phase is such that it floats on top of the aqueous phase, which facilitates easy sampling of the organic phase. The organic phase sample is then shaken with an aqueous phase containing cobalt, thus forming a cobalt soap in the organic phase. The cobalt in the organic phase is then complexed with an indicator, and the complex is estimated spectrophotometrically. The assay is summarised in fig 2.1.

Stock Solutions

The following stock solutions were made up; solutions 1 to 6 were stored at room temperature.

1) Modified Dole's extraction mixture; Propan-2-ol 1000 ml
   Heptane 1000 ml
   2 N H_2SO_4 80 ml

2) Chloroform : Heptane, 55:45 v/v

3) Saturated (at room temperature) aqueous solution of Na_2SO_4

4) Saturated (at room temperature) aqueous solution of K_2SO_4

5) Indicator stock solution - a saturated (at room temperature) solution of α-nitroso-β-naphthol in ethanol

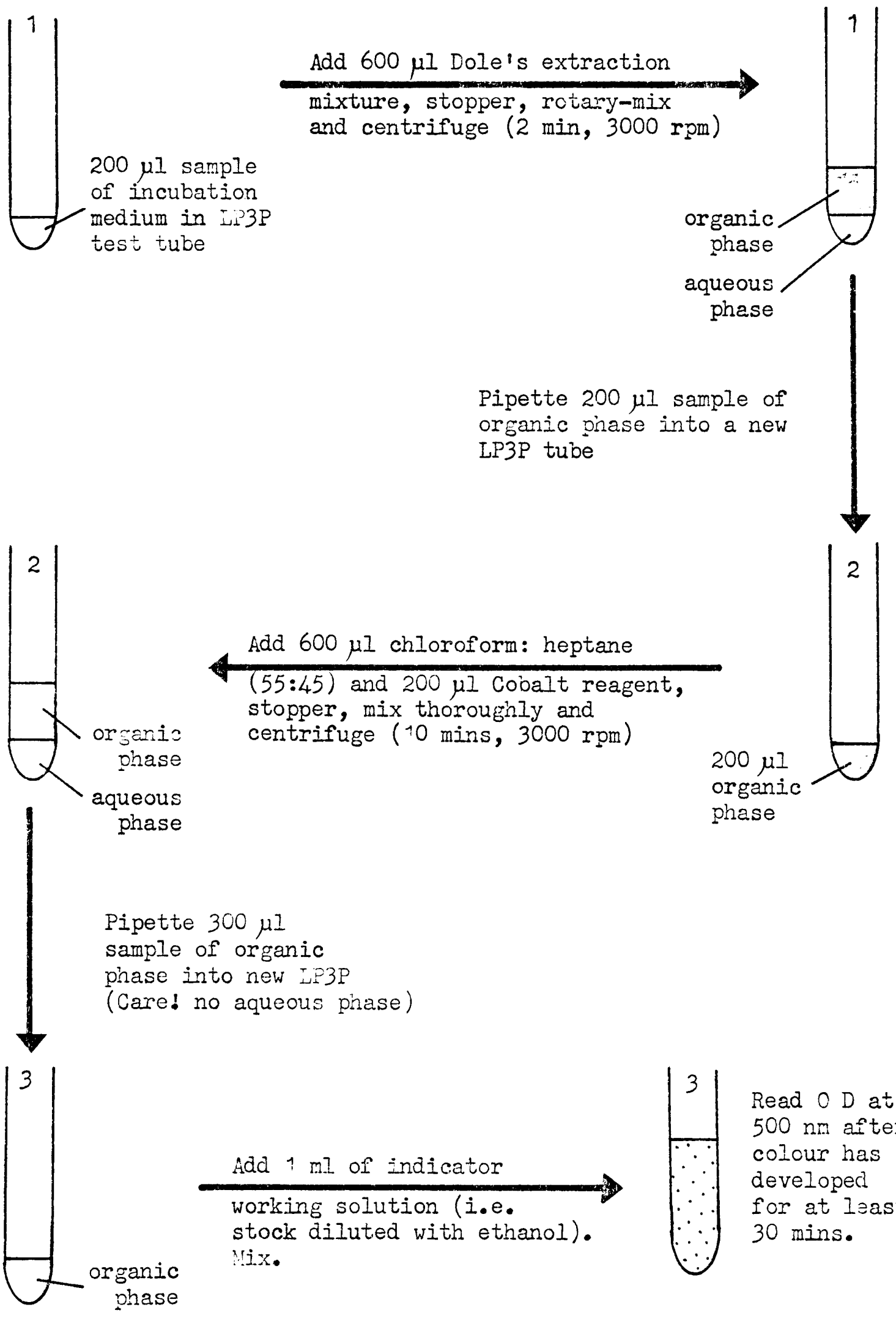
6) Solution A: Cobalt nitrate. $6H_2O$ 6 g
   Glacial acetic acid 0.8 ml
   Made up to 100 ml with the saturated Potassium sulphate

7) FFA standard solutions -

   These can either be made up in the modified Dole's extraction mixture or in 4% w/v defatted BSA solution. The latter are easier to handle and were used in preference to the former. Standards of 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0,

Fig 2.1 Summary of the FFA Assay

Three LP3P test tubes are used in this assay - they are numbered near the mouth for clarity. All centrifugation is in a bench centrifuge.



1.5 and 2.0 mMolar palmitic acid in BSA were frozen in aliquots at -20°C. They were prepared by first making a 2 mM solution of palmitic acid - this was done by dissolving palmitic acid in the minimum amount of ethanol, and then adding this to the BSA solution. The 2 mM palmitate was diluted to make the lower concentrations.

Preparation of cobalt working solution

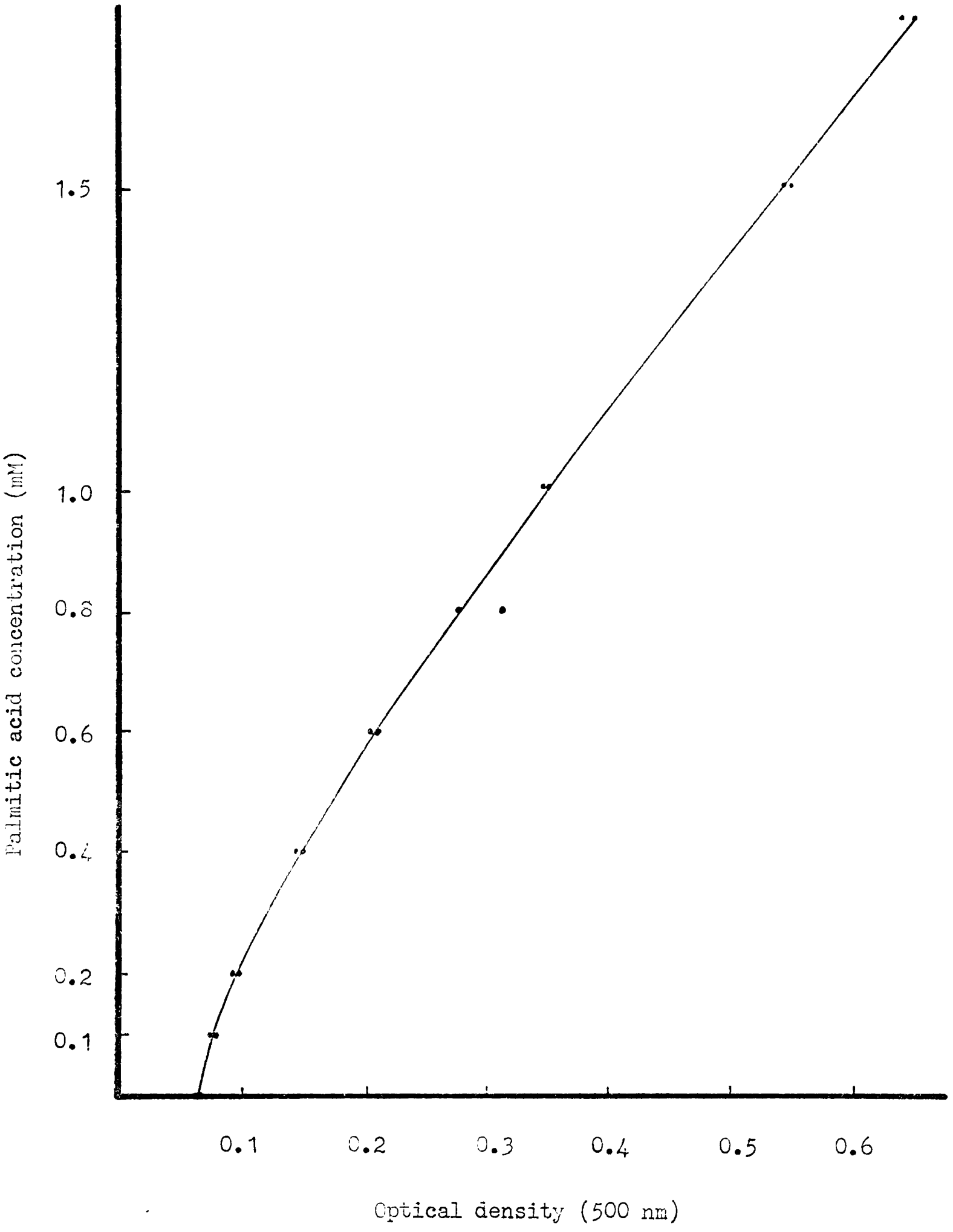
On the day of assay, the cobalt working solution was prepared as follows:- Triethanolamine (approx. 2.7 ml) was made up to approx. 20 ml with solution A. Saturated sodium sulphate was then added to make a total volume of approx. 34 ml. This reagent is not stable and must be made up fresh immediately before use.

Assay procedure

In the case of measuring the FFA content of fat pad incubation medium the incubation was stopped by the removal of the fat pad; hence the sample for the FFA assay was pure incubation medium. In the case of fat cell incubations, the incubation was stopped using H_2SO_4 and hence a sample of the acidified incubation medium was used. The palmitic acid standards were also acidified accordingly.

Samples of the incubation medium (200 µl) were pipetted into a polypropylene LP3P test tube together with Dole's extraction mixture (600 µl). This was mixed on a rotary mixer and then spun for two minutes at maximum rpm in a bench centrifuge. A sample of the organic supernatant phase (200 µl) was pipetted into another LP3P tube, and 600 µl of the chloroform: heptane 55:45 v/v plus 200 µl of cobalt reagent were added. The tube was capped, and mixed in such a way as to form an emulsion with the aqueous phase - this increases the surface area available for formation of the cobalt soap. It was then spun for ten minutes at maximum rpm in a bench centrifuge.

Fig 2.2 Typical standard curve for FFA assay



A sample (300 µl) of the top organic phase was pipetted into a third LP3P tube and 1 ml of indicator working solution (made by diluting the indicator stock solution 1:10 in ethanol) added. After mixing, the solution was left for half an hour for the complex to form - the complex is stable for several hours. Optical density at 500 nm was measured using a Gilford through-flow Stasar spectrophotometer. Palmitic acid standards were taken through the whole procedure and used to construct a standard curve - this was done for each assay because standard curves differed from experiment to experiment. A typical standard curve is shown in fig 2.2.

The whole assay was done on one day since the organic phases cannot be stored in the polypropylene tubes, due to the leaching out of solvent. The volatile organic phases may be handled using a Finn pipette as long as the tip is saturated with vapour before starting the pipetting. This can be achieved by drawing the organic phase up and down the tip a few times before starting handling the samples.

2.10 Tissue and cell preparation and incubations

1) Incubation of intact fat pads

Rats were killed by stunning and cervical dislocation, and the peri-genital fat pad was removed. In larger rats, only the thin distal portion of fat pad was used. The pad was weighed and up to 200 mg of fresh tissue was incubated in 2 ml Krebs-Ringer bicarbonate buffer containing defatted BSA. Pre-incubation of fat pads was for 10-20 minutes in the presence of all hormones used in the main incubation. The main incubation was done in capped polythene scintillation vials for one hour. The incubation was stopped by removal of the fat pad. Samples (100-200 µl) of the incubation

medium were taken and frozen in polypropylene LP3P test tubes. A further 1 ml sample was precipitated using 200 µl 40% TCA, mixed, and spun in a bench centrifuge for 5 minutes at maximum rpm. A 500 µl sample of the supernatant was removed and 10 µl Universal Indicator added. The pH was adjusted to neutral using a microsyringe with 7 M KOH. The neutralised samples were frozen for later glycerol assay.

In experiments in which the results are expressed as a rate per gram of fat-free dry tissue (i.e. experiments involving <u>in vivo</u> treatment of animals) the fat pad was extracted as follows. The pad was placed in a glass scintillation vial and 10 ml hexane: isopropanol 3:2 v/v added. (This mixture has been found to be as efficient at lipid extraction as the more common chloroform: methanol 2:1 v/v extraction mixture, but is not as toxic - chloroform damages the liver and methanol damages the visual system (Hara & Radin, 1978)). The fat pad was left standing in the extraction solvent for 1-2 days. The solvent was then poured off and a second 10 ml added. This procedure has been validated by weighing the fat pads at each stage and after further extraction. After 1-2 days, the pad (to all external appearances) does not appear to have lost any lipid at all; however this is misleading, and removal of the pad and leaving it to dry gives a fat-free dry residue which can then be weighed. Note that this lipid extraction method requires no more than the standing of the tissue in the extraction solvent.

2) <u>Preparation of isolated adipocytes</u>

Adipocytes were prepared essentially according to the method of Rodbell (1964). This method involves the liberation of intact fat cells from fat pads treated with collagenase. The fat cells are concentrated by centrifugation - the high fat content of adipocytes

means that they float to the top of the centrifuge tube.

Rats were killed by stunning and cervical dislocation, and the thin distal portion of the epididymal fat pads were removed. They were immediately placed in a 50 ml conical flask containing 10 ml Krebs-Ringer bicarbonate buffer with 4% w/v defatted BSA and 10 mg collagenase (Boehringer). Prior to the experiment, the conical flask had been silicon-coated using either 'silicote' or 'repelcote' (see section 2.1) to minimise the tendancy of fat cells to stick to the glass. The flask was capped and shaken at about 150 cycles/ minute in a water bath at 37°C.

When the fat pads were broken up (usually about 45 minutes) the digest was filtered through a piece of muslin into a centrifuge tube. A length of plastic tube was placed in the centrifuge tube, which was then accelerated to 1000 rpm in a bench centrifuge. After 5 seconds at this speed the centrifuge was stopped, and the liquid and precipitate lying below the supernatant cell plug was drawn out through the tube using a syringe. A 10 ml volume of pre-warmed buffer was then poured in and the tube inverted a few times before repeating the centrifugation. This washing procedure was repeated, and the fat cells were then washed into a conical flask containing the appropriate amount of buffer. The suspension was swirled continuously by hand in order to stop the adipocytes floating to the surface, and aliquots of cell suspension were dispensed using a Finn pipette. The tips of the pipettes had had a few millimetres of the end removed in order to prevent damage to the fat cells due to pipetting through a small hole.

Experiments in which glucose was present in all incubations had glucose present during the whole procedure of the isolation of cells; otherwise no glucose was present during cell preparation. A rule of

thumb in determining the number of rats to use to make a cell preparation is to use one rat weighing 120g for every five fat cell incubations.

Certain problems can arise in the preparation of adipocytes. Some preparations of collagenase produce cells which are not sensitive to hormones - the stimulation of glucose uptake by insulin is particularly sensitive to different collagenases. It is not possible to use purified collagenase, since this does not break up the tissue; a fairly crude preparation is necessary. The only method of finding a suitable batch of collagenase is by trial and error.

Difficulty may be experienced in breaking up the tissue. This is particularly the case with older rats, which need a longer time for digestion of the fat pads. A better solution is to use younger rats: fat cells from young rats are more responsive to hormones.

Fat cells are sensitive to mechanical agitation. Vega & Kono (1978) have shown that if fat cells are centrifuged too hard the basal uptake and utilisation of glucose is increased. Factors such as centrifugation and shaking during fat pad digestion should therefore be standardised wherever possible.

3) Incubation of isolated adipocytes

Hormones to be used in the incubation were diluted and added to scintillation vials during the preparation of the fat cells. Aliquots of the fat cell suspension were then added, the scintillation vials were capped, and the cells were incubated for an hour in a water bath with gentle shaking. The final volume of the incubation was 1.3 ml.

Termination of the incubation was by adding H_2SO_4 (300 µl, 2 M). Sulphuric acid stops the incubation but does not precipitate the albumin; this means that samples for the FFA assay can be taken. (In contrast, if TCA is used to stop the incubation, the albumin is precipitated into clumps; this leads to very variable FFA samples.)

After taking FFA samples (2x 200 µl samples into LP3P test tubes) a 1 ml sample was taken for glycerol assay. The cells and albumin were precipitated from this sample using TCA (200 µl of 40% w/v) followed by centrifugation. Most of the supernatant (799 µl) was removed, 15 µl of Universal Indicator added and the mixture was neutralised by adding 7 M KOH from a microsyringe. The resulting solution was used in the glycerol assay.

2.11 Experiments using tritiated water in vitro

i) System used

Experiments comparing the rate of incorporation of ^{3}H from tritiated water or ^{14}C from U-^{14}C-glucose were done using fat pads (100-200 mg in 1.5 ml buffer) incubated in glass vials as described above. Fat pads rather than fat cells were used in these experiments since these give a large amount of triglyceride for the determination of the incorporation of radioactivity into the triglyceride-glycerol and -fatty acid moieties. Each pad was cut into two and one half incubated with tritiated water and the other with ^{14}C-glucose. The specific activity of tritium was 1-2 mCi/ ml incubation buffer, and that of the U-^{14}C-glucose was 100 µCi/ mmol glucose. All other conditions are given in the tables.

ii) Extraction and hydrolysis of triglyceride

Incubations were stopped by dropping the fat pads into 1.5 ml ice-cold perchloric acid. Petroleum ether (5 ml of 40-60 fraction) was added and the tissue was homogenised using a Polytron tissue grinder. The petroleum ether fraction was aspirated and kept in a ground glass stoppered test-tube, and the homogenisation procedure was repeated with a further 5 ml petroleum ether. This gives an organic phase containing the tissue lipid plus either tritiated water

or ^{14}C-glucose from the tissue. These were removed by washing the organic phase by shaking it with a mixture of NaCl and H_2SO_4 (both 0.05 M, both in same solution). After shaking the tubes were centrifuged (10 seconds, 1000 rpm bench centrifuge) to sharpen the phase boundary, and the aqueous layer was aspirated and discarded, taking care not to remove any of the organic phase. This washing procedure was performed a total of three times (see section 2.12). The petrol ether phase was evaporated to dryness and the triglyceride hydrolysed in 1.5 ml of 3:1 v/v ethanol:KOH (60% w/v) for three hours at 60-70°C. This was done in stoppered glass test tubes. After cooling, the hydrolysate was acidified using 6 molar H_2SO_4. Fatty acids were removed from the hydrolysate by two extractions using 5 ml 40-60 fraction petroleum ether. The petroleum ether washings were combined and evaporated to dryness on top of a dry block inside a glass scintillation vial. Scintillant (10 ml of toluene:PPO:POPOP 1000 ml:4 g: 0.1 g) was added and the radioactivity incorporated into triglyceride-fatty acids was counted. Triglyceride-glycerol radioactivity was determined by counting the maximum amount (about 250 µl) of the hydrolysate aqueous phase that could be dissolved in 10 ml of scintillant (toluene:triton:PPO:POPOP, 1500 ml:750 ml:6 g: 0.15 g). During the hydrolysis a variable amount of yellow colour was produced; this caused a large variation in the degree of quenching during counting. For this reason. all values were corrected for quench by using internal standardisation. After the hydrolysis, the aqueous phase was found to vary in volume between different tubes (probable due to differences in the fit of the stoppers). In order to know the fraction of the hydrolysate phase that is used for counting triglyceride-glycerol, the volume of the hydrolysate phase was determined by isotope dilution of ^{14}C-glycerol added to the aqueous phase after the triglyceride-glycerol sample had been taken.

iii) Equations used to determine triglyceride-glycerol and fatty acid synthesis

The following equations were used to calculate rates of triglyceride and fatty acid synthesis from measurements of tritiated water incorporation (adapted from Hems et al., 1975);

Micromoles fatty acid synthesised =

$$\frac{\text{Tritium dpm incorporated into fatty acids} \times 1111.1}{\text{Number of tritium atoms per fatty acid} \times \text{tritium dpm in 10 } \mu\text{l water}}$$

Micromoles triglyceride-glycerol synthesised =

$$\frac{\text{Tritium dpm incorporated into triglyceride-glycerol} \times 111.1}{\text{Number of tritium atoms per triglyceride-glycerol} \times \text{tritium dpm in 10 } \mu\text{l water}}$$

Values for the number of tritium atoms incorporated per fatty acid and triglyceride-glycerol moiety were 13.3 and 3.3 respectively (these values are discussed in chapter 3).

2.12 Experiments using tritiated water in vivo

Mice were used in these experiments since they are smaller than rats and therefore the amounts of tritiated water required are less. Female mice were used because they do not fight amongst themselves and hence are easier to handle. Details of each experiment are outlined in the tables of results; essentially each experiment consisted of the animal being injected with tritiated water and then killed an hour later. The fat tissue was dissected out and the triglyceride extracted for determination of radioactivity in triglyceride-glycerol and fatty acids.

One of the assumptions used in the tritiated water method of

measuring the rate of fatty acid and triglyceride synthesis is that tritium is only incorporated into fatty acids or glyceride-glycerol during their synthesis (as opposed to hydrogen exchange between water and the already-formed triglyceride molecule). The following experiment was performed in order to assess how significant this latter exchange might be.

Mice were injected with tritiated water. After 15 mins (sufficient time for the tritiated water to equilibrate throughout the animal - see Hems et al., 1975) they were killed and left at room temperature. Perigenital fat pads were removed at various times after death, and the lipid extracted for hydrolysis and determination of tritium incorporated into triglyceride-glycerol and fatty acids. The results (fig 2.3) show that there is no detectable rate of incorporation of tritium into triglyceride-glycerol or fatty acids after the animal has been killed. Thus the rate of exchange of tritium with the already-formed triglyceride molecule can be assumed to be negligible in comparison to the rate of incorporation of tritium during triglyceride synthesis. These results also show that it is not necessary to homogenise the fat pad immediately upon the death of the animal, since the enzymes of the esterification pathway do not appear to be active once the animal is killed (fig 2.3).

In the <u>in vivo</u> experiments the fat pads (up to 500 mg) were homogenised in 10 ml chloroform:methanol (2:1 v/v) using hand homogenisers. This extraction solvent will dissolve appreciable amounts of tritiated water, and the large amounts of tritiated water from the adipose tissue must therefore be removed in order to be able to detect the radioactivity present in the lipid. This was done by washing the chloroform:methanol phase with a salt solution (a solution of 0.05 M NaCl plus 0.05 M sulphuric acid) - see Folch et al.,

Fig 2.3a Rate of Triglyceride-glycerol synthesis in white adipose tissue dissected and homogenised at different times after death

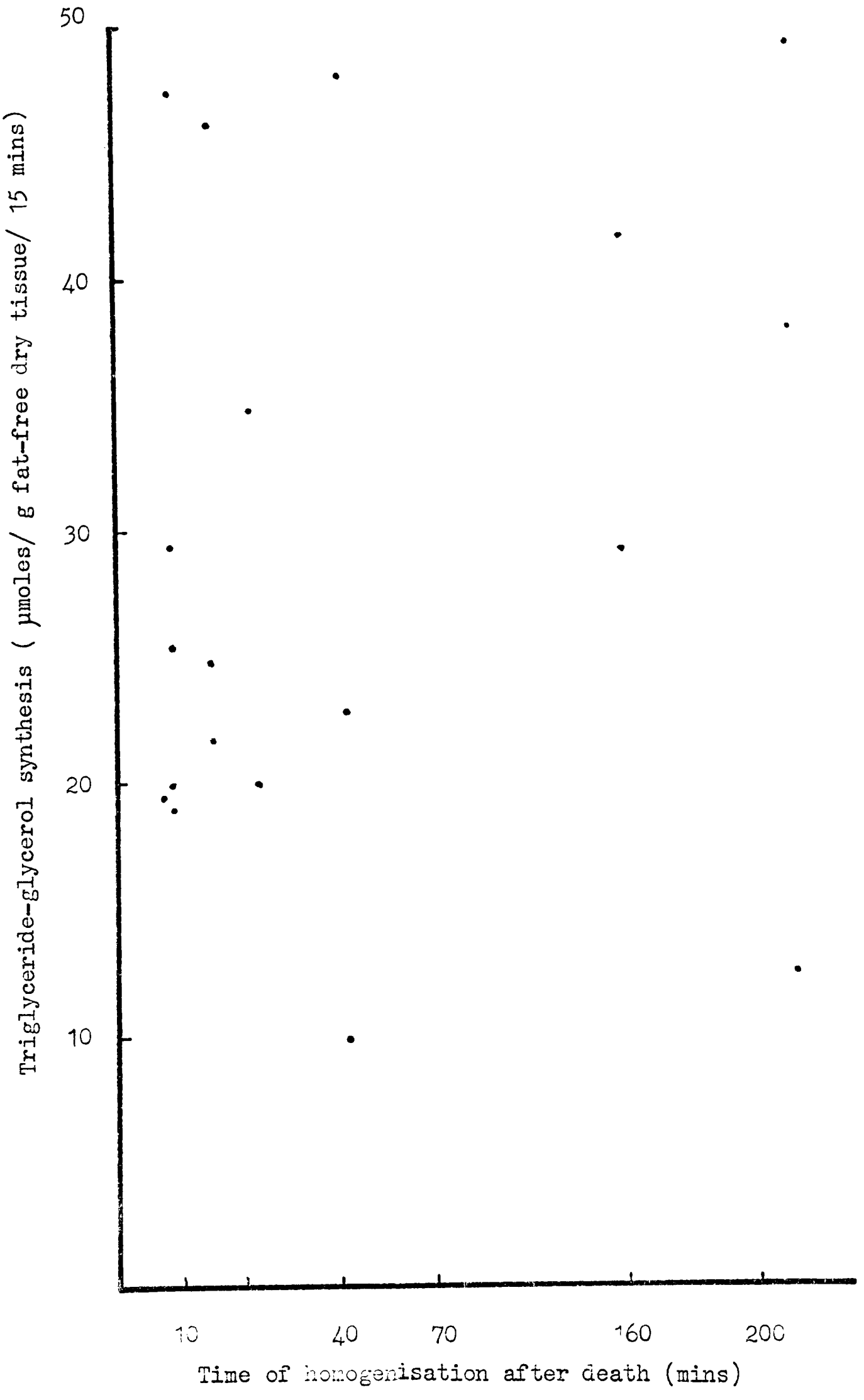
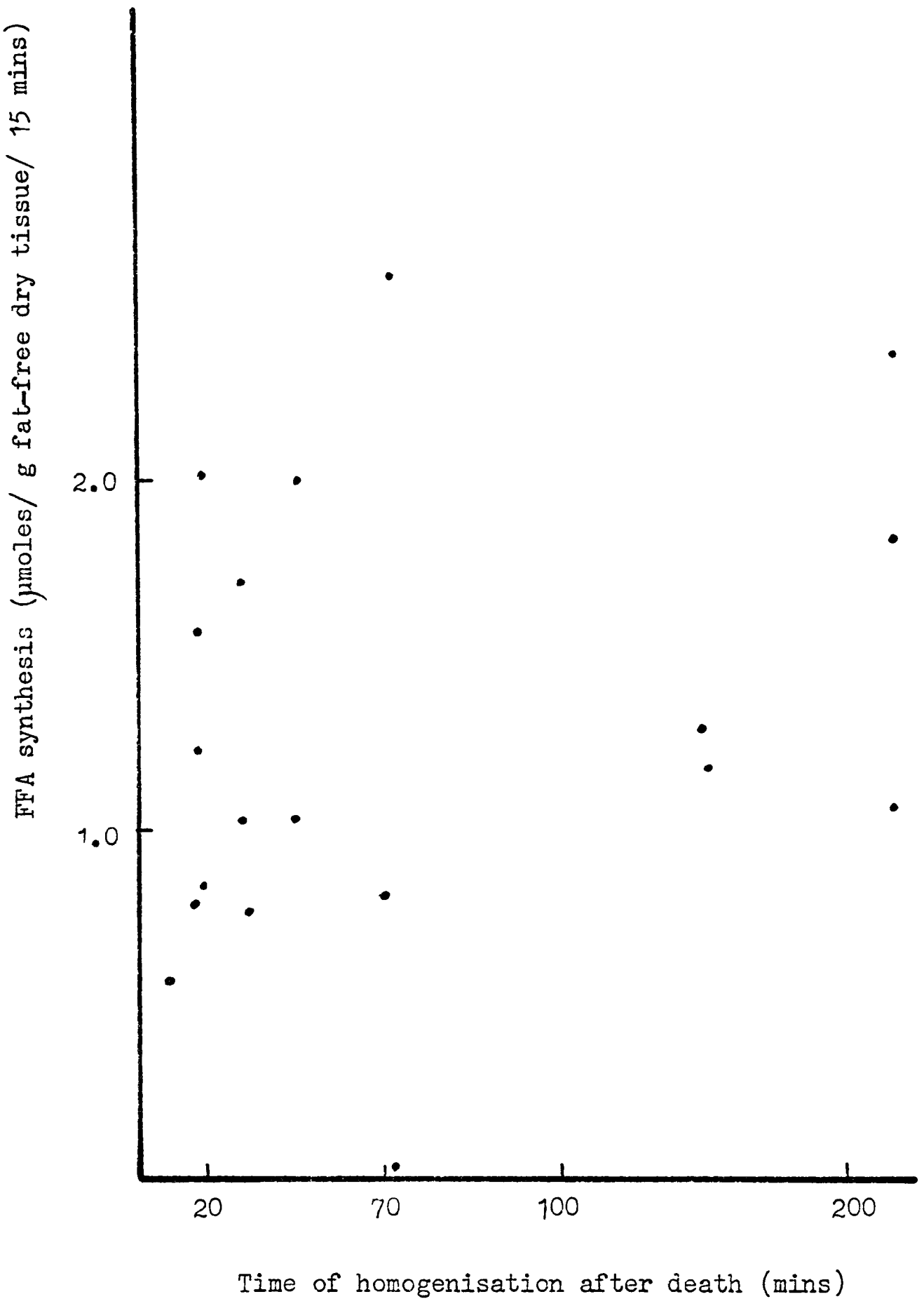


Fig 2.3b Rate of FFA synthesis in white adipose tissue dissected and homogenised at different times after death.



1957. To find the number of times that the choroform:methanol phase had to be washed in order to remove the tritiated water, the following experiment was performed.

At least twice as much tritiated water as is present in a fat pad in the *in vivo* experiments was added to the chloroform:methanol extraction mixture. The mixture was then repeatedly washed with an equal volume of the $NaCl/H_2SO_4$ solution. After each washing, a sample of the organic phase was taken and the tritium activity present was determined by scintillation counting; the results are shown below.

	DPM in sample of organic phase (mean ± S.D.)
Before washing	45710 ± 870
After 1st wash	770 ± 72
2nd wash	-5.8 ± 7.0
3rd wash	-5.1 ± 9.8
4th wash	-5.6 ± 13.1

This experiment shows that two washings are sufficient to reduce the level of tritiated water to below detectable levels. Thus the procedure used to remove tritiated water from chloroform:methanol extracts was to wash the extract three times with $NaCl/H_2SO_4$. Note that during the removal of the aqueous washing phase, care was taken not to aspirate off any of the organic phase (thus losing lipid), and also to remove all droplets of water left on the side of the tube.

A further experiment was performed to validate the use of three washings of the chloroform:methanol phase for the removal of tritiated water. Fat tissue from a non-radioactive mouse was extracted as normal into chloroform:methanol. Tritiated water was then added to an activity of at least 10 times that observed in the chloroform:methanol extract from fat tissue of radioactive mice. The organic phase was

then washed against three lots of the $NaCl/H_2SO_4$ washing solution and the resulting chloroform phase evaporated to dryness on a dry block. Scintillant was added to the remaining lipid fraction and the radioactivity present was determined. Control tubes of tissue extract but without any tritiated water present were taken through the whole procedure. The results are shown below.

Activity in sample of chloroform:methanol phase of extract from tritiated mouse	2.6×10^5 dpm per ml extract
Activity in extract of non-tritiated mouse fat tissue after addition of tritiated water	2.6×10^6 dpm per ml extract
Activity in control tubes (non-tritiated taken through whole procedure)	23.6 cpm
Activity in total lipid extract of non-tritiated mouse (extract treated with tritiated water)	26.5 cpm
Difference between control and tritium-treated tissue extracts	6 dpm

This experiment shows that the difference between the tritiated water treated and control tissue extracts is about 6 dpm; this difference is at most about 0.1% of the activity incorporated into the triglyceride-glycerol moiety. Hence the washing of the chloroform: methanol extract three times is sufficient to remove the tritiated water present in the tissue. This experiment also shows that there is no hydrogen exchange between the lipid and the tritiated water when it is in the chloroform:methanol extract.

The procedure followed for the hydrolysis of triglyceride in the *in vivo* experiments was the same as that for the *in vitro* experiments

(see above). One of the problems of the direct measurement of the radioactivity in the triglyceride-glycerol moiety by counting an aliquot of the hydrolysate aqueous phase is that the volume of hydrolysate that can be added to the scintillant is limited by the ability of the scintillant to dissolve the high amounts of salts present in the hydrolysate after the KOH has been acidified. In later experiments, therefore, the radioactivity present in triglyceride-glycerol was determined by difference between the total-triglyceride activity and the activity present in the fatty acid hydrolysate extract. This was achieved by dividing the washed chloroform phase; part of the extract (40%) was used to count total-triglyceride activity, and the rest was hydrolysed and used to count fatty acid activity. The fraction of the triglyceride used for counting total-triglyceride and for hydrolysis was determined by weight. This method has the advantage that it is quicker (there is no need to determine the volume of the hydrolysate aqueous phase) and the efficiency of counting of radioactivity is higher.

The scintillation mixture used for counting the <u>in vivo</u> work was toluene:ethoxyethenol:butyl PBD, 625 ml:375 ml:6 g (for aqueous samples) or toluene:butyl PBD, 1000 ml: 6 g (for non-aqueous samples). Chemical and colour quenching was corrected for by internal standardisation (except for some experiments using the Beckmann 8100 scintillation counter in which the H-number method for quench-correction was found to be as good as internal standardisation). Care was taken that all dissection of animals, phase separations etc. of the control and test treatments were performed in a non-biased order (e.g. in the order treatments 1, 2, 1, 2).

2.13 <u>Statistics</u>

Statistical significance was determined by first determining the

variance ratio (F-test). If this gave a significant result, then a t-test assuming the variance of the two samples to be different was used; if the F-test was not significant, then the variance of the two treatments was pooled to give a joint estimate of the population variance (Bailey, 1964).

CHAPTER THREE

Methods of measuring the rate of Triglyceride- Free fatty acid cycling

3.1 Introduction

There are two ways in which the rate of reesterification of FFA can be measured. These can be referred to as the 'breakdown' and 'synthesis' methods, since they rely on the measurement of the breakdown and synthesis of triglyceride. The basis of these methods, together with the assumptions that they require, are discussed below.

3.2 Determination of cycling by measuring glycerol and FFA release: The Breakdown Method

When a molecule of triglyceride is hydrolysed, one glycerol and three FFA molecules are released. If, however, the TG-FFA cycle is operative, then some of the FFA released by lipolysis will be reesterified, meaning that fewer than three FFA molecules per glycerol will be detected. Hence by finding the difference between the FFA released (as predicted by 3x glycerol production) and the actual amount of FFA released (by direct measurement) the amount of FFA reesterified can be estimated.

The main criterion that has to be met for the measurement of cycling by the breakdown of triglyceride is that three molecules of FFA must be released for every molecule of glycerol released. This requires that the glycerol and FFA released from adipose tissue is derived from the breakdown of triglyceride (rather than diglyceride or phospholipid). Also, adipose tissue must not utilise the glycerol or FFA after it has been released by lipolysis (except that FFA can be used for reesterification). The evidence which supports each of these assumptions is given in the next three sections.

3.2.1 FFA must not be used except for reesterification

The only real alternative to esterification for FFA in adipose tissue is oxidation. The tissue is known to oxidise FFA and this oxidation increases during starvation. However, rates of FFA oxidation are low compared to rates of lipolysis and esterification and hence the oxidation of FFA is usually considered as insignificant (Steinberg & Vaughan, 1965; Harper & Saggerson, 1976).

3.2.2 Glycerol released must not be reutilised

For glycerol to be reutilised it has first to be phosphorylated to give glycerol phosphate, and this step is catalysed by the enzyme glycerol kinase. The activity of this enzyme in rat epididymal adipose tissue is low when compared with the rate of release of glycerol (Margolis & Vaughan, 1962; Robinson & Newsholme, 1967) and a similar level of activity has been reported for mouse adipose tissue (Treble & Mayer, 1963). Recent work by Barrera & Ho (1979) has shown that glycerol kinase activity in a fraction purified from adipose tissue was much higher than previously reported and that the enzyme exhibited two K_m's with respect to glycerol, one in the micromolar range and one in the millimolar range. However, studies with intact tissue imply that even if the glycerol kinase is present in white adipose tissue it is strongly inhibited in the intact cell. Incubation of adipose tissue, even with high concentrations of glycerol, leads only to a very low rate of metabolism of glycerol (Steinberg & Vaughan, 1965). Belfrage et al. (1979) found no glycerol reutilisation in dog fat pads *in vivo*. Hence it appears that glycerol is not significantly reutilised in white adipose tissue.

3.2.3 Glycerol and FFA released must come from triglyceride

If glycerol should originate from some source other than triglyceride then the assumption that the rate of FFA release = 3x rate of glycerol released will not be valid. Glycerol could originate from the hydrolysis of the glycerol phosphate produced in glycolysis. However, this phosphatase action does not appear to accur to any significant extent; when adipose tissue is incubated with radioactive glucose the glycerol appearing in the medium has a very low specific radioactivity, implying that little originates directly from the glycolytic pathway (Lynn et al., 1960; Denton & Randle, 1967).

FFA released from adipose tissue could originate from de novo fatty acid synthesis, the newly formed fatty acid being released without it being esterified. However, by incubating fat pads with radioactive glucose and then determining the incorporation of radioactivity into FFA bound to albumin, Denton & Halperin (1968) showed that the release of newly-formed FFA from incubated fat pads is very low, being only 1% of the FFA that is esterified. Hence the release of FFA derived from de novo fatty acid synthesis is insignificant (except perhaps when the rate of lipolysis is very low and the rate of fatty acid synthesis is very high).

Glycerol could be released from the hydrolysis of di- or monoglyceride, meaning that fewer than three FFA molecules would be released per glycerol molecule. Conversely, FFA release could result from the incomplete hydrolysis of triglyceride or diglyceride, with the consequence that more than three FFA would be released per glycerol molecule. Either of these possibilities would invalidate the assumption that three FFA molecules are liberated per glycerol released. Both possibilities would require that the amount of di-

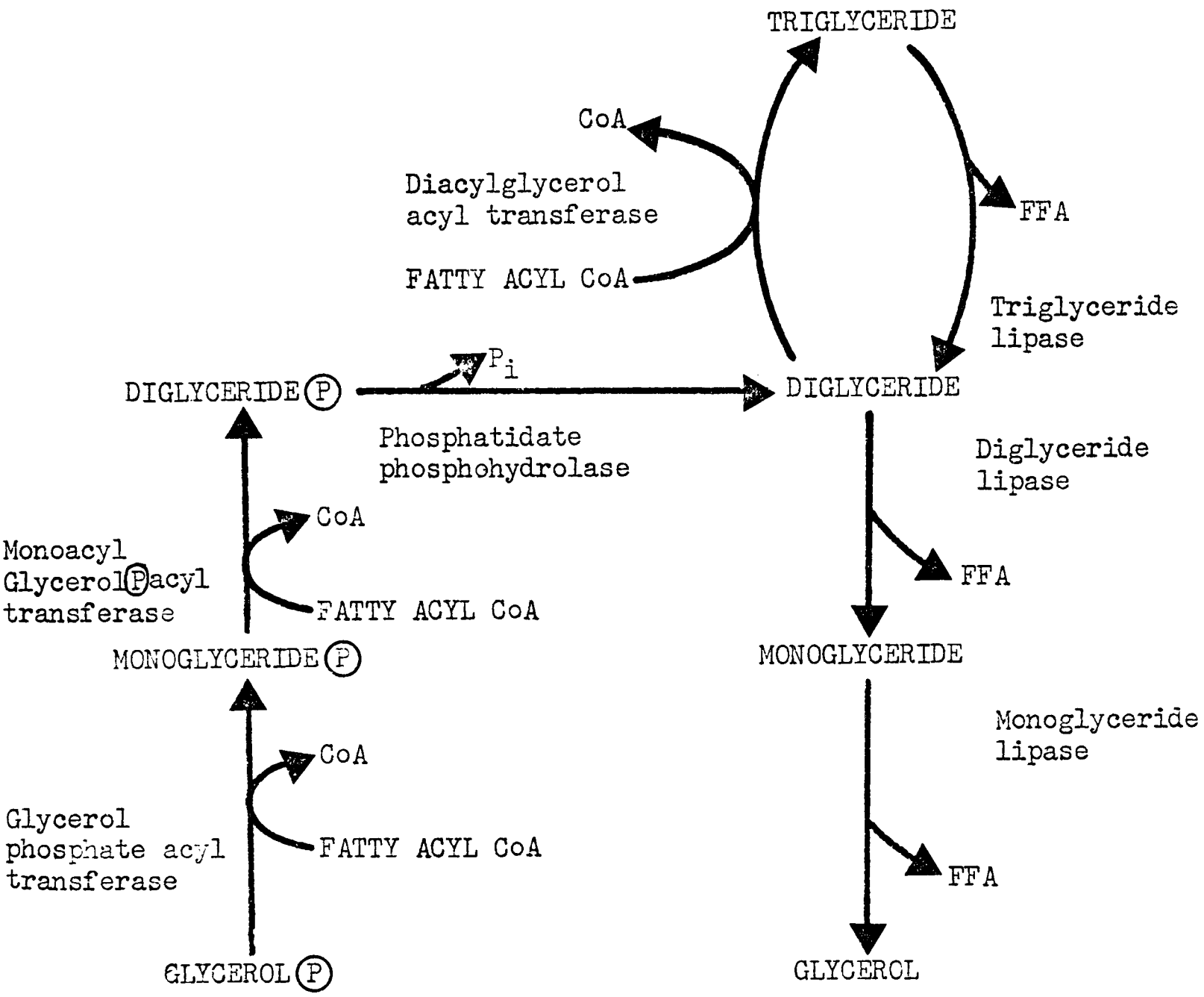
or monoglyceride present in the fat cell should change during an incubation; in other words, that the intermediates in the lipolytic pathway are not in a steady state. If the measurements of the output of FFA and glycerol from a tissue are made whilst it is in a steady state, then the assumption that FFA release is equal to three times glycerol release will be true. Vaughan & Steinberg (1963) found that incubation of adipose tissue with adrenocorticotrophic hormone (ACTH) gave no change in the concentration of lower glycerides, and Angel (1970) found that the concentration of diglyceride varied very little over a 60 minute incubation. However, Scow (1965) found that in perfused parametrial rat fat pad the level of diglyceride was increased by ACTH treatment, suggesting that it is possible to have errors due to changes in the concentrations of lower glycerides. Care should therefore be taken to ensure that adipose tissue is studied whilst in a steady state. If the tissue is able to reach a steady state in a time that is a small proportion of the total incubation time, then the errors involved in assuming that the tissue is in a steady state over the whole incubation will be small. With incubated isolated fat cells, the output of both FFA and glycerol reaches a steady state in under 5 minutes after the addition of noradrenaline, and remains at a constant rate for at least an hour (Angel et al., 1971a; Allen et al., 1973; Allen, 1979). Similarly, rates of FFA and glycerol release from incubated fat pads are linear over an hour (Denton et al., 1966). These results therefore suggest that preincubation of fat cells in order to achieve a steady state after addition of hormones is unnecessary if the incubation is continued for an hour. In the experiments reported in this thesis, isolated fat cells were not pre-incubated. However, pre-incubation of fat pads for about 10 minutes under the same hormonal

conditions as the main incubation was considered to be wise since this should lessen any problems of diffusion of the hormones into, and the glycerol and FFA out of, the fat pad.

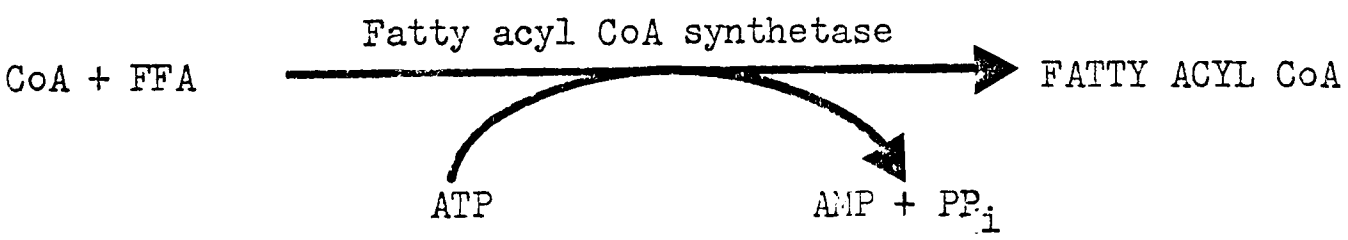
3.3 Determination of cycling by measuring the rates of triglyceride and fatty acid synthesis; The Synthesis Method

Glycerol phosphate is the predominant carbon precursor with which FFA in adipose tissue are esterified to give triglyceride (Vaughan & Steinberg, 1965). The intermediates in the synthesis of triglyceride are shown in fig 3.1. The rate of incorporation of FFA into triglyceride will be three times the rate of incorporation of glycerol phosphate since three FFA are esterified with one glycerol phosphate molecule. When adipose tissue is incubated *in vitro* either the FFA for esterification can be supplied by fatty acid synthesis or they may come from the fatty acids released by lipolysis. Thus if the rate of incorporation of glycerol phosphate (GOP) and newly-formed FFA into triglyceride is measured, then the rate of esterification during an incubation involves the extraction of lipid from the adipose tissue. In the experiments in this thesis the lipid extract was not split into the various intermediates of the triglyceride synthesis pathway, and the amount of glycerol phosphate in each of these intermediates was therefore not determined. Hence it has to be assumed that each glycerol phosphate molecule had three FFA esterified to it (rather than two or one, thus forming di- or mono-glycerides). This condition will be satisfied if the concentration of the intermediates in the esterification pathway remains constant (i.e. they are in a steady state over the incubation period). The author is not aware of any reports on the levels of the phospho-lipids of this pathway, but Angel (1970) found that the diglyceride pool size varied very little over a 60 minute incubation, which

Fig 3.1 The relationship between the pathways of triglyceride synthesis and degradation in adipose tissue



Formation of Fatty acyl CoA:



implies that he was studying a steady state. Angel found several indications that steady state of the esterification pathway was reached very quickly (in under five minutes) in incubated adipocytes. He followed the incorporation of glucose, acetate or palmitate into phospholipids, diglyceride and triglyceride. After five minutes of incubation, over 85% of radioactivity was located in triglycerides and over 90% of this triglyceride activity was already associated with the central lipid droplet. The radioactivity in the phospholipid fraction was always very much less than that in the di- and triglyceride pools, which implies that the error involved in assuming that the phospholipid concentration stays constant will be small in comparison to the overall flux. Similarly, in experiments with adipose tissue homogenates, Angel & Roncari (1967) found that 90% of palmitate incorporated into lipid was in the form of triglyceride, showing that the pool sizes of each of the esterification pathway intermediates were small in comparison to the flux through the pathway. This in turn suggests that the degree of error involved in assuming that the concentration of phospholipid intermediates does not change is small.

3.4 Are the synthesis and breakdown methods of measuring cycling independent of each other's assumptions?

The breakdown and synthesis methods of determining the rate of TG-FFA cycling offer two completely different ways to measure the rate of cycling. It is possible to compare the results of the two methods for the same piece of incubated adipose tissue. The aim of this section is to find to what extent the rate of TG-FFA cycling as determined by the breakdown method can be used as a check on the validity of the assumptions made for the synthesis method (and vice versa). Ideally, therefore, the two methods must not involve the

Table 3.1 Assumptions necessary for Synthesis and Breakdown methods of measuring TG-FFA cycle, together with error expected if assumption is not valid

BREAKDOWN CYCLING = 3x Glycerol release - FFA release	ERROR IN ESTIMATING CYCLING IF ASSUMPTION IS UNTRUE		SYNTHESIS CYCLING = 3x Triglyceride synthesis - FFA synthesis
a) Pool size of lower glycerides must not increase	Under-estimate	Over-estimate	Pool size of esterification intermediates must not increase
b) Pool size of lower glycerides must not decrease	Over-estimate	Under-estimate	Pool size of esterification intermediates must not decrease
c) FFA are not used by adipocyte except for esterification	Over-estimate	Over-estimate	No esterification of FFA from sources other than lipolysis and de novo synthesis, unless measured
d) Glycerol released by lipolysis is not utilised by the tissue	Under-estimate	Under-estimate	Newly-synthesised triglyceride is not broken down again
e) FFA released must come from triglyceride hydrolysis	Under-estimate	Under-estimate	Monoacylglycerol pathway of triglyceride synthesis is insignificant
f) Glycerol released must come from triglyceride hydrolysis	Over-estimate		

same assumption. The assumptions necessary for the breakdown and synthesis methods of measuring the rate of the TG-FFA cycle are summarised in table 3.1, together with the expected error if the assumption is not valid.

With one exception, none of the assumptions listed in table 3.1 is common to both the synthesis and breakdown methods of measuring TG-FFA cycling; the exception involves the concentration of diglyceride. Both methods require that the levels of the intermediates in the triglyceride synthesis and breakdown pathways do not change over the incubation period. The pathways for the synthesis and degradation of triglyceride (fig 3.1) are different except that they share diglyceride as an intermediate. However, should the level of diglyceride change during an incubation, then the breakdown and synthesis methods will be in error in opposite directions (i.e. if one overestimates then the other underestimates: cases (a) and (b) in table 3.1. Hence the two methods do provide a check on each other, though it is possible that both methods could simultaneously give an over- or underestimate due to a combination of unrelated assumptions being violated.

3.5 Methods of measuring the rate of fatty acid and triglyceride synthesis

Two methods were used to measure the rate of fatty acid and triglyceride synthesis; these involved the use of either ^{14}C-labelled glucose or tritiated water. The use of these isotopes in measuring the rates of fatty acid synthesis and glycerol phosphate incorporation into triglyceride is discussed in the subsequent sections.

3.5.1 Use of ^{14}C-Glucose to measure fatty acid and triglyceride synthesis

3.5.1.1 Triglyceride Synthesis

The incorporation of ^{14}C-glucose into the triglyceride-glycerol

(TG-glycerol) moiety has often been used to measure triglyceride synthesis. Glucose is metabolised via the glycolytic pathway to give glycerol phosphate and acetyl CoA. To measure the rates of TG and FA synthesis the specific activity of the GOP and acetyl CoA has to be known, since the possibility exists that endogenous glycogen could be broken down thus diluting the specific activity of the glycerol phosphate and acetyl CoA. Denton & Randle, (1967) found that when fat pads were incubated with ^{14}C-glucose and a variety of hormones, the specific activity of lactate in the medium was the same as the starting glucose specific activity. From this they reasoned that the breakdown of endogenous glycogen must be low, and that the specific activity of the glycerol phosphate should be similar to the glucose in the medium. In the experiments in this thesis in which fat pads are incubated with ^{14}C-labelled glucose, it is assumed that the specific activity of the glycolytic intermediates is the same as the glucose in the incubation medium. However, under certain circumstances breakdown of glycogen does occur in adipose tissue and this leads to dilution of the specific activity of the glycolytic intermediates (Jungas, 1968, 1970; Leboeuf, 1965). This is a problem with the use of ^{14}C-labelled glucose (see section 3.5.2).

3.5.1.2 Fatty Acid Synthesis

The incorporation of glucose from the medium can also be used to follow the synthesis of fatty acids. It is possible that the oxidation of endogenous fatty acids could dilute the specific activity of the acetyl CoA pool giving an underestimate of the rate of fatty acid synthesis. However, Bally et al. (1960) showed that the oxidation of exogenous FFA was greatly inhibited by low concentrations of glucose. This suggests that the dilution of the acetyl CoA

specific activity should be small when fatty acids are being synthesised from glucose.

3.5.2 Use of tritiated water to measure fatty acid and triglyceride synthesis

3.5.2.1 Fatty Acid Synthesis

In 1936, Schoenheimer & Rittenburg reported the use of deuterated water to follow the synthesis of fatty acids. Since then the method has been extended to the use of hydrogen's radioactive isotope, tritium. The advantage of the use of labelled water is that it is easy to maintain a constant specific activity within the cell, which (if it is assumed that the water has equilibrated throughout the intracellular and extracellular fluids) can easily be measured by taking a sample of extracellular water. In contrast, the use of carbon-14 to follow rates of reactions has the problem that the specific activity of the intermediates within the cell can be diluted by the breakdown of endogenous carbon sources, leading to an underestimate of the flux rates (Jungas, 1968, 1970; Leboeuf, 1965).

During synthesis of fatty acids, tritium (which must have originated from tritiated water) is incorporated into the molecule. Three possible sources of hydrogen atoms may have been originally present as the methyl hydrogen atoms of acetyl CoA (or the equivalent methylene hydrogen atoms of malonyl CoA), or they may have been gained either as hydride ions from reduced pyridine nucleotides (e.g. NADPH) or as protons from water. From a knowledge of the enzyme mechanisms it can be predicted that the hydrogen atoms on the even-numbered carbon atoms of the fatty acid should arise from acetyl CoA and protons, whereas those on the odd-numbered carbon atoms would be expected to originate in the reductive hydrogen of reduced pyridine

nucleotide. In practice, the situation is not as clear cut as this (Foster & Bloom, 1963; Jungas, 1968) since fatty acids synthesised from tritiated acetate contain fewer hydrogen atoms from carbon 2 of acetyl CoA than expected, this loss being ascribed to exchange with protons from water.

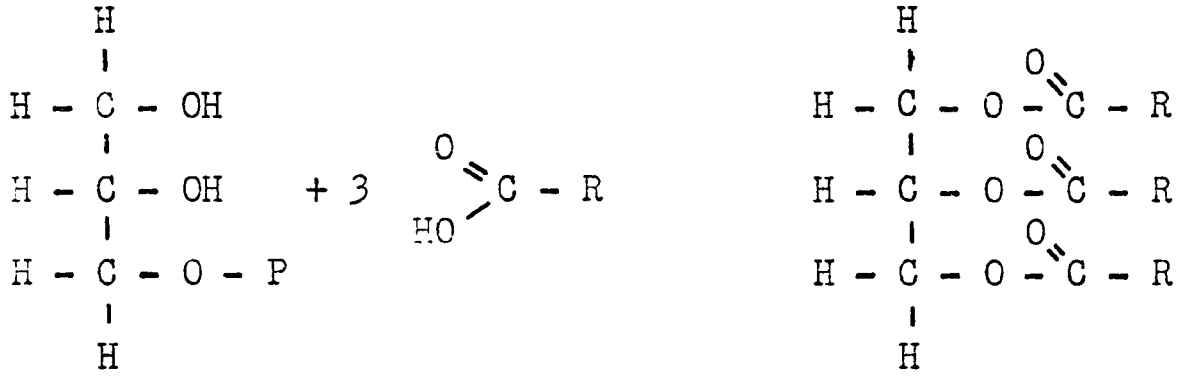
The mean number of carbon atoms in fatty acids synthesised in rat adipose tissue is 16, and these have been found to have 13.9 tritium atoms bound to them (see Jungas, 1968). Similarly, 13.3 tritium atoms per fatty acid synthesised have been found for fatty acids in liver (Windmueller & Spaeth, 1966). Isotope discrimination is known to occur in the synthesis of fatty acids, since incubating tissue with deuterated plus tritiated water gives a greater number of tritium atoms incorporated per fatty acid synthesised than in tissue incubated with water plus tritium (Jungas, 1968).

Owing to the stability of the carbon-hydrogen bond in fatty acids, hydrogen exchange with water is extremely low; tritium is therefore incorporated into fatty acids only during their synthesis (Van Heyningen et al., 1938; Windmueller & Spaeth, 1966). Hence the incorporation of tritium from tritiated water is a very useful method for measuring the rate of fatty acid synthesis, and it has the advantage of being a method of quantifying the rate of this process when fatty acid synthesis is being carried out from several different substrates simultaneously (see for example Hems et al., 1975; Stansbie et al., 1976).

3.5.2.2 Triglyceride Synthesis

The glycerol moiety of the triglyceride molecule has five hydrogen atoms in it (see fig 3.2). Because these hydrogen atoms are bound to carbon atoms, they are very resistant to proton exchange with water. As discussed previously, the major precursor of the

glyceride-glycerol moiety in adipose tissue is glycerol phosphate (see section 3.3). Hence, if any of the hydrogen atoms on glycerol phosphate are gained from water during the synthesis of the glycerol phosphate, then it should be possible to use tritiated water to measure the rate of incorporation of glycerol phosphate into triglyceride.



Glycerol phosphate Fatty Acid Triglyceride

Fig 3.2 The structures of triglyceride and its precursors.
R = an alkyl group

Jungas (1968) found that when adipose tissue was incubated with tritiated water and insulin, 3.3 tritium atoms were incorporated into each triglyceride-glycerol moiety formed (the rate of triglyceride synthesis was measured using ^{14}C-glucose). Hence it appears that at least three of the five hydrogen atoms bound to the glycerol phosphate carbon atoms can be derived, directly or indirectly, from water. It is not immediately apparent how these hydrogens are derived from water, and in order to see how these hydrogen atoms are gained, the mechanisms of the relevant reactions involved in glycerol phosphate production must be understood. In Appendix II the mechanisms of these reactions are discussed with respect to the gain of carbon-bound hydrogen atoms by glycerol phosphate. The information in this appendix is summarised as follows. Consider the conversion of one molecule of glucose to give two molecules of

glycerol phosphate (GOP); of the ten carbon-bound hydrogen atoms present on the two GOP molecules, only seven of them can exchange with water. Six of them (i.e. three per GOP molecule) will be gained if the triose phosphate isomerase and aldolase reactions are at equilibrium; the seventh tritium atom will be gained by only one of the two GOP molecules (the old carbons 1,2 and 3 of glucose) in the phosphoglucose isomerase reaction. Hence an average of 3.5 tritium atoms per glycerol phosphate can be expected. The experimental value found by Jungas (1968) of 3.3 tritium atoms per glycerol phosphate fits very well with this predicted value.

It is possible that at high rates of glycolysis or during times of high demand for glycerol phosphate the phosphoglucose isomerase, aldolase and triose phosphate isomerase reactions might depart from equilibrium. If the departure from equilibrium is pronounced, then this would lead to a smaller number of tritium atoms incorporated per glycerol phosphate molecule. However, this number would not be expected to drop below about $2\frac{1}{4}$ tritium atoms per glycerol phosphate. This is because only one extra tritium atom on each of glyceraldehyde phosphate and DHAP is gained if the triose phosphate isomerase and aldolase reactions are at equilibrium, and only about a half a tritium is gained per F6P molecule (equal to a quarter of a tritium per glycerol phosphate) if the phosphoglucose isomerase reaction is at equilibrium. Hence the maximum error possible in estimating the rate of triglyceride-glycerol synthesis by using a figure of 3.3 tritium atoms per glycerol phosphate is to underestimate the rate of triglyceride-glycerol synthesis by about 30%. The extent of departure of the above reactions from equilibrium can only be determined by experiment. The results of these investigations are given in the next section.

3.5.3 Measurement of tritium incorporation into glycerol phosphate and comparison of the breakdown and synthesis methods of measuring TG-FFA cycling

Ideally, the specific activity of glycerol phosphate in adipose tissue incubated in tritiated water should be measured directly. However, due to the low levels of intracellular water in adipose tissue, and the difficulty in separating glycerol phosphate from the other sugar phosphates, the measurement of the specific activity of glycerol phosphate is very difficult (see Denton & Randle, 1967). For this reason, the specific activity of tritium in glycerol phosphate was measured by incubating epididymal adipose tissue with ^{14}C-glucose and tritiated water and comparing the radioactivity incorporated into the triglyceride-glycerol moiety.

Several experiments were done attempting to use fat cells to investigate the incorporation of tritiated water into triglyceride-glycerol. Unfortunately, it was not possible to find a satisfactory method for separating the small amounts of lipid in the isolated fat cells from the vast tritiated water radioactivity. However, it was found that the greater quantities of triglyceride that are obtained from fat pads made the counting of tritium incorporated easier. Hence fat pads were used in these experiments. Each fat pad was cut into two pieces; one piece was incubated with ^{14}C-glucose and the other with tritiated water. These radioisotopes were present in different flasks so as to avoid the errors inherent in dual isotope counting. After an hour of incubation, triglyceride was extracted from the tissue and radioactivity present in fatty acids and the triglyceride-glycerol moiety was determined as described in chapter 2. Knowledge of these two parameters can be used to derive the rate of TG-FFA cycling by the synthesis method (see section 3.3). The release of glycerol and FFA from the tissue was also measured, and

these two parameters can be used to determine the rate of TG-FFA cycling by the breakdown method (see section 3.2). Hence the results of these experiments can be used to make two comparisons. The first is to compare the rates of incorporation of ^{14}C-glucose and tritiated water into the triglyceride-glycerol moiety. The second is to compare the synthesis and output methods of measuring the TG-FFA cycle. The former comparison will give the number of tritium atoms per glycerol phosphate molecule synthesised. The data from each experiment is presented in tables which show the rates of glycerol and FFA release, the rates of TG-FFA cycling as measured by the synthesis and breakdown methods (tables 3.2, 3.4 and 3.6). The rates of cycling, together with the results of statistical tests, are summarised in separate tables (tables 3.3, 3.5 and 3.7). A graphical summary of these experiments is shown in fig 3.5.

3.5.3.1 Comparison of ^{14}C-glucose and tritiated water incorporation

The aim of the comparison of the rates of incorporation of tritiated water and ^{14}C-glucose into the triglyceride-glycerol moiety is to find the number of tritium atoms incorporated into glycerol phosphate. Incubation of a fat pad with ^{14}C-glucose provides a means of estimating the rate of triglyceride-glycerol synthesis. A simultaneous incubation under the same conditions but with tritiated water instead of ^{14}C-glucose will enable the number of tritium atoms incorporated per glycerol phosphate to be calculated by means of straightforward comparison between the two rates of radiolabel incorporation. If this is done for the results presented in tables 3.2, 3.4 and 3.6, then a figure of 3.1 $\pm$ 0.4 (mean $\pm$ SEM, N = 10) tritium atoms per glycerol phosphate is found. This value agrees well with that found by Jungas (1968) of 3.3 $\pm$ 0.33 (mean $\pm$ SEM, N = 6).

TABLE 3.2 RATE OF TG-FFA CYCLING IN INCUBATED MOUSE EPIDIDYMAL FAT PADS AS MEASURED BY BREAKDOWN AND SYNTHESIS METHODS (using tritiated water and ^{14}C Glucose)

INCUBATION CONDITIONS AND ISOTOPES USED		BREAKDOWN METHOD			SYNTHESIS METHOD		
		Rate Glycerol release	Rate FFA release	Rate TG-FFA cycling	Rate TG-Glycerol synthesis	Rate FA synthesis	Rate TG-FFA cycling
Ins, 1 mM Gluc	^{3}H	1.83 ± 0.18	2.21 ± 0.36	3.28 ± 0.27	0.95 ± 0.11	0.057 ± 0.017	2.78 ± 0.32
	^{14}C	1.72 ± 0.17	2.46 ± 0.31	2.70 ± 0.32	0.52 ± 0.09	0.015 ± 0.005	1.54 ± 0.28
Ins, 10mM Gluc	^{3}H	1.73 ± 0.11	1.20 ± 0.13	3.99 ± 0.27	1.18 ± 0.08	0.15 ± 0.05	3.29 ± 0.20
	^{14}C	1.63 ± 0.11	1.16 ± 0.31	3.58 ±0.39	1.00 ± 0.08	0.12 ± 0.05	2.87 ± 0.19
NA Ins, 10mM Gluc	^{3}H	7.53 ± 1.06	13.5 ± 2.51	9.16 ± 1.51	2.08 ± 0.30	0.176 ± 0.110	6.06 ± 0.82
	^{14}C	7.46 ± 1.05	13.6 ± 2.37	8.77 ± 1.67	2.81 ± 0.47	0.122 ± 0.067	8.31 ± 1.35

For legend see table 3.3

TABLE 3.3 STATISTICAL SIGNIFICANCE BETWEEN RATES OF TG-FFA CYCLING AS MEASURED BY BREAKDOWN AND SYNTHESIS METHODS (data from table 3.2)

Incubation Conditions	^3H_2O INCUBATIONS				^{14}C GLUCOSE INCUBATIONS			
	Rate of cycling measured by breakdown method		Rate of cycling measured by synthesis method		Rate of cycling measured by breakdown method		Rate of cycling measured by synthesis method	
1 mM Glucose Insulin	3.28 ± 0.27	NS	2.78 ± 0.32	*	1.54 ± 0.28	*	2.70 ± 0.32	
10 mM Glucose Insulin	3.99 ± 0.27	NS	3.29 ± 0.20	NS	2.87 ± 0.19	NS	3.58 ± 0.39	
10 mM Glucose Insulin Noradrenaline	9.16 ± 1.51	NS	6.06 ± 0.82	NS	8.31 ± 1.35	NS	8.77 ± 1.67	

The two epididymal fat pads were removed from Pathology Oxford mice fed ad libitum. Both pads were incubated under exactly the same conditions except that one pad was incubated with tritiated water present and the other with ^{14}C-glucose. Concentrations used were: Insulin, 500 µU/ ml; Noradrenaline, 5 µMolar. Calculations assume 3.3 tritium atoms per glycerol phosphate synthesised. Values are for medium changes and are given as mean ± SEM (7 mice per treatment). Units are µmoles per gram wet weight tissue per hour.

Statistical results are for t-tests between columns either side of, and immediately adjacent to, the statistics column. Significance indicated by:- NS = $p > 0.05$
* = $p < 0.05$

TABLE 3.4 RATE OF TG-FFA CYCLING IN INCUBATED RAT EPIDIDYMAL FAT PADS AS MEASURED BY BREAKDOWN AND SYNTHESIS METHODS (using tritiated water and ^{14}C Glucose). For legend see table 3.5.

INCUBATION CONDITIONS AND ISOTOPE USED		BREAKDOWN METHOD			SYNTHESIS METHOD		
		Rate Glycerol release	Rate FFA release	Rate TG-FFA cycling	Rate TG-Glycerol synthesis	Rate FA synthesis	Rate TG-FFA cycling
CONTROL	^{3}H	0.63 ± 0.05	1.39 ± 0.22	0.49 ± 0.13	1.37 ± 0.19	0.80 ± 0.08	3.29 ± 0.51
	^{14}C	0.56 ±0.08	1.60 ± 0.22	0.08 ± 0.32	0.93 ± 0.13	0.46 ± 0.09	2.34 ± 0.31
GLUCAGON	^{3}H	4.50 ± 0.37	3.15 ± 0.76	10.3 ± 0.62	3.50 ± 0.22	0.98 ± 0.12	9.51 ± 0.57
	^{14}C	4.05 ± 0.40	3.45 ± 0.46	8.71 ± 0.87	4.01 ± 0.29	0.53 ± 0.06	11.5 ± 0.84
ACTH	^{3}H	6.01 ± 0.57	10.1 ± 0.67	7.96 ± 1.40	2.64 ± 0.25	0.42 ± 0.04	7.51 ± 0.72
	^{14}C	5.75 ± 0.27	10.7 ± 0.77	6.53 ± 0.61	3.04 ± 0.15	0.17 ± 0.02	8.96 ± 0.93
ACTH PGE_1	^{3}H	6.84 ± 0.42	10.5 ± 1.23	10.0 ± 0.70	3.57 ± 0.26	0.58 ± 0.06	10.1 ± 0.76
	^{14}C	5.46 ± 0.39	7.96 ± 0.39	8.43 ± 1.01	3.69 ± 0.02	0.28 ± 0.04	10.8 ± 0.64

TABLE 3.5 STATISTICAL SIGNIFICANCE BETWEEN RATES OF TG-FFA CYCLING AS MEASURED BY BREAKDOWN AND SYNTHESIS METHODS (data from table 3.4)

Incubation Conditions	$^{3}H_2O$ INCUBATIONS				^{14}C INCUBATIONS			
	Rate of cycling measured by breakdown method		Rate of cycling measured by synthesis method		Rate of cycling measured by breakdown method		Rate of cycling measured by synthesis method	
CONTROL	0.49 ± 0.13	***	3.29 ± 0.51	NS	2.34 ± 0.31	***	0.084 ± 0.32	
GLUCAGON	10.3 ± 0.62	NS	9.51 ± 0.57	NS	11.5 ± 0.84	*	8.71 ± 0.87	
ACTH	7.96 ± 1.40	NS	7.51 ± 0.72	NS	8.96 ± 0.43	**	6.53 ± 0.61	
ACTH + PGE_1	10.0 ± 0.70	NS	10.1 ± 0.76	NS	10.8 ± 0.64	NS	8.43 ± 1.01	

Glucose was present in all incubations. Fat pads were removed from Sprague-Dawley rats (250-300g) fed ad libitum. Each pad was cut into two for two treatments (^{14}C glucose and $^{3}H_2O$ incubations for each hormonal treatment). The pads were pre-incubated for 10 minutes in buffer containing 1% adbumin under the same hormonal conditions as in the main incubation. The main incubation (with 4% albumin) was for 1 hour. Concentrations used were: glucose, 5 mM; glucagon, 5 µg/ ml; ACTH, 5 µg/ ml; PGE_1, 5 µM. Calculations assume 3.0 tritium atoms per glycerol phosphate synthesised. Values are in µmoles per gram wet weight per hour and are given as mean ± SEM (9 incubations per treatment).

Statistical results are for t-tests between adjacent columns. Significance indicated by:- NS = $p > 0.05$
* = $p < 0.05$
** = $p < 0.01$
*** = $p < 0.001$

TABLE 3.6 RATE OF TG-FFA CYCLING IN INCUBATED RAT EPIDIDYMAL FAT PADS AS MEASURED BY BREAKDOWN AND SYNTHESIS METHODS (using tritiated water and ^{14}C Glucose)

INCUBATION CONDITIONS AND ISOTOPE USED		BREAKDOWN CYCLING			SYNTHESIS CYCLING		
		Rate Glycerol release	Rate FFA release	Rate TG-FFA cycling	Rate TG-Glycerol synthesis	Rate FA synthesis	Rate TG-FFA cycling
CONTROL	^{3}H	2.24 ± 0.41	1.32 ± 0.10	5.39 ± 1.19	2.07 ± 0.28	1.03 ± 0.15	5.18 ± 0.71
	^{14}C	2.13 ± 0.20	1.33 ± 0.18	5.05 ± 0.57	3.37 ± 0.41	2.18 ± 0.36	7.94 ± 0.94
TRIIODO-THYRONINE	^{3}H	2.25 ± 0.17	2.74 ± 0.32	4.00 ± 0.44	2.01 ± 0.17	0.95 ± 0.10	5.09 ± 0.44
	^{14}C	1.65 ± 0.25	2.39 ± 0.43	2.57 ± 0.65	2.81 ± 0.41	1.79 ± 0.45	6.64 ± 0.80
CORTISOL	^{3}H	1.71 ± 0.23	3.35 ± 0.49	1.77 ± 0.39	1.55 ± 0.10	0.69 ± 0.05	3.96 ± 0.25
	^{14}C	1.41 ± 0.11	3.30 ± 0.42	0.94 ± 0.19	2.72 ± 0.25	1.44 ± 0.14	6.74 ± 0.68

For legend see table 3.7

TABLE 3.7 STATISTICAL SIGNIFICANCE BETWEEN RATES OF TG-FFA CYCLING AS MEASURED BY BREAKDOWN AND SYNTHESIS METHODS (data from table 3.6)

Incubation Conditions	$^{3}H_2O$ INCUBATIONS				^{14}C GLUCOSE INCUBATIONS			
	Rate of cycling measured by breakdown method		Rate of cycling measured by synthesis method		Rate of cycling measured by breakdown method		Rate of cycling measured by synthesis method	
CONTROL	5.39 ± 1.19	NS	5.18 ± 0.71	*	7.94 ± 0.94	*	5.05 ± 0.57	
TRIIODOTHYRONINE	4.00 ± 0.44	NS	5.09 ± 0.44	NS	6.64 ± 0.80	**	2.57 ± 0.65	
CORTISOL	1.77 ± 0.39	***	3.96 ± 0.25	**	6.74 ± 0.68	***	0.94 ± 0.19	

Glucose was present in all incubations. Fat pads were removed from Sprague-Dawley rats (150-190g) fed ad libitum. Each pad was cut into two for two treatments (^{14}C glucose and $^{3}H_2O$ incubations for each hormonal treatment). The pads were pre-incubated for 10 minutes in buffer containing 1% albumin under the same hormonal conditions as in the main incubation. The main incubation (with 4% albumin) was for one hour. Concentrations used were: glucose, 5 mM; triiodothyronine, 1.25 µg/ ml; cortisol acetate, 5 µg/ ml. Calculations assume 3.0 tritium atoms per glycerol phosphate synthesised. Values are in µmoles per gram wet weight per hour and are given as mean ± SEM (10 incubations per treatment).

Statistical results are for t-tests between adjacent columns. Significance is indicated by:- NS = $p > 0.05$
* = $p < 0.05$
** = $p < 0.01$
*** = $p < 0.001$

Fig 3.5 Bar chart showing rate of cycling as measured by breakdown and synthesis methods - values are from tables 3.3, 3.5 and 3.7.

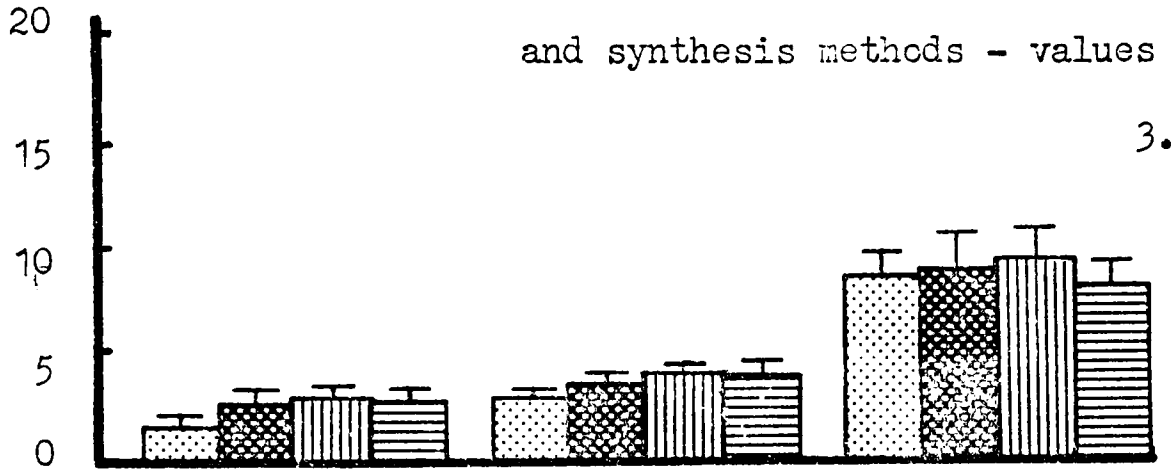


Table 3.3 (N=7)

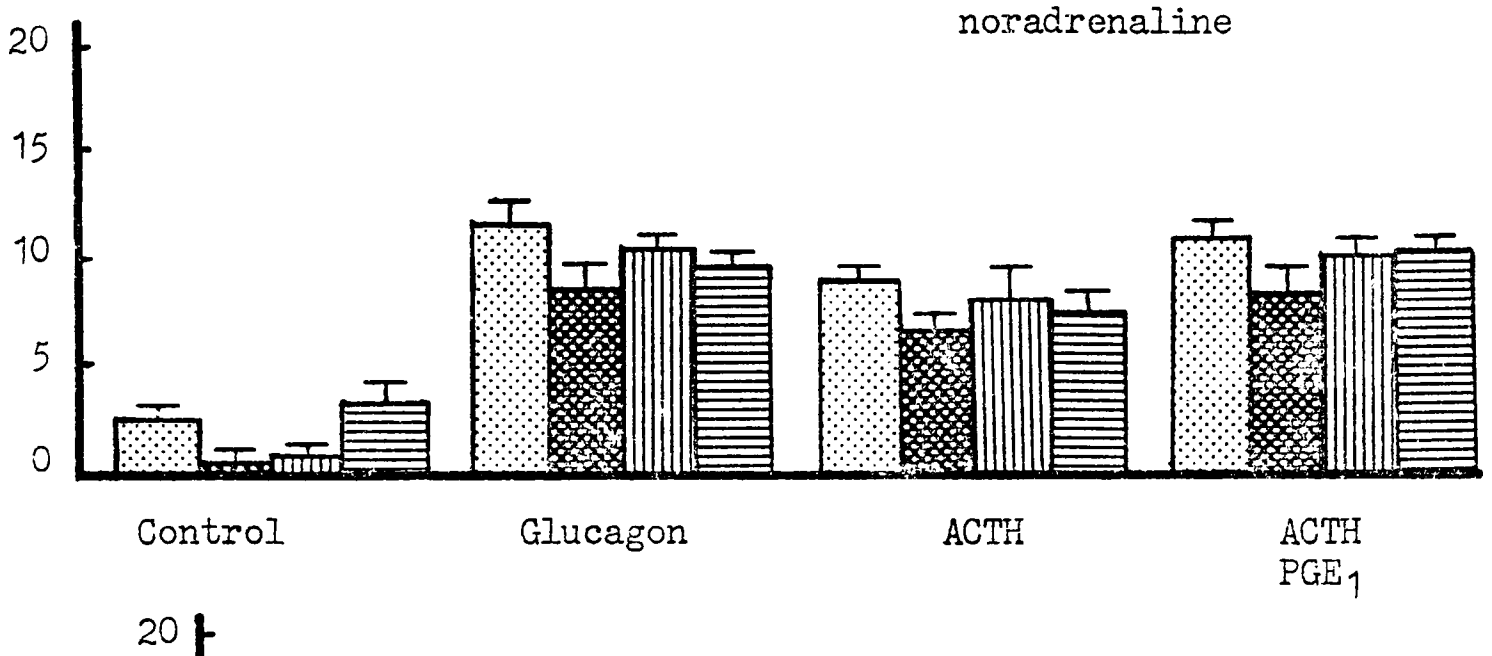


Table 3.5 (N=9)

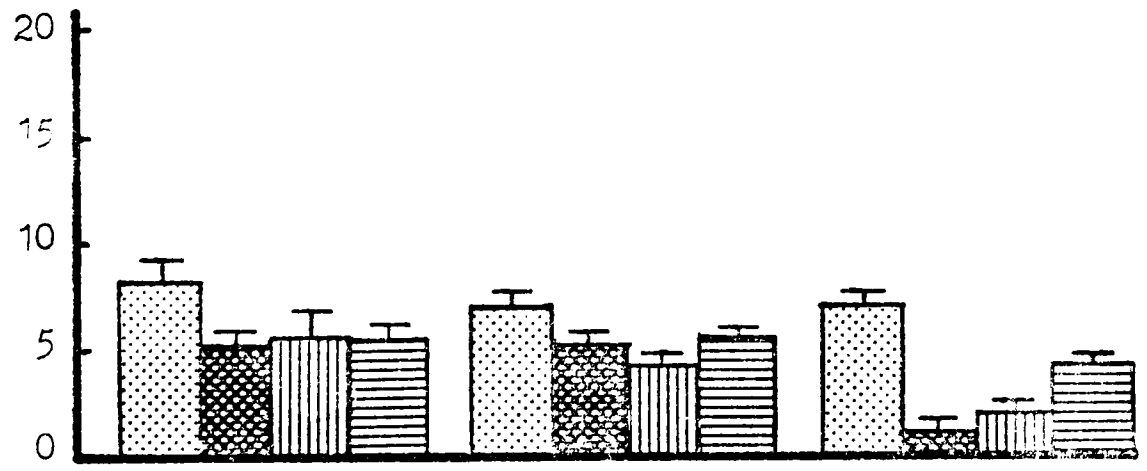


Table 3.7 (N=10)

Synthesis cycling, measured using ^{14}C-glucose

Breakdown cycling in ^{14}C incubation

Breakdown cycling in $^{3}H_2O$ incubation

Synthesis cycling measured using $^{3}H_2O$

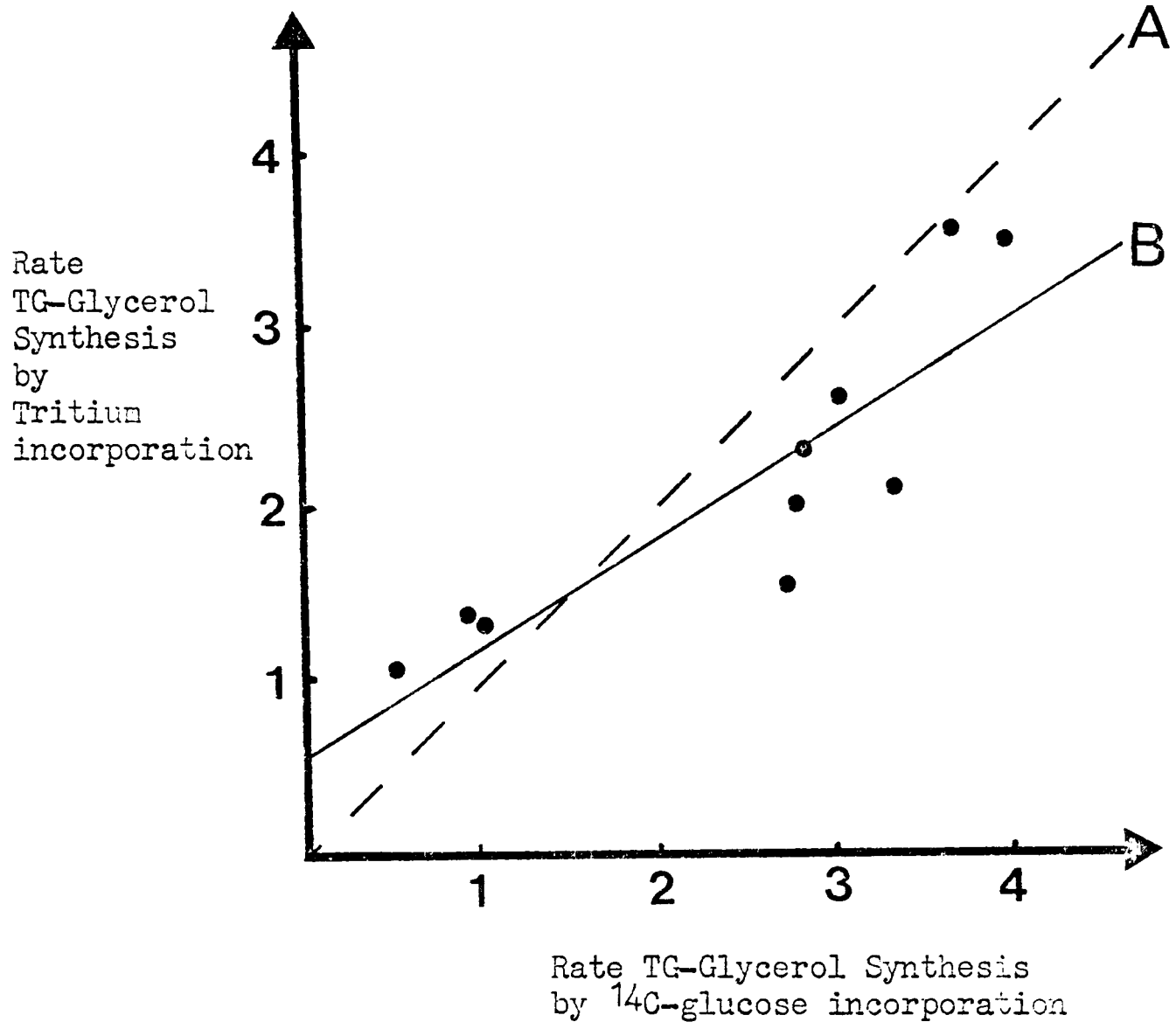
Units are in μmoles per gram wet weight per hour. Error bars are for SEM.

Another way in which the number of tritium atoms per triglyceride-glycerol can be calculated is to compare the breakdown and synthesis methods of measuring the TG-FFA cycle. Knowing the rate of TG-FFA cycling (by the breakdown method) and the rate of fatty acid synthesis (by tritium incorporation) the rate of triglyceride synthesis necessary to give agreement between the breakdown and synthesis methods can be calculated. Using all of the treatments shown in tables 3.2, 3.4 and 3.6, an average of 3.8 ± 0.7 (mean ± SEM, N = 10) tritium atoms per glycerol phosphate is found. This high value for the incorporation of tritium is due to one result being very high. This result (for the control incubations in table 3.4) shows a large discrepancy between the breakdown and synthesis methods of measuring the rate of FFA reesterification. The difference persists with both the ^{14}C-glucose and tritium methods of measuring triglyceride-glycerol synthesis, and it is possible that the low estimate for the rate of output cycling is due to errors in measuring the small amount of glycerol released in the control incubation. If this result is omitted, then a figure of 3.2 ± 0.3 (mean ± SEM, N = 9) tritium atoms per triglyceride-glycerol moiety is obtained.

As discussed in section 3.5.2.2, it is possible that as the demand for glycerol phosphate increases, the number of tritium atoms incorporated per glycerol phosphate molecule may decrease. This is because when the glycolytic flux is high, the phosphoglucose isomerase, aldolase, triose phosphate isomerase and glycerol phosphate dehydrogenase reactions might depart from equilibrium, meaning that certain hydrogen atoms on the substrates/products of the above reactions would not get the chance to equilibrate with tritium in the aqueous phase. If the rate of triglyceride synthesis is calculated assuming a constant number of tritium atoms per triglyceride-glycerol moiety, then a graph of the rate of triglyceride-glycerol synthesis (as

determined by tritium incorporation) against the rate as determined by ^{14}C-glucose incorporation should show whether this disequilibrium occurs to any significant extent. If the above reactions do not move from equilibrium, then this graph should have a gradient of unity; if the reactions do shift from equilibrium then the gradient of the graph should be less than unity. Fig 3.6 shows that the experiments reported in this chapter provide an indication that the reactions catalysed by the enzymes given above do move from equilibrium as the rate of triglyceride-glycerol synthesis increases, since the gradient of the least-squares best fit straight line is less than unity. This therefore suggests that as the rate of triglyceride-glycerol synthesis is increased a lower value for the number of tritium atoms per glycerol phosphate should be used. However, it would require many more experiments to determine the relationship between the incorporation of tritium and the rate of triglyceride synthesis; the experiments reported in this chapter are not sufficient to establish this relationship with accuracy. Hence in all of the calculations of the rate of triglyceride-glycerol synthesis a constant number of tritium atoms has been assumed. As discussed in section 3.5.2.2 and appendix II this assumption can lead to the rate of triglyceride synthesis being underestimated by up to 30%. The experiments reported above suggest that an average number of 3.1 (based on comparison of tritium incorporation and breakdown cycling) tritium atoms are incorporated per glycerol phosphate molecule. These figures are similar to the value of 3.3 tritium atoms per triglyceride-glycerol moiety as found by Jungas (1968). It was therefore decided to use a value of 3.3 tritium atoms per glycerol phosphate in all of the calculations of the rate of triglyceride-glycerol synthesis, since this figure should ensure that the rate of triglyceride synthesis is

Fig 3.6 Graph of rate of triglyceride-glycerol synthesis as measured by tritium incorporation versus the rate of triglyceride-glycerol synthesis as measured by ^{14}C-glucose incorporation



All rates are in μmoles per gram wet tissue per hour. Calculations assume 3.0 tritium atoms per glyceride-glycerol moiety.

Line A = Line for rate of TG-glycerol synthesis being equal when measured by both tritium and ^{14}C-glucose methods (ie gradient = one).

Line B = Least-squares best fit straight line through all points.

not overestimated.

3.5.3.2 Comparison of breakdown and synthesis cycling

The bar-chart summary shown in fig 3.5 compares the rate of TG-FFA cycling as measured by the synthesis and breakdown methods. In most of the treatments shown there is very good agreement, both qualitatively and quantitatively, between the two methods. As discussed in section 3.4, the possibility that both of the methods of measuring TG-FFA cycling give a simultaneous under- or over-estimate cannot be excluded. However, the agreement seen here between two completely different methods of measuring the rate of the TG-FFA cycle is impressive.

3.6 The triglyceride-diglyceride (TG-DG) cycle

A substrate cycle could exist between diglyceride and triglyceride without the involvement of phospholipids or monoglycerides (see fig 3.1). Measurement of this cycle would be very difficult since FFA incorporation into triglyceride could be due to the TG-DG cycle or due to the completely new synthesis of triglyceride starting from glycerol phosphate.

The TG-DG cycle would require a competition for substrate between the diglyceride lipase and diacylglycerol acyl transferase. For the TG-DG cycle to operate, the diglyceride molecule needs to have access to both hormone-sensitive lipase and diacylglycerol acyl transferase. Hormone-sensitive lipase is found in the soluble fraction of the cell (see Schwartz & Jungas, 1971; Heller & Steinberg, 1972) whereas diacylglycerol acyl transferase is found in the microsomal fraction (see table 4.1). It would therefore seem that the diglyceride molecule would have to cross between different cellular compartments in order to reach both enzymes. (FFA involved in the TG-FFA cycle would also have to cross between these

compartments). If this movement between compartments is not fast then the diglyceride would be broken down by diacylglycerol lipase. An indication that this transfer of diglyceride may not be fast can be deduced from the fact that tissue does not release diglyceride into the incubation medium. This implies that diglycerides are unable to cross the plasma membrane cf FFA which do cross the plasma membrane. However, the plasma membrane and membranes dividing intracellular compartments need not be permeable to the same molecule to a similar extent. Hence, due to the difficulty in measuring the TG-DG cycle, together with the lack of knowledge concerning lipid transport within the cell, it is difficult to determine the likely extent of the TG-DG cycle.

3.7 The Dihydroxyacetone phosphate and Monoacylglycerol pathways

It has been demonstrated that in addition to glycerol phosphate both dihydroxyacetone phosphate (DHAP) and monoacylglycerol (MG) may act as the backbone with which FFA may be esterified to form triglyceride (see Dodds et al., 1976). They found that the effectiveness as FFA acceptor decreased in the order glycerol phosphate - DHAP - monoglyceride. Schlossman & Bell (1976) concluded that DHAP acyl transferase and glycerol phosphate acyl transferase were the same enzyme. They also decided that *in vivo* glycerol phosphate should be at least 24 times more efficient as a FFA acceptor than DHAP. It therefore appears that the DHAP and MG acyl transferase pathways for triglyceride synthesis are insignificant when compared to the glycerol phosphate pathway. Nevertheless, it is of interest to see how they would affect the breakdown and synthesis methods of measuring the TG-FFA cycle.

DHAP is produced in glycolysis. Use of ^{14}C-glucose to measure the rate of triglyceride synthesis would still give the correct

rate of cycling. The tritium method of measuring triglyceride-glycerol synthesis might be affected by the use of DHAP for FFA esterification. The number of tritium atoms present of DHAP would be the same as on glycerol phosphate only if a tritium atom is gained when the ketone-oxygen on DHAP is reduced. The argument is perhaps academic since the overall number of tritium atoms incorporated per triglyceride-glycerol synthesised is determined by comparison with ^{14}C-glucose anyway. This method will take into account both the glycerol phosphate and DHAP pathways of synthesising triglyceride. Hence the synthesis method is not affected by the DHAP pathway. The breakdown method is also not affected by the DHAP pathway since for this method for measuring TG-FFA cycling no assumptions concerning triglyceride synthesis are necessary.

The monoglyceride pathway would be expected to give an underestimate of the rate of cycling by the breakdown method. If the monoglyceride for the pathway is derived from lipolysis then an underestimate of the total amount of FFA release would result since glycerol would not be released. The synthesis method would also give an underestimate of the rate of cycling, since the use of monoacylglycerol for FFA esterification would not be detected as triglyceride synthesis by using either the ^{14}C-glucose or the tritium incorporation method. However, the low rates reported for this pathway imply that the possible errors due to the monoacylglycerol pathway should be small.

CHAPTER FOUR

The action of various effectors on the rate of triglyceride-fatty acid cycling in white adipose tissue

4.1 Introduction

The primary role of white adipose tissue is to store triglyceride for use as a fuel for other tissues in the animal. During times of demand for energy, there is net hydrolysis of triglyceride and FFA are released into the blood for use by other tissues (see Newsholme & Start, 1973). Conversely, when the fuel stores of the animal are low and the animal is in the fed state, there is net deposition of triglyceride within adipose tissue. Various agents are known to affect the rate of lipolysis and esterification, but there has been no systematic study of the effects of these agents on the rate of TG-FFA cycling. This chapter therefore investigates the acute effects of a selection of agents on the rate of TG-FFA cycling and on the sensitivity of the TG-FFA cycle. Before the results are presented, the action of each of these agents on adipose tissue will be briefly reviewed.

4.2.1 Effects of catecholamines on adipose tissue

The two catecholamine hormones, adrenaline and noradrenaline, are thought to be of prime importance to the adipocyte. Adrenaline is considered to be a humorally-delivered agent acting on fat cells, whilst noradrenaline is the transmitter molecule released by the nerve-endings of the sympathetic nervous system which are known to terminate near fat cells (see Hales et al., 1978). In rat adipose tissue, adrenaline and noradrenaline are alike in actions and are active at similar concentrations (Vaughan, 1961a), and hence investigations *in vitro* were only done using noradrenaline. The action of catecholamines on adipose tissue can be divided into effects

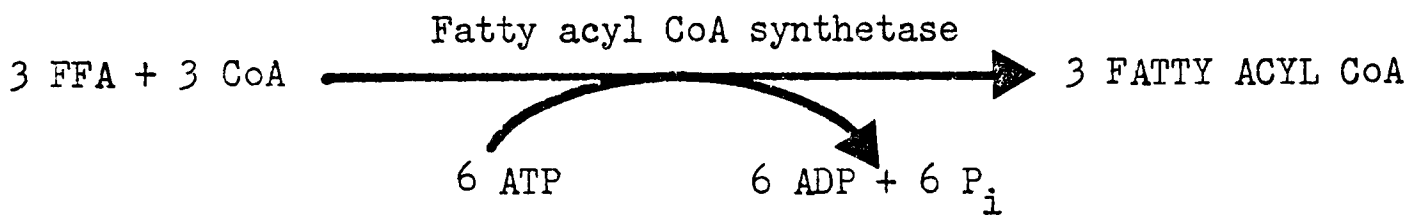
on lipid metabolism and effects on the metabolism of carbohydrate.

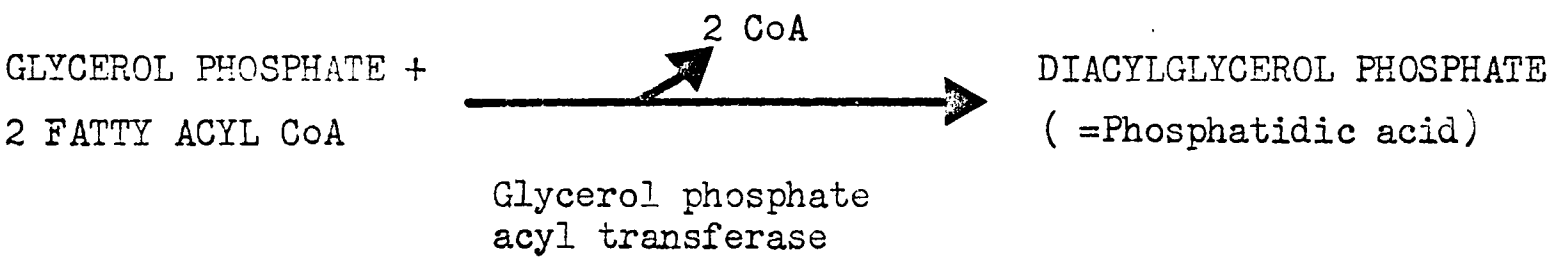
4.2.1.1 Catecholamines and lipid metabolism

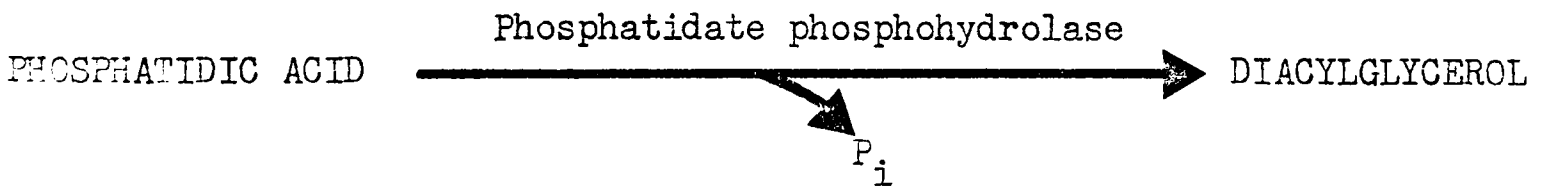
The primary action of catecholamines on adipose tissue is that of initiating triglyceride breakdown. Current understanding of the mechanism of action of catecholamines is based on the secondary-messenger concept of hormone action and analogy with hormone-stimulated glycogenolysis, and has been extensively reviewed (see Steinberg 1976; Hales et al., 1978). Interaction of the hormone with a receptor on the exterior of the cell increases the activity of adenylate cyclase (which is also thought to be plasma-membrane-bound, see Rodbell, 1967; Luzio et al., 1976). This raises the concentration of cAMP inside the cell, thus activating protein kinase, which then phosphorylates and activates the triglyceride lipase (Robison et al., 1971).

The pathway of triglyceride synthesis from glycerol phosphate and FFA involves four enzymes; fatty acyl CoA synthetase, glycerol phosphate acyl transferase, phosphatidate phosphohydrolase and diacylglycerol acyltransferase. These enzymes, together with the reactions that they catalyse, are shown in fig 4.1. The activity of each of the enzymes has been shown to be decreased when it is measured subsequent to incubation of fat cells in the presence of catecholamines (see table 4.1, which is a summary of the results of Saggerson et al., 1979). The action of catecholamines in increasing the activity of triglyceride lipase and simultaneously decreasing the activity of the esterification enzymes is consistent with the 'classical' idea of reciprocal inhibition and activation of opposing reactions (see chapter 1) and suggests that the rate of TG-FFA cycling would be decreased by catecholamines. However, the control of the flux through the esterification pathway is by no means as

Fig 4.1 Reactions and enzymes of triglyceride synthesis in adipose tissue







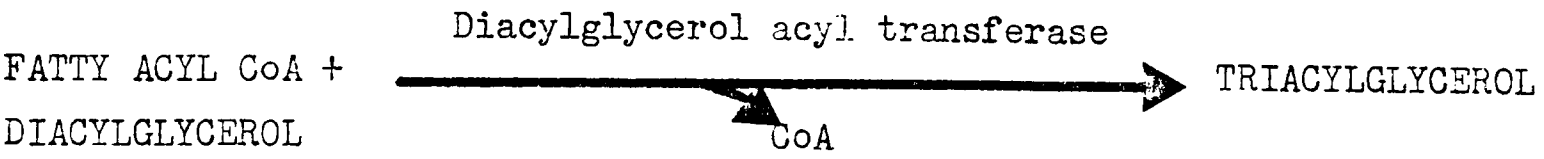


TABLE 4.1 SUMMARY OF THE EFFECT OF INCUBATION OF ADIPOCYTES WITH CATECHOLAMINES ON THE MAXIMUM ACTIVITY OF ENZYMES OF FFA ESTERIFICATION PATHWAY (from Saggerson et al., 1979)

ENZYME (ABBREVIATION) Cellular location	INCUBATION CONDITIONS	% CHANGE IN ENZYME ACTIVITY	COMMENTS
Fatty Acyl CoA Synthetase microsomal	Adrenaline 15 mins	30-40% decrease	Effect is - - opposed by insulin - blocked by propranolol
Glycerol Phosphate Acyl Transferase (GPAT) 90% microsomal	Adrenaline 60 mins	50% decrease	Effect is - - dose dependent - opposed by insulin - blocked by propranolol - slow in onset - progressive with time - not mimicked by FFA addition - probably due to phosphorylation of enzyme - probably on microsomal enzyme
Phosphatidate Phosphohydrolase (PPH) soluble fraction	Noradrenaline 20 mins	45% decrease	Effect is - - dose dependent - opposed by insulin - blocked by propranolol - mimicked by FFA to some extent (gives 20% decrease in activity) - confined to a decrease in the Mg^{2+} dependent PPH activity (cf no. effect on Mg^{2+}independent)
Diacylglycerol Acyl Transferase (DGAT) microsomal	Adrenaline 20 mins	25% decrease	Effect is - - dose dependent - opposed by insulin

clear cut as these changes in enzyme activity might suggest, since when adipose tissue is incubated with catecholamines an increase in the rate of triglyceride-glycerol formation is observed (Denton & Randle, 1967; Denton & Halperin, 1968; Saggerson & Greenbaum, 1970, 1970a; Saggerson, 1972). The problem of the regulation of the esterification pathway is discussed in more detail in section 4.4.2.

4.2.1.2 Catecholamines and glucose metabolism

Catecholamines have marked effects on the metabolism of glucose by adipose tissue. Glucose uptake, its conversion to glyceride-glycerol and its oxidation to carbon dioxide are all increased, whereas fatty acid synthesis from glucose is decreased in adipose tissue incubated with catecholamines (Vaughan, 1961, 1961a). Catecholamines have been shown to reduce the activity of two enzymes in the fatty acid synthetic pathway; pyruvate dehydrogenase (Coore et al., 1971; Smith & Saggerson, 1978) and acetyl CoA carboxylase (Brownsey et al., 1979). Several workers have suggested that the effects of catecholamines on the above enzymes and on the metabolism of glucose are not due to a direct effect of the catecholamine, but rather that they are due to the increased concentration of FFA seen during catecholamine stimulation. However, it is not yet clear to what extent a raised intracellular concentration of FFA can account for the observed effects of catecholamines on glucose uptake (Rodbell et al., 1968) and its conversion to glyceride-glycerol, fatty acids and carbon dioxide (Turtle & Kipnis, 1967; Smith & Saggerson, 1978).

Catecholamines increase the proportion of phosphorylase in the active form in adipose tissue (Frerichs & Ball, 1962) and they also lead to a conversion of glycogen synthetase into a form requiring a high concentration of glucose-6-phosphate for activity (Kaslow et al., 1979). Both of these effects are believed to be mediated

via the cAMP-dependent protein kinase system.

4.2.2 Effect of insulin on adipose tissue

In man and in the rat, insulin and the catecholamines are the two most important acutely-acting hormones which regulate adipose tissue metabolism (see Hales et al., 1978). Insulin affects the metabolism of lipid and carbohydrate in adipose tissue, but its actions are usually antagonistic (an exception is the uptake of glucose) to those of the catecholamines. Hence catecholamines can be regarded as the primary catabolic hormone in human and rat adipose tissue, whereas insulin is anabolic.

Insulin stimulates the transport of glucose across the fat cell plasma membrane (see for example Blecher, 1967; Livingston et al., 1978) and it does this by increasing the V_{max} of the transport process without affecting the K_m (Halperin et al., 1978). Insulin increases the rate of glycolysis in adipose tissue, partly because by increasing glucose transport it increases the concentration of glucose-6-phosphate and fructose-6-phosphate (Denton et al., 1966; Saggerson & Greenbaum, 1970) and partly because it increases the amount of type II (insulin-induced) hexokinase (Katzen, 1967).

Insulin promotes glycogen storage in fat cells by means of its effects on the glycogen-metabolising enzymes (Steinberg, 1976). Adipose tissue does not have a glucose-6-phosphate (G6P)-independent form of glycogen synthetase, but instead has more than one form of G6P-dependent activity (Haslow et al., 1979). These different forms of glycogen synthetase are activated by different concentrations of G6P, and interconversion between the forms of the enzyme is thought to be via a phosphorylation/dephosphorylation mechanism. Insulin increases the amount of enzyme that requires a relatively low concentration of G6P for activity (Haslow et al., 1979). Insulin

either decreases or does not affect the proportion of phosphorylase in the active (phosphorylated) form, and is able to oppose the stimulatory action of adrenaline on phosphorylase activity (Jungas, 1966; Lawrence et al., 1977).

Insulin increases the synthesis of fatty acids from glucose and pyruvate (Weingrad & Renold, 1958; Fain, 1964; Halperin, 1970; Saggerson, 1972). It was thought that the effects of insulin on fatty acid synthesis were secondary to its effect on glucose transport (see Vaughan, 1961a). However, the fact that the transport of pyruvate is not affected by insulin whereas fatty acid synthesis from pyruvate is increased by insulin implies that insulin has a more direct effect on fatty acid synthesis (Sooranna & Saggerson, 1975). The rate of fatty acid synthesis is thought to be regulated at the pyruvate dehydrogenase (Coore et al., 1971; Jungas, 1971) and acetyl CoA carboxylase stages (Halestrap & Denton, 1973, 1974). Both of these enzymes exist in interconvertible forms, and insulin increases the proportion of each enzyme in its higher-activity form (phosphorylated in the case of pyruvate dehydrogenase, polymeric in the case of acetyl CoA carboxylase - see Denton et al., 1977).

The incorporation of ^{14}C-glucose into the glycerol moiety of triglyceride is stimulated by insulin (Vaughan, 1961), and this is often assumed to indicate that insulin increases the rate of the esterification pathway. However, such results must be interpreted with caution, since the rate of triglyceride synthesis can only be measured if the specific activity of glycerol phosphate is known. When insulin is absent, the glycerol phosphate required for esterification in fat cells can come from endogenous glycogen (Jungas, 1968). When insulin is added, glycogen breakdown is inhibited and the glycerol phosphate required for esterification is derived from extracellular

glucose, thus increasing the specific activity of glycerol phosphate. Hence an apparent increase in the rate of triglyceride synthesis can be obtained by addition of insulin even if the actual rate of synthesis is decreased (see Jungas, 1968; Leboeuf, 1965). If the rate of triglyceride synthesis is measured by following tritiated water incorporation into the triglyceride-glycerol moiety, then a decrease in the rate of triglyceride synthesis is observed in response to insulin (Jungas, 1968, 1970; Sooranna & Saggerson, 1975). This decrease in the rate of triglyceride synthesis produced by insulin appears to be an indirect result of the antilipolytic action of insulin (see below) on the intracellular FFA levels, since if adipose tissue is incubated with palmitic acid, the rate of triglyceride synthesis (as measured by tritiated water incorporation into glyceride-glycerol) is increased by insulin (Sooranna & Saggerson, 1975). Similarly, the incorporation of labelled FFA from the incubation medium into triglyceride is increased by insulin (Bally et al., 1960). Hence, insulin does in fact stimulate the esterification pathway, and it is possible that it does this by increasing the activities of the enzymes catalysing esterification.

Adipose tissue is able to store fatty acids derived from very-low-density lipoprotein (VLDL) present in the blood (see Fielding & Havel, 1977). Fatty acids are released from VLDL by the action of the enzyme lipoprotein lipase, and the activity of this enzyme in adipose tissue is increased by insulin (Robinson & Wing, 1971; Garfinkel et al., 1976).

In 1963, Jungas & Ball showed that insulin inhibited basal and hormone-stimulated lipolysis in adipose tissue, and this has been extensively confirmed (see review by Hales et al., 1978). It has been suggested that insulin might inhibit lipolysis by lowering cAMP

levels, thus inactivating cAMP-dependent protein kinase and hence the triglyceride lipase (see section 4.2.2). Insulin has been reported to decrease adenylate cyclase activity (Renner et al., 1974) and increase the activity of the low K_m phosphodiesterase in adipose tissue (Kono et al., 1975). Some investigators find that insulin's inhibition of lipolysis is accompanied by a lowering of cAMP (Manganiello et al., 1971). However, others find that insulin can inhibit lipolysis with little or no change in the concentration of cAMP (Jarett et al., 1972; Siddle & Hales, 1974). Another suggestion for the mechanism of action of insulin on lipolysis in fat cells is that calcium ions act as secondary messengers. Deposits of precipitated calcium have been observed in the smooth endoplasmic reticulum of fat cells, and it is speculated that this structure may serve a function analogous to that of the sarcoplasmic reticulum in muscle (see Hales et al., 1974). However, despite extensive investigations the mechanism of the antilipolytic action of insulin remains unknown.

4.2.3 Effects of Glucagon, ACTH and TSH on adipose tissue

Glucagon is secreted by the alpha cells of the pancreas, whereas both ACTH (adrenocorticotrophic hormone) and TSH (thyroid-stimulating hormone) are secreted by the anterior pituitary (see Bell et al., 1972; DiGirolamo et al., 1968). The effects of each one of these hormones on adipose tissue are very similar to those of the catecholamines (Vaughan, 1961a; Vaughan & Steinberg, 1965). Thus each hormone is found to have the following action in adipose tissue:-

(a) increases adenylate cyclase activity and cAMP concentrations

(b) increases the rates of glycerol and FFA release

(c) increases the rate of glucose uptake and oxidation

(d) increases phosphorylase activity

(e) increases the rate of triglyceride-glycerol synthesis.

Each of these hormones is a polypeptide (Bell et al., 1972) and is thought to bind to a receptor on the outside of the cell membrane, thus activating adenylate cyclase (Sutherland et al., 1962; Rall & Sutherland, 1962; Rodbell et al., 1968; Birnbaumer et al., 1969). The ability to maximally-stimulate adenyl cyclase decreases in the order (adrenaline), ACTH, glucagon, TSH, (Birnbaumer & Rodbell, 1969). However, there is some doubt as to whether these hormones are physiologically significant regulators of adipose tissue in the rat (see Hales et al., 1978).

4.2.4 Effects of prostaglandins on adipose tissue

Steinberg et al. (1964) were the first to report that prostaglandins are able to inhibit hormone-stimulated lipolysis in adipose tissue. Since then the release of both prostaglandins and of arachidonic acid (this is the essential fatty acid precursor required for prostaglandin biosynthesis) by adipose tissue has been shown to be increased by nervous or hormonal stimulation (Shaw & Ramwell, 1968; Christ & Nugteren, 1970). The precursors of prostaglandins are present in the triglycerides of the fat cell and are released as free fatty acids during lipolysis; this led to the suggestion that prostaglandins might be feedback regulators of lipolysis (see Christ & Nugteren, 1970). However, acetyl choline and histamine at concentrations which had no effect on lipolysis were as potent as catecholamines in stimulating prostaglandin release (Shaw & Ramwell, 1968) which casts doubt on the significance of prostaglandins as feedback regulators of lipolysis. Similarly, the proposal that prostaglandins could account for the refractoriness that is shown by adipose tissue to a second dose of lipolytic hormone (Illiano & Cuatrecasas, 1971) remains very speculative (Fain, 1973; Hales et al., 1978).

Prostaglandin E_1 increases glucose uptake, oxidation and conversion to fatty acids in fat cells. However, the maximal stimulation of glucose metabolism by PGE_1 is only about 10% of the stimulation seen with insulin, and in general PGE_1 is more potent as an antilipolytic agent than as a stimulator of glucose metabolism (Fain, 1973).

4.2.5 Effects of glucose on adipose tissue

Glucose is a major precursor of glycerol phosphate in adipose tissue (see chapter 3; Francendese & DiGirolamo, 1981). It has been suggested that the availability of glucose to the adipocyte could control the concentration of glycerol phosphate, hence regulating the rate of esterification of FFA in adipose tissue and thus the release of FFA into the blood for oxidation by the other tissues of the body (see Randle et al., 1963; Lisch et al., 1973). The problem with this suggestion is that nobody has been able to demonstrate that the rate of esterification of FFA is dependent on the concentration of glycerol phosphate in the adipocyte (this is discussed in more detail in section 4.4.3). However, there is some experimental support for the idea that the release of FFA from adipose tissue can be influenced by the availability of glucose. The release of FFA from adipose tissue incubated with catecholamines is decreased by glucose(Jungas & Ball, 1963; Hall & Ball, 1970; Knight & Iliffe, 1973). The action of glucose in decreasing the rate of hormone-induced FFA release does not appear to be a result of a decreased rate of lipolysis, since glucose does not affect or even enhances the rate of hormone-induced glycerol release (Hall & Ball, 1970; Knight & Iliffe, 1973; Smith, 1974; Thomas et al., 1979; Allen, 1979). Since hormone-stimulated FFA release is inhibited by glucose but there is no change in the rate of lipolysis it seems that glucose

has stimulated the re-esterification of FFA. Addition of glucose to hormone-stimulated adipocytes is known to lower the intracellular FFA level (Angel et al., 1971). However, it is not possible to say whether this effect of glucose is due to an increase in the availability of glycerol phosphate, since the concentration of this intermediate does not appear to have been compared in hormone-stimulated adipose tissue incubated with and without glucose.

4.2.6 Effects of adenosine on adipose tissue

4.2.6.1 Introduction to adenosine

Adenosine has an important role as a local hormone (i.e. an informational link between different cells within one organ) in many tissues of the body (see review by Arch & Newsholme, 1978). It is suggested that adipose tissue may be one such tissue, since adenosine has marked effects on the metabolism of both lipid and carbohydrate in fat cells. However, there is some debate as to the physiological significance of these effects (see later).

Adenosine probably exerts its effects on adipose tissue metabolism by acting extracellularly with a specific receptor on the cell membrane. Control of the concentration of adenosine is by the enzymes 5' nucleotidase, adenosine kinase and adenosine deaminase, the first two enzymes forming a substrate cycle between AMP and adenosine (see fig 4.2; for an in depth review, see Arch & Newsholme, 1978; Fischer, 1979).

4.2.6.2 Effects of adenosine on lipolysis in adipose tissue

Adenosine inhibits the basal rate of lipolysis in isolated adipocytes (Schwabe et al., 1975; Turpin et al., 1977). Thus addition of adenosine deaminase (ADA), which deaminates endogenous adenosine to the less active inosine, increases the rate of lipolysis in isolated fat cells to near maximal levels (see Schwabe et al., 1975). This

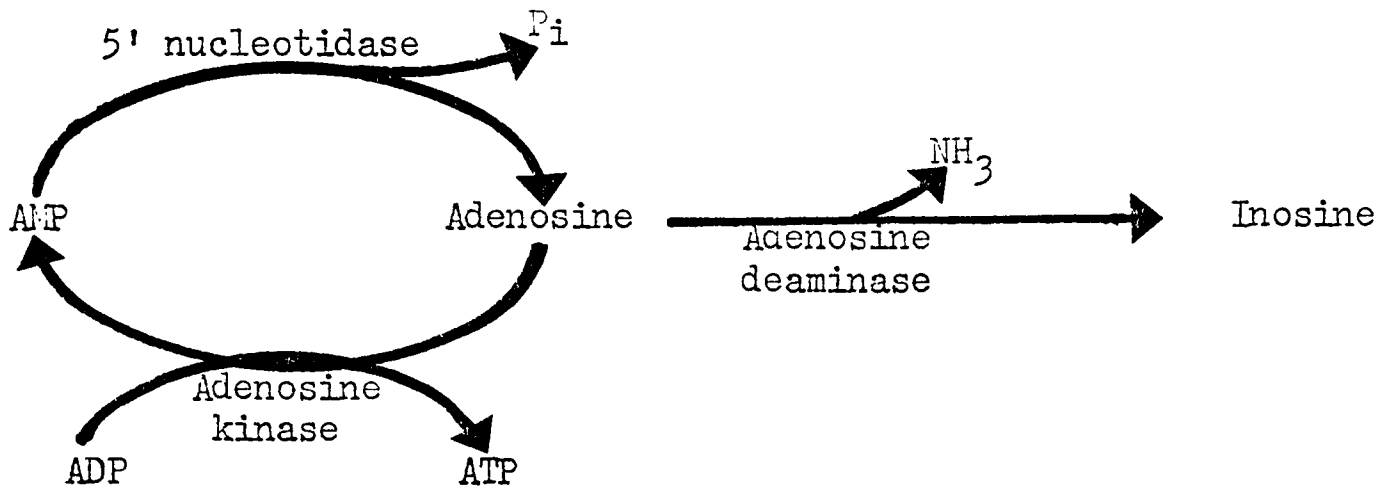


Fig 4.2 Enzymes involved in the regulation of the concentration of adenosine (from Arch & Newsholme, 1978)

stimulation of lipolysis by the removal of adenosine is due to the relief of adenosine's inhibition of adenylate cyclase (Fain et al., 1972). Adenosine only slightly inhibits the rate of catecholamine-induced lipolysis; however, it does cause a dramatic decrease in cAMP levels. This lack of correlation between lipolysis and the concentration of cAMP is well documented (see Schwabe et al., 1974; Hales et al., 1978; Fredholm, 1978).

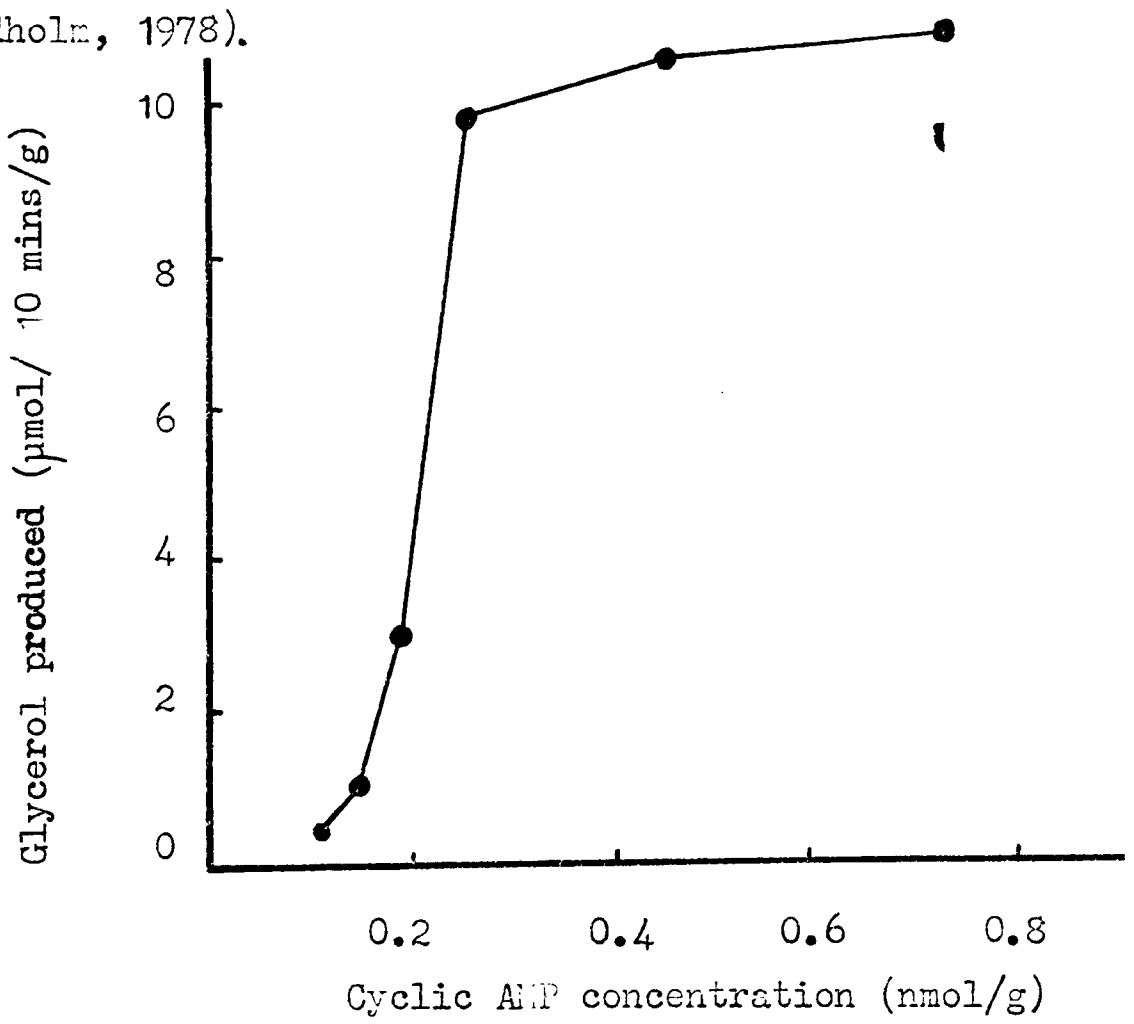


Fig 4.3 Relationship between cAMP and lipolysis in adipocytes stimulated with adrenaline (from Hales et al., 1978)

It seems that lipolysis in rat fat cells is directly related to cAMP concentration only over a fraction (up to approximately three times basal) of the potential maximum cAMP content. Greater increases in cAMP do not further activate lipolysis, presumably because some later step is rate-limiting (see Hales et al., 1978). Insulin lowers the concentration of cAMP and inhibits lipolysis in fat cells stimulated with noradrenaline, providing that the cAMP concentration is not above a critical level (Schwabe et al., 1974). Presumably, this critical level of cAMP is such that the action of insulin can lower the cAMP concentration to a point where it is limiting for lipolysis (see fig 4.3). High concentrations of catecholamines raise the concentration of cAMP well above this critical level, so that insulin is unable to inhibit lipolysis. However, if adenosine is added (or allowed to accumulate - this happens faster in more concentrated cell suspensions), the concentration of cAMP is lowered to below the critical level, and insulin is able to inhibit lipolysis (see Schwabe et al., 1974).

Another problem in the control of lipolysis is the existence of the inhibitory factor first reported by Manganiello et al. (1971) and Ho & Sutherland (1971). This factor accumulates during the incubation of adipocytes with lipolytic hormones, and causes refractoriness to the action of further additions of hormone. Responsiveness to the lipolytic hormone **can** be regained by washing the cells and resuspending them in new buffer. It has been suggested that this inhibitory factor could be prostaglandins (see section 4.2.4) or that it could be adenosine (Schwabe et al., 1975). The release of adenosine from fat cells does not appear to be related to the rate of lipolysis and hence adenosine is not thought to be a feedback regulator that is directly linked to lipolysis (Fredholm & Hjemdahl, 1979; Hales et al., 1978; Fain, 1979). However, the production of adenosine is

increased by activation of sympathetic nerves or anoxia, and this release is thought to be a consequence of ATP breakdown (Fredholm & Hjemdahl, 1979). It is therefore possible that adenosine might act as a modulator of lipolysis even though it does not serve as a feedback regulator in the proper sense (Fredholm, 1978).

4.2.6.3 Effects of adenosine on glucose metabolism in adipose tissue

Adenosine increases glucose transport into adipocytes (Taylor & Halperin, 1979) and increases glucose utilisation by fat pads and fat cells (Dole, 1962; Schwabe et al., 1974). In these respects the effects of adenosine are similar to those of insulin (see section 4.2.2).

4.2.6.4 Adenosine and insulin sensitivity in adipose tissue

The effects of adenosine on the metabolism of lipid and carbohydrate in adipose tissue are similar to those of insulin (see above). Adenosine is also able to potentiate the effects of insulin on adipose tissue metabolism (see Schwabe et al., 1975). It is therefore possible that changes in the concentration of adenosine could alter the sensitivity of adipose tissue to insulin. Support for this idea comes from Fernandez & Saggerson (1979) who found that fat cells from adrenalectomised animals are more sensitive to insulin and less responsive to noradrenaline or glucagon stimulation of lipolysis. These differences could be abolished by the addition of adenosine deaminase, suggesting a raised ability of the adrenalectomised fat cells to produce adenosine, or an increased sensitivity to adenosine, or a decreased capacity of the cells to metabolise adenosine. Study of the enzymes involved in the metabolism of adenosine showed that the changes seen in adrenalectomised animals could be accounted for by a decreased ability to metabolise adenosine, since the activity of adenosine kinase was decreased in adipose tissue from adrenalectomised animals (see Green, 1979). In addition, Green's (1979) results suggested

that the concentration of adenosine could be changed in adipose tissue from ob/ob mice (decreased adenosine concentration), starvation (increase in adenosine) and diabetes (possible decrease in adenosine). Each of these conditions is characterised by a parallel change in insulin sensitivity in adipose tissue, suggesting a physiological role for adenosine in the control of insulin action on fat cells.

It has been suggested that the adenosine released during incubation of fat cells is not due to a specific mechanism, but rather simply due to the rupture of fat cells during pipetting leading to release of nucleotides into the incubation medium (Schwabe et al., 1975; Fain, 1979). If this suggestion is correct, then it argues against the idea that adenosine is physiologically important in adipose tissue. However, the demonstration that perfusion in situ of canine subcutaneous adipose tissue with adenosine deaminase leads to an increase in the rate of lipolysis (Fredholm, 1978) provides strong evidence that adenosine inhibits lipolysis in vivo. Furthermore, recent work has shown that stimulation of sympathetic nerves, or perfusion with noradrenaline, induces a marked release of adenosine in perfused white adipose tissue (Fredholm & Sollevie, 1981). The levels of adenosine reached during this stimulation were high enough to be of physiological relevance to the control of adipose tissue metabolism. Hence it does seem that adenosine is a local hormone of physiological significance in white adipose tissue.

4.2.7 Effects of alpha and beta adrenergic agonists and antagonists on adipose tissue

It was observed that a series of catecholamines arranged themselves into two different orders of potency when tested for their ability to provide a variety of adrenergic responses (such as muscle contractions or relaxation) in different tissues. To explain this,

Ahlquist (1958) proposed the existence of two types of receptor for catecholamines, designated alpha and beta receptors. Adrenaline, noradrenaline and the drug isoproterenol could therefore be arranged in the following orders of potency for action on alpha- and beta-adrenergic receptors (Levy & Wilkenfield, 1970).

For alpha effects adrenaline > noradrenaline > isoproterenol

For beta effects isoproterenol > adrenaline > noradrenaline
(depending on the tissue)

In the time since it was first proposed, the alpha-beta classification of adrenoreceptors has been extended. In a statistical comparison of the potencies of a large number of catecholamine derivatives, Lands et al. (1967) concluded that one type of receptor - $beta_2$ - mediates vasodilation and bronchodilation whilst a second type - $beta_1$ - mediates cardiac stimulation and lipolysis in adipose tissue. Until recently, the classification of the beta-receptor in rat white adipose tissue was by no means clear since it appeared to have both $beta_1$ and $beta_2$ characteristics (Himms-Hagen, 1970), the weight of evidence suggesting that the receptor was $beta_1$ (Lefkowitz, 1975). However, recent investigations have shown that the beta-receptor in rat adipose tissue is in fact different from that in rat heart (Harms et al., 1977). Further work has shown that the interaction site of the rat adipocyte β-adrenoreceptor for the aromatic moiety of catecholamines has β_2-characteristics whereas the alkanolamine side chain interaction site has a $beta_1$-nature (De Vente et al., 1980). These workers have also discounted the suggestion that both $beta_1$ and $beta_2$ receptors are simultaneously present on the same cell, which has been reported for a number of tissues (Minneman et al., 1979).

In the same way that the division between alpha and beta receptors

was made on the basis of agonist potencies, results have accumulated which suggest the existence of two types of alpha-adrenergic receptor (see Berthelsen & Pettinger, 1977). Agonists such as methoxamine and phenylephrine are relatively selective for one type of receptor, designated alpha$_1$ receptors. Agonists such as methylnoradrenaline, clonidine and tramazoline are relatively selective for another type of receptor, called alpha$_2$. It has been suggested that alpha$_1$ receptors are stimulatory in action (for example, alpha$_1$ receptors cause vasodilation in vascular smooth muscle) and act by altering the ion-permeability of the cell membrane, whereas alpha$_2$ receptors are inhibitory in action (for example, alpha$_2$ receptors on the pre-synaptic membrane of sympathetic nerve terminals inhibit noradrenaline release) and act via adenylate cylase - see Berthelsen & Pottinger, 1977; Fain & Garcia-Sainz, 1980. The author has been unable to find any studies on the type of alpha- adrenergic receptor in rat white adipose tissue. However, studies in hamsters (Schimmel, 1976), humans (Lafontan et al., 1980), rabbits and dogs (Lafontan et al., 1980a) all report that the receptor causing inhibition of lipolysis in white adipose tissue was the alpha$_2$ receptor.

The effects of alpha-receptor stimulation in adipose tissue are to inhibit adenylate cyclase, thus lowering cellular cAMP and leading to inhibition of cAMP-dependent kinase. The effects of beta-adrenergic stimulation in adipose tissue are to activate adenylate cyclase, thus raising the cAMP concentration and activating cAMP-dependent protein kinase (Himms-Hagen, 1970). Adipose tissue has both alpha and beta receptors and there is considerable species variation in the proportions of alpha to beta. Rat white adipose tissue has a high proportion of beta receptors and only a few alpha receptors - in contrast, rabbit adipose tissue is the opposite (see Hales et al., 1978). Hamsters

and humans have an equal proportion of alpha and beta.

Adrenaline and noradrenaline have both alpha and beta activity and hence will stimulate both receptors simultaneously if they are present. If a specific alpha- or beta- blocking drug is used in the presence of adrenaline or noradrenaline, then the characteristics of the unblocked receptor will be manifested (see for example, Turtle & Kipnis, 1967a).

4.2.8 Summary of introduction

Each of the agents reviewed in section 4.2 is known to have an effect on the rate of lipolysis or esterification in white adipose tissue. Since these two sets of reactions comprise the two halves of the TG-FFA cycle, it is possible that any of these agents could affect the rate of TG-FFA cycling. A comprehensive study of the effects of these agents on the rate of FFA esterification in white adipose tissue has not been done. Hence the action of each effector on the rate of TG-FFA cycling was determined by incubating either fat pads or fat cells with the agent and then measuring the accumulation of glycerol and FFA. Thus the rate of FFA reesterification could be determined using the breakdown method of measuring the cycle (see chapter 3).

4.3 Results

The majority of the agents used in this chapter stimulated the rate of TG-FFA cycling. Noradrenaline, adenosine deaminase, glucagon, ACTH, TSH and beta-adrenergic drugs all significantly enhanced the rate of FFA reesterification in white adipose tissue as compared to control incubations (tables 4.2 to 4.6). The rates of TG-FFA cycling in control incubations were very low, thus making it difficult to observe an inhibition of the rate of cycling. Insulin was the only hormone to significantly reduce the control rate of cycling, and

even then this was only observed using fat pads - these exhibit a higher basal rate of cycling (as compared to the maximal rate) than fat cells (table 4.2, 4.4). However, when the fat tissue was stimulated using either adenosine deaminase or noradrenaline, insulin markedly inhibited the rate of TG-FFA cycling (table 4.4). Similarly, prostaglandin E_1 slightly inhibited the noradrenaline-stimulated rate of cycling (table 4.5).

As perhaps might have been expected, propranolol inhibited the noradrenaline-stimulated rate of TG-FFA cycling, but had no effect on the basal rate (tables 4.6, 4.7). Alpha-agonist and antagonist drugs had no effect on the rate of TG-FFA cycling in rat isolated fat cells (tables 4.8, 4.9).

The effect of insulin on the rate of noradrenaline-stimulated TG-FFA cycling was variable depending on whether fat pads or isolated fat cells were used for the incubation. In fat pads, insulin stimulated the rate of TG-FFA cycling (table 4.2), whereas in fat cells, insulin decreased the rate of FFA reesterification (table 4.4). The rate of lipolysis in the two preparations showed a similar trend; insulin enhanced lipolysis in fat pads but inhibited lipolysis in fat cells. However, these differences between the two preparations in the response to insulin were lost if adenosine deaminase was added to the isolated fat cells. Thus in the presence of ADA insulin stimulated both the rate of lipolysis and the rate of TG-FFA cycling in incubated adipocytes (table 4.4).

4.4 Discussion

4.4.1 Rates of TG-FFA cycling

Glucagon, ACTH, TSH, noradrenaline and adenosine deaminase each stimulated the rates of TG-FFA cycling, glycerol and FFA release (tables 4.2, 4.3 and 4.5). From the data presented in this thesis, it

TABLE 4.2 THE EFFECT OF INSULIN AND NORADRENALINE ON THE RATE OF TG-FFA CYCLING IN RAT INCUBATED EPIDIDYMAL FAT PADS

INCUBATION CONDITIONS	micromoles/g wet tissue/hour		
	TG-FFA CYCLING	GLYCEROL RELEASE	FFA RELEASE
Control	3.94 ± 0.25	1.54 ± 0.10	0.66 ± 0.13
	**	**	NS
Insulin	2.12 ± 0.37	0.87 ± 0.15	0.48 ± 0.09
	***	***	**
Noradrenaline + Insulin	30.7 ± 1.81	12.8 ± 0.8	7.42 ± 0.82
	*	*	NS
Noradrenaline	22.5 ± 1.9	10.1 ± 0.8	7.90 ± 1.01

Fat pads from Sprague-Dawley rats (150-210g, fed ad libitum) were incubated as described in chapter two. Concentrations used were; glucose (present in all incubations), 10mM; insulin, 1mU/ml; noradrenaline, 10μM.

Results are given as mean ± SEM of five experiments, and are for changes in medium concentrations. Results of t-tests between treatments immediately above and below each symbol are indicated by:

NS = $p > 0.05$
* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

TABLE 4.3 THE EFFECT OF NORADRENALINE AND ADENOSINE DEAMINASE (ADA) ON THE RATE OF TG-FFA CYCLING IN RAT ISOLATED EPIDIDYMAL FAT CELLS

INCUBATION CONDITIONS	micromoles/g dry cells/hour		
	TG-FFA CYCLING	GLYCEROL RELEASE	FFA RELEASE
Control	-5.61 ± 2.83	-1.08 ± 1.04	2.38 ± 3.51
	***	***	***
ADA	131 ± 10	102 ± 2	175 ± 7
	NS	***	*
Noradrenaline	128 ± 10	91.5 ± 1.56	146 ± 9
	**	***	*
Noradrenaline + ADA	163 ± 4	112 ± 2	174 ± 2
	*	**	NS

Fat cells were prepared from Sprague-Dawley rats (100-125g, fed ad libitum) and incubated as described in chapter two. Concentrations used were; glucose (present in all incubations), 5mM; noradrenaline, 1μM; adenosine deaminase (ADA), 10μg/ml.

Results are given as mean ± SEM (n=6). Results for t-tests between treatments immediately above and below the symbol (except the ADA versus noradrenaline + ADA t-test, results of which are given below the latter treatment) are indicated by the following symbols:

NS = $p > 0.05$
* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

TABLE 4.4 THE EFFECT OF INSULIN ON BASAL AND STIMULATED TG-FFA CYCLING IN RAT ISOLATED EPIDIDYMAL FAT CELLS

INCUBATION CONDITIONS	micromoles/g dry cells/hour		
	TG-FFA CYCLING	GLYCEROL RELEASE	FFA RELEASE
Control	-5.61 ± 2.83	-1.08 ± 1.04	2.38 ± 3.5
	NS	NS	NS
Insulin	0.45 ± 2.25	0.06 ± 0.61	-0.28 ± 1.12
ADA	72.9 ± 3.1	101 ± 1	231 ± 3
	***	***	***
ADA + Insulin	47.1 ± 3.4	27.4 ± 1.7	35.0 ± 2
NA	128 ± 10	91.5 ± 1.5	146 ± 9
	***	***	***
NA + Insulin	34.5 ± 6.8	12.7 ± 1.9	3.63 ± 2.06
NA + ADA	106 ± 3	109 ± 1	221 ± 1
	***	***	NS
NA + ADA + Insulin	153 ± 6	125 + 2	222 ± 4

Fat cells were prepared from Sprague-Dawley rats (100-125g, fed ad libitum) and incubated as described in chapter two. Concentrations used were; glucose (present in all incubations), 5mM; noradrenaline (NA), 1μM; insulin, 100μU/ml; adenosine deominase (ADA), 10μg/ml.

Results are given as mean ± SEM; incubations with ADA are from one experiment (n=6), incubations without ADA are from another experiment (n=5). Format as for table 4.2, NS = $p > 0.05$
*** $p < 0.001$

TABLE 4.5 EFFECT OF GLUCAGON, ADRENOCORTICOTROPHIC HORMONE (ACTH), THYROID STIMULATING HORMONE (TSH) AND PROSTAGLANDIN E_1 (PGE_1) ON THE RATE OF TG-FFA CYCLING IN RAT ISOLATED EPIDIDYMAL FAT CELLS

INCUBATION CONDITIONS	micromoles/g dry cells/hour		
	TG-FFA CYCLING	GLYCEROL RELEASE	FFA RELEASE
Control	-5.61 ± 2.83	-1.08 ± 1.04	2.38 ± 3.51
Glucagon	102 ± 4	42.8 ± 1.6	25.9 ± 3.2
ACTH	174 ± 6	122 ± 1	191 ± 4
TSH	165 ± 4	112 ± 1	170 ± 2
Noradrenaline	128 ± 10	91.5 ± 1.5	146 ± 9
	NS	***	***
NA + PGE_1	112 ± 5	67.3 ± 3.5	90.3 ± 6.1

Fat cells were prepared from Sprague-Dawley rats (100-125g, fed ad libitum) and incubated as described in chapter two. Concentrations used were; glucose (present in all incubations), 5mM; glucagon, 5μg/ml; ACTH, 5μg/ml; TSH, 10mU/ml; noradrenaline (NA), 1μM; PGE_1, 5μM.

Results are given as mean ± SEM (n=6). Results of t-tests between treatments immediately above and below each symbol are indicated by NS = $p > 0.05$
*** $p < 0.001$

TABLE 4.6 THE EFFECT OF FENOTEROL (A BETA AGONIST) AND PROPRANOLOL (A BETA-BLOCKER) ON THE RATE OF TG-FFA CYCLING IN RAT ISOLATED EPIDIDYMAL FAT CELLS

INCUBATION CONDITIONS	micromoles/g dry cells/hour		
	TG-FFA CYCLING	GLYCEROL RELEASE	FFA RELEASE
Control	-0.07 ± 0.75	0.35 ± 0.17	1.13 ± 0.66
	***	***	***
Fenoterol	96.7 ± 2.3	91.2 ± 2.9	117 ± 9
	***	***	***
Propranolol	1.27 ± 1.33	0.62 ± 0.21	0.59 ± 0.93
	NS	NS	NS
ADA Control	34.1 ± 4.3	44.0 ± 2.5	97.8 ± 5.4
	***	***	***
ADA + Fenoterol	77.9 ± 3.7	82.1 ± 2.3	168 ± 10
	***	***	***
ADA + Propranolol	40.7 ± 2.6	45.2 ± 3.3	94.8 ± 7.5
	NS	NS	NS

Fat cells were prepared from Sprague-Dawley rats (130-150g, fed ad libitum) and incubated as described in chapter two. Concentrations used were; glucose (present in all incubations), 5mM; ADA, 10μg/ml; fenoterol, 10μM; propranolol, 10μM.

Results are given as mean ± SEM (n=6). Format as for table 4.3, NS = $p > 0.05$
*** $p < 0.001$

TABLE 4.7 THE EFFECT OF PROPRANOLOL (A BETA BLOCKER) ON THE NORADRENALINE-STIMULATED RATE OF TG-FFA CYCLING IN RAT ISOLATED EPIDIDYMAL FAT CELLS

INCUBATION CONDITIONS	Micromoles/g dry cells/hour		
	TG-FFA CYCLING	GLYCEROL RELEASE	FFA RELEASE
Noradrenaline	114 ± 6	95.8 ± 3.8	174 ± 7
	***	***	***
NA + Propranolol	27.8 ± 1.9	13.1 ± 4.5	11.2 ± 1.5
	***	***	***
Control	-0.07 ± 0.75	0.35 ± 0.17	1.13 ± 0.66
ADA + NA	89.0 ± 8.2	88.4 ± 4.5	176 ± 7
	NS	NS	NS
ADA + NA + Propranolol	72.2 ± 3.4	78.3 ± 1.8	163 ± 4
	***	***	***
ADA control	34.1 ± 4.3	44.0 ± 2.5	97.8 ± 5.4

Fat cells were prepared from Sprague-Dawley rats (130-150g, fed ad libitum) and incubated as described in chapter two. Concentrations used were; glucose (present in all incubations), 5mM; ADA, 10µg/ml; noradrenaline (NA), 10µM; propranolol, 10µM.

Results are given as mean ± SEM (n=6). Format as for table 4.2 NS = $p > 0.05$
*** $p < 0.001$

TABLE 4.8 THE EFFECT OF PHENYLEPHRINE (AN ALPHA AGONIST) AND PHENTOLAMINE (AN ALPHA BLOCKER) ON THE RATE OF TG-FFA CYCLING IN RAT ISOLATED EPIDIDYMAL FAT CELLS

INCUBATION CONDITIONS	micromoles/g dry cells/hour		
	TG-FFA CYCLING	GLYCEROL RELEASE	FFA RELEASE
Control	-0.07 ± 0.75	0.35 ± 0.17	1.13 ± 0.66
	NS	**	NS
Phenylephrine	1.14 ± 0.80	1.16 ± 0.17	2.34 ± 0.50
	NS	NS	NS
Phentolamine	-1.02 ± 2.05	0.62 ± 0.21	2.88 ± 1.50
	NS	NS	NS
ADA Control	34.1 ± 4.3	44.0 ± 2.5	97.8 ± 5.4
	*	**	***
ADA + Phenylephrine	58.9 ± 7.5	72.2 ± 6.0	158 ± 11
	NS	NS	NS
ADA + Phentolamine	53.5 ± 11.8	63.3 ± 7.6	137 ± 12
	NS	NS	*

Fat cells were prepared from Sprague-Dawley rats (130-150g, fed ad libitum) and incubated as described in chapter two. Concentrations used were; glucose (present in all incubations), 5mM; adenosine deaminase (ADA), 10µg/ml; phenylephrine, 10µM; phentolamine, 10µM.

Results are given as mean ± SEM (n=6). Format as for table 4.3;

NS = $p > 0.05$
* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

TABLE 4.9 THE EFFECT OF PHENTOLAMINE (AN ALPHA BLOCKER) ON THE NORADRENALINE-STIMULATED RATE OF TG-FFA CYCLING IN RAT ISOLATED EPIDIDYMAL FAT CELLS

INCUBATION CONDITIONS	micromoles/g dry cells/hour		
	TG-FFA CYCLING	GLYCEROL RELEASE	FFA RELEASE
Noradrenaline	114 ± 6	95.8 ± 3.8	174 ± 7
	NS	NS	NS
NA + Phento-lamine	99.7 ± 5.0	85.3 ± 3.1	156 ± 6
	***	***	***
Control	-0.07 ± 0.75	0.35 ± 0.17	1.13 ± 0.66
ADA + NA	89.0 ± 8.2	88.4 ± 4.5	176 ± 7
	NS	NS	NS
ADA + NA + Phentolamine	81.6 ± 3.0	79.6 ± 2.5	157 ± 6
	***	***	***
ADA control	34.1 ± 4.3	44.0 ± 2.5	97.8 ± 5.4

Fat cells were prepared from Sprague-Dawley rats (130-150g, fed ad libitum) and incubated as described in chapter two. Concentrations used were; glucose (present in all incubations), 5mM; ADA, 10μg/ml; noradrenaline (NA), 10μM; phentolamine, 10μM.

Results are given as mean ± SEM (n=6). Results of t-tests between treatments immediately above and below each symbol - as in table 4.2 - are indicated by: NS = $p > 0.05$
*** $p < 0.001$

is not possible to determine which of these is the most effective at stimulating the rate of TG-FFA cycling (i.e. the intrinsic activities relative to, say, noradrenaline). This is because such comparisons require that a concentration of hormone which gives a maximal response is used, and this concentration has to be determined by incubating adipose tissue with a range of concentrations of hormone. In these experiments, the concentrations of each hormone used was selected from the literature as giving a maximal rate of lipolysis (Bray, 1967; Blecher et al., 1969; Angel et al., 1971). However, owing to the variability between different fat cell suspensions and between different collagenase preparations (Jamdar, 1978; Green, 1979) the use of these concentrations of hormones must be validated in a given system before comparisons of intrinsic activities are made.

The increase in the rate of lipolysis (glycerol release) in response to glucagon was much greater than the increase in the rate of FFA release (glycerol release increased by 43 μmoles/g dry cells/hour whereas FFA release increased by 24 - see table 4.5). This contrasts with the response of incubated adipocytes to ACTH, TSH or noradrenaline, in all of which the increase in the rate of FFA release is greater than the increase in glycerol release. Hence in the glucagon incubation, more than two of the three FFA released in lipolysis must be reesterified, which suggests that glucagon is a relatively good stimulus for TG-FFA cycling. However, it is possible that this high percentage reesterification of FFA is due to the glucagon preparation being contaminated by traces of insulin; Vaughan (1961) reports a high percentage reesterification of FFA in fat pads incubated with glucagon and speculates on this being due to insulin impurities.

Fenoterol, a beta agonist, greatly increased the rate of TG-FFA cycling, lipolysis and FFA release in incubated fat cells (table 4.6),

and in this respect it is similar to noradrenaline. Propranolol, a beta blocking drug, had no effect on the rate of TG-FFA cycling or glycerol release when fat cells were incubated under either basal conditions or with adenosine deaminase (this enzyme lowers the concentration of adenosine in the incubation medium, thus stimulating the adipocyte by raising the intracellular cAMP levels; see section 4.2.6) - see table 4.6. As expected from the fact that it is a beta-blocker, propranolol inhibited the stimulatory action of noradrenaline on the rate of lipolysis and cycling (table 4.7). However, at the concentration used in this experiment, propranolol did not appear to be able to inhibit the action of noradrenaline if adenosine deaminase was also present in the incubation medium. It therefore appears that adenosine can in some way synergise with propranolol to inhibit noradrenaline-stimulated lipolysis.

Incubation of rat adipocytes with the alpha agonist phenylephrine did not affect the rate of TG-FFA cycling (table 4.8). Similarly, the alpha blocking drug phentolamine did not change the rate of cycling in fat cells incubated under basal or noradrenaline-stimulated conditions (tables 4.8, 4.9). It therefore appears that alpha-adrenergic receptors have no role in the modulation of the TG-FFA cycle in rat adipose tissue.

The results shown in table 4.8 show that phenylephrine can actually stimulate lipolysis, this effect being particularly pronounced when fat cells are incubated with adenosine deaminase. However, it is likely that this is due to phenylephrine stimulating the beta receptors on the fat cell; Fain (1973) points out that no drug has an activity which is absolutely specific for a given receptor. When high concentrations of drugs are used, problems can therefore occur with the specificity of a drug, and this is particularly so in a tissue with a high population of one type of receptor and a low population

of another, as is the case with rat adipose tissue (see section 4.2.7). Other problems also occur when adrenergic drugs are used in high concentrations. Himms-Hagen points out that all of the adrenergic drugs will inhibit lipolysis when they are present at high concentration; the effect is a non-competitive one which does not appear to be directed at the receptor or at cAMP formation, but at a later stage of lipolysis, possibly the inhibition of the triglyceride lipase (Lech & Calvert, 1968). Fain (1973) also discusses the non-specific effects of adrenergic drugs on adipose tissue; he ascribes these effects to an anaesthetic action of the drugs. Thus caution is required in the design and interpretation of experiments to investigate the role of alpha receptors in rat adipose tissue (see Himms-Hagen, 1970).

4.4.2 TG-FFA cycle and sensitivity of control of FFA release

In chapter 1, the properties of substrate cycles were discussed and it was shown that they were a means for increasing the sensitivity of control of the flux through a reaction. It was shown that the sensitivity of a substrate cycle was proportional to the ratio of the cycling rate to the flux through the pathway (C/J). Since both the rate of TG-FFA cycling and the flux through the cycle (FFA release) have been measured in these experiments, it is possible to study the effects of the agents used in this chapter on the sensitivity properties of the cycle.

Under conditions of net breakdown of triglyceride the TG-FFA cycle is as shown in fig 4.4. If the sensitivity of the lipolytic response to a change in concentration of effector is denoted by 'n', then operation of the TG-FFA cycle will increase the sensitivity of the release of FFA in response to a change in the effector molecule such that:

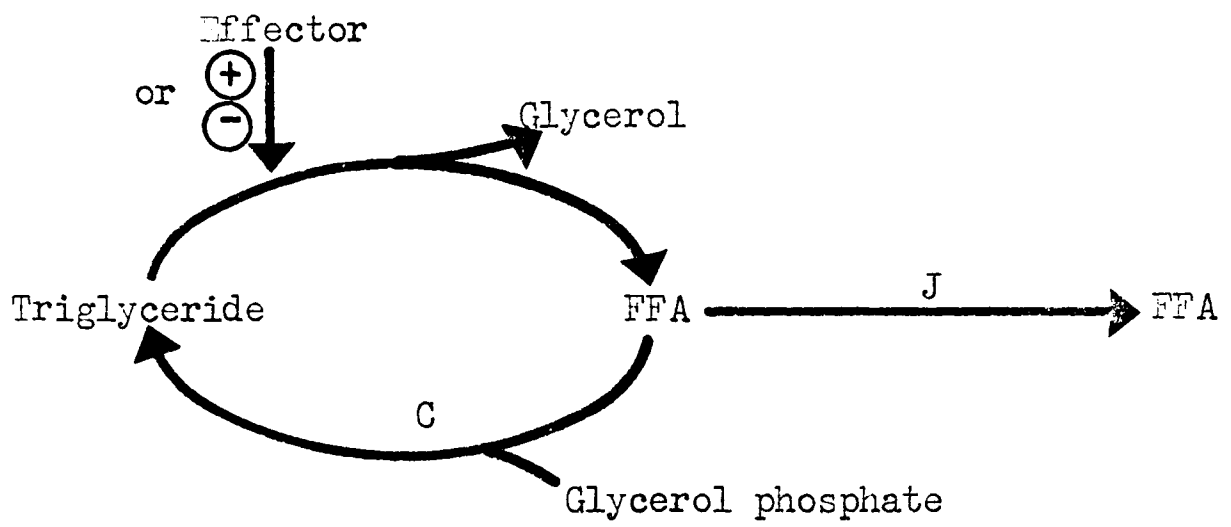


Fig 4.4 TG-FFA cycle during net FFA release

C = cycling rate = FFA reesterification

J = pathway flux = FFA release

$$\text{Sensitivity} = \frac{\% \text{ change in FFA release}}{\% \text{ change in effector concentration}}$$

$$= n \left[1 + \frac{\text{FFA reesterification}}{\text{FFA release}} \right]$$

The results from a single fat cell experiment are shown in table 4.10. The table shows the rates of FFA release and the rate of TG-FFA cycling and also the increase in sensitivity that this rate of cycling would give to the release of FFA. (Results from this experiment have been used earlier in the chapter). Under most of the incubation conditions, 1 + C/J is about 2, and it rises to as high as 10. Hence on average the TG-FFA cycle could contribute a two-fold increase in the sensitivity of control of FFA release, and there are indications that much higher levels of sensitivity can be attained. On the other hand, it is possible that the TG-FFA cycle does not make any contribution to the sensitivity of control of FFA release, and in order to appreciate why this is the case, the derivation of the parameter 1 + C/J must be considered.

It should be noted that in the derivation of the sensitivity

TABLE 4.10 THE PATHWAY FLUX, RATE OF TG-FFA CYCLING AND $(1+C/J)$ UNDER DIFFERENT INCUBATION CONDITIONS

INCUBATION CONDITIONS	PATHWAY FLUX = rate of FFA release = J	CYCLING FLUX = rate FFA re-esterification = C	SENSITIVITY INCREASED BY FACTOR OF $(1 + C/J)$
ADA	168 ± 2	106 ± 6	1.63
Glucose	2.38 ± 3.51	-5.61 ± 2.83	-1.36
Glucose + ADA	175 ± 7	131 ± 10	1.75
Glucose + Insulin	-0.28 ± 1.12	0.17 ± 1.83	1.61*
NA	130 ± 7	68.7 ± 6.2	1.53
Glucose + NA	146 ± 9	128 ± 10	1.88
Glucose + NA + Insulin	3.63 ± 2.06	34.5 ± 6.8	10.5
Glucose + NA + PGE_1	90.3 ± 6.1	112 ± 5	2.24
Glucsoe + NA + ADA	174 ± 2	163 ± 4	1.94
Glucose + Glucagon	25.9 ± 3.2	102 ± 4	4.94
Glucose + TSH	170 ± 2	165 ± 4	1.97
Glucose + ACTH	191 ± 4	174 ± 6	1.91

* Since there is net FFA uptake in this incubation, the rate of cycling simply equals 3 x rate glycerol release.

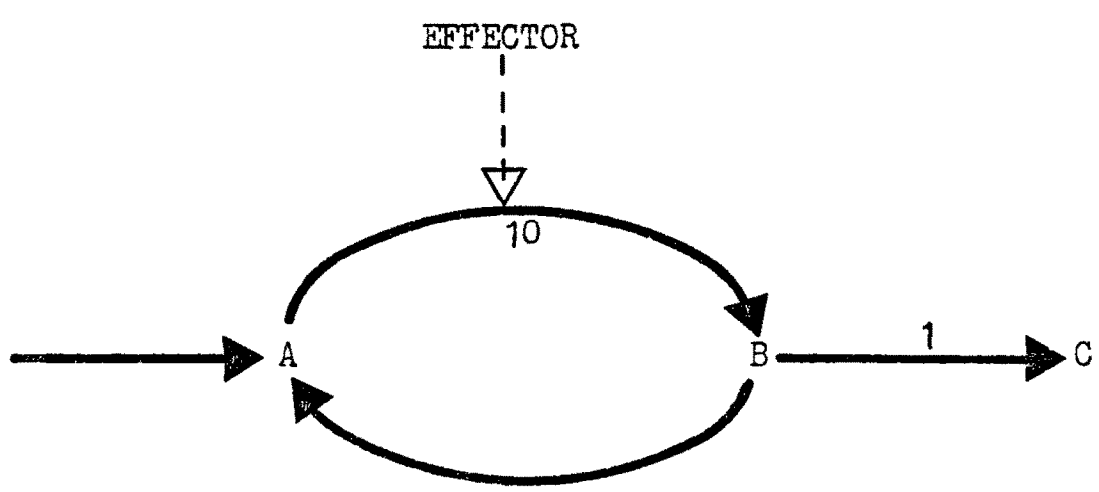
Fat cells were prepared from Sprague-Dawley rats (100-125g, fed ad libitum) and incubated as described in chapter two. Concentrations used were; glucose, 5mM; ADA, 10µg/ml; insulin, 100µU/ml; noradrenaline, 1µM; PGE_1, 5µM; glucagon, 5µg/ml; TSH, 10µU/ml; ACTH, 5µg/ml.

Results are given as mean ± S.E.M. (n=6), units are in µmoles/g dry cells/hour.

attained by substrate cycling (i.e. 1 + C/J, see Newsholme & Crabtree, 1976; also appendix I) it is assumed that the rate of cycling remains constant. To put it another way, the assumption is made that when the forwards reaction of the substrate cycle is stimulated, there is no change in the rate of the back reaction. However, this assumption need not necessarily be true. Stimulation of the forwards reaction of the cycle will lead to an increase in the concentration of B. As can be seem from table 4.11, if this rise in the concentration of B leads to an increase in the rate of the back-reaction of the cycle, then the sensitivity actually exhibited by the substrate cycle to a change in the concentration of effector will be less than 1 + C/J. The situation in case A of table 4.11 shows the substrate cycle after a 10% stimulation of the forwards reaction, there being no change in the rate of cycling. The actual sensitivity observed to be due to the substrate cycle (i.e. the % increase in pathway flux divided by the % increase in the concentration of effector) is 10, and this agrees with the sensitivity predicted by 1 + C/J for the cycle prior to the change in effector concentration. However, cases B and C of table 4.11 illustrate the actual sensitivity obtained by the operation of the substrate cycle if the back-reaction of the cycle is stimulated in addition to the rate of the forward reaction. In case B, where the forward and backward reactions are both stimulated by 10%, the operation of a substrate cycle has not lead to any increase in the sensitivity of control of pathway flux. Case C illustrates how the cycle will give an intermediate value for the sensitivity obtained by cycling (that is, in between cases A and B) if the percentage increase in the forwards reaction is greater than the percentage increase in the back reaction.

Hence the equation 1 + C/J only predicts the sensitivity obtainable at a given rate of cycling if there is no change in the

Table 4.11 Illustration of how the sensitivity property of a substrate cycle may be decreased or lost by an increase in the rate of the back reaction of the cycle



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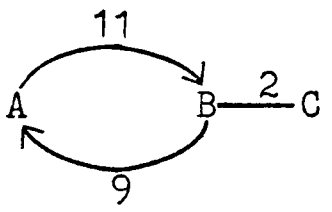
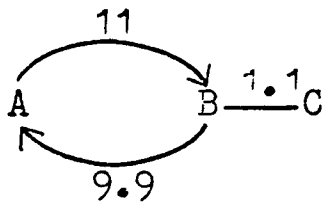
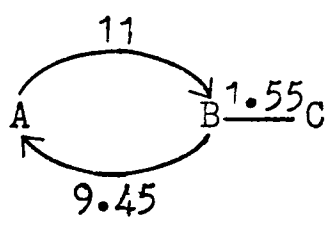
Initially, the fluxes through and around the substrate cycle are as shown in the diagram. In this substrate cycle, the increase in sensitivity due to cycling is ten-fold (i.e. 1 + C/J = 10). Stimulation of the forwards reaction of the cycle by 10% will increase the concentration of the intermediate B. The table opposite shows the actual sensitivity obtained by the substrate cycle if the rise in the concentration of B leads to an increase in the rate of the back-reaction.

Table 4.11

Changes due to 10% change in effector concentration	New fluxes in the system after changes due to effector	Sensitivity = $\frac{\%\ \Delta\ \text{pathway flux}}{\%\ \Delta\ \text{effector}}$	Ratio = $\frac{\%\ \Delta\ \text{forward}}{\%\ \Delta\ \text{cycling}}$
A 10% increase in forward reaction only		$\frac{100\%}{10\%} = 10$	$\frac{100\%}{0\%} = \infty$
B 10% increase in both forward and back reactions		$\frac{10\%}{10\%} = 1$	$\frac{10\%}{10\%} = 1$
C 10% increase in forwards reaction, 5% increase in back reaction		$\frac{55\%}{10\%} = 5.5$	$\frac{10\%}{5\%} = 2$

rate of the back-reaction in response to an alteration in the concentration of effector. If there is a change in the rate of the back-reaction, then this can either increase or decrease the sensitivity properties of the cycle, depending on whether the back-reaction is inhibited or stimulated by the change in effector. It is demonstrated in appendix III that if the sensitivity of the forwards reaction of a cycle to a change in the concentration of effector is given by n_f, and the sensitivity of the back-reaction is given by n_b, then the sensitivity of the control of pathway flux is given by:

$$\text{Sensitivity} = n_f \left[1 + \frac{C}{J}\left(\frac{n_f - n_b}{n_f}\right)\right]$$

If the substrate cycle did not exist, then the sensitivity of control of the pathway flux would be simply n_f. Hence, operation of a substrate cycle increases the sensitivity of control by a factor of

$$1 + \frac{C}{J}\left(\frac{n_f - n_b}{n_f}\right)$$

Note that if a regulator stimulates the backward reaction, then n_b is positive and there is a decrease in the sensitivity of the cycle. Conversely, if a regulator inhibits the back-reaction, then n_b is negative and there is an increase in the sensitivity of the response of the pathway flux to changes in the concentration of regulator.

Knowledge of the ratio C/J under any given set of conditions, therefore, does not mean that the actual increase in sensitivity that is achieved by operation of a substrate cycle (i.e. the sensitivity which is manifested in response to an actual change in the concentration of effector) is known. In order to describe the complete response of a substrate cycle to changes in the concentration

of a regulator, the sensitivities of the forward and backward reactions must be known. Unfortunately, in the case of the TG-FFA cycle, such information is not available; indeed, the mechanism of regulation of the rate of esterification is still unknown (see later). Hence, knowledge of the rate of TG-FFA cycling cannot be used to estimate the increase in the sensitivity of the control of FFA flux which is due to the TG-FFA cycle. However, it is possible to measure by experiment the percentage increases in the rates of lipolysis and reesterification in response to incubation with a hormone. If the ratio

$$\frac{\%\text{ increase in lipolysis}}{\%\text{ increase in reesterification}}$$

is greater than unity, then this means that the sensitivity of the lipolytic process to a change in regulator is greater than the sensitivity of the esterification pathway. Hence the ratio $\left(\frac{n_f - n_b}{n_f}\right)$ in the equation given above is greater than zero, and thus the TG-FFA cycle has increased the sensitivity of the response to the regulator. The values of the ratio

$$\frac{\%\text{ increase in forward reaction}}{\%\text{ increase in back-reaction}}$$

for a variety of hypothetical responses of a substrate cycle to changes in regulator are shown in table 4.11. The cycle illustrated in case B shows that when the sensitivities of the forwards and backwards reactions are the same, the ratio

$$\frac{\%\text{ increase in forwards}}{\%\text{ increase in backwards}}$$

is unity. In both of the other examples shown, this ratio is greater than unity, and in both cases the substrate cycles increase the control of pathway flux.

In Appendix III it is shown that if a tissue is incubated with two different concentrations of hormones and the ratio of the percentage increase in the forward reaction to the percentage

increase in the backward reaction of the cycle is determined (this ratio being denoted by 'Q'), then the actual observed sensitivity of the response of the pathway flux to the change in the concentration of regulator is given by

$$\text{Sensitivity} = n_f\left(1 + \frac{C}{J}\left(\frac{Q-1}{Q}\right)\right) \quad \text{where } Q = \left[\frac{\%\text{ increase in forwards reaction}}{\%\text{ increase in backwards reaction}}\right]$$

Hence the actual increase in the sensitivity of the response of the pathway flux to a change in concentration of regulator that was actually due to the operation of the substrate cycle is given by

$$1 + \frac{C}{J}\left(\frac{Q-1}{Q}\right)$$

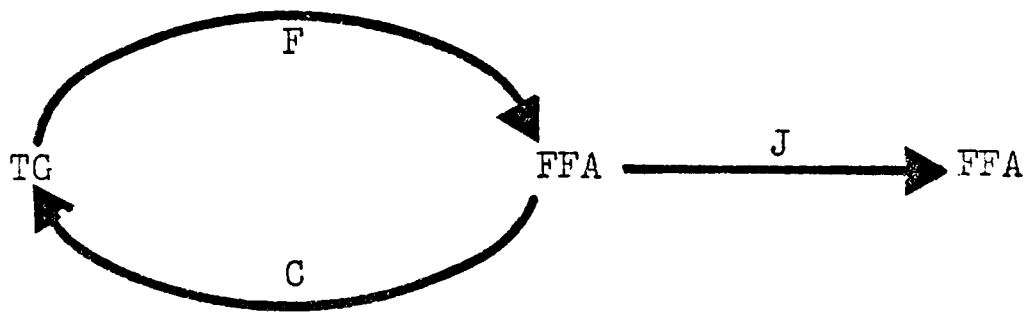
In this equation, each of the parameters can be determined relatively easily by experiment; C is the rate of cycling under one of the incubation conditions, and J is the pathway flux under these conditions. Q is the ratio (defined above) that is observed for the changes in the rates of the forwards and backwards reactions of the substrate cycle in response to the concentration of hormone in the second incubation. Note that this equation gives the actual value of the increase in sensitivity of the control of pathway flux, but does not require knowledge of the actual increase in the concentration of the hormone between the two incubations. Nor does it require any knowledge of the changes in the concentration of intracellular secondary messengers or substrates for either the forward or the backward reaction of the cycle. The equation therefore offers a direct means of assessing the sensitivity increases that are due to the operation of a substrate cycle.

The results obtained by applying $1 + \frac{C}{J}\left(\frac{Q-1}{Q}\right)$ to the results of two incubation experiments are shown in tables 4.12 and 4.13. In each of these tables, an incubation has been selected against which the other incubations are compared. In the selected incubation, the value of

Table 4.12 Actual sensitivity of FFA release that is due to the TG-FFA cycle in response to different hormones

Incubation Conditions	Rate of FFA release = pathway flux = J	Rate of FFA reesterification = cycling flux = C	$\frac{\%\text{ increase in F}}{\%\text{ increase in C}}$ (relative to control) = Q	TG-FFA cycle increases sensitivity of control of FFA release by a factor of $1 + \frac{C}{J}\left(\frac{Q-1}{Q}\right)$
Control	0.66 ± 0.13	3.94 ± 0.25		
Insulin	0.48 ± 0.09	2.12 ± 0.37	0.94	0.63
NA + Ins	7.42 ± 0.82	30.7 ± 1.81	1.07	1.41
NA	7.90 ± 1.01	22.5 ± 1.9	1.19	1.96

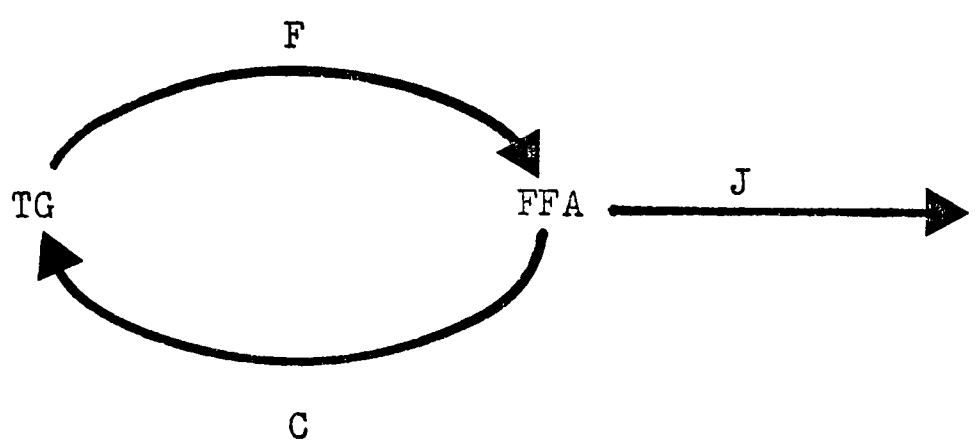
Data is from table 4.1; rates are in μmoles FFA/ g wet tissue/ hour



For the control incubation, (1 + C/J) = 6.97. This is the degree of the increase in sensitivity of the control of FFA release by TG-FFA cycling if an effector does not affect the rate of the back reaction of the cycle. The actual sensitivity afforded by the TG-FFA cycle in response to the regulators shown in the incubation conditions column is shown in the right hand column, $1 + \frac{C}{J}\left(\frac{Q-1}{Q}\right)$

Table 4.13(a). The response of fat cells incubated under various conditions and the degree to which the TG-FFA substrate cycle has increased the sensitivity of the response

The fluxes through the TG-FFA cycle are labelled thus:



Epididymal fat cells were prepared from Sprague-Dawley rats (2 days fasted, 100g body weight at start of fast) and incubated as described in chapter two. Concentrations used were; glucose, 5 mM; adenosine deaminase (ADA), 10 μg/ ml; insulin (Ins), 151 μU/ ml; noradrenaline (NA), 1 μM. Rates are in μmoles FFA/ g dry cells/ hour and are given as mean ± SEM (N=5).

For the control incubation, $(1 + C/J) = 4.13$. This is the degree of the increase in the sensitivity of the control of FFA release by TG-FFA cycling if an effector does not affect the rate of the back reaction of the cycle. The actual sensitivity afforded by the TG-FFA cycle in response to the regulators shown in the incubation conditions column is shown in the right hand column, $1 + \frac{C}{J}\left(\frac{Q-1}{Q}\right)$.

Table 4.13(a)

Incubation Conditions	Rate of FFA release = pathway flux =J	Rate of FFA reesterification = cycling flux =C	$\frac{\% \Delta F}{\% \Delta C}$ = Q	Sensitivity = $1 + \frac{C}{J}\left(\frac{Q-1}{Q}\right)$
Control	2.26 ± 1.06	7.07 ± 6.31		
Glucose	7.91 ± 3.39	40.9 ± 5.3	0.88	0.59
ADA	80.3 ± 12	44.0 ± 15.0	2.36	2.80
Glucose + ADA	328 ± 29	197 ± 20	2.06	2.61
Glucose + Insulin	6.22 ± 2.26	41.4 ± 3.7	0.85	0.43
Glucose + NA	222 ± 12	173 ± 11	1.76	2.35
Glucose + ADA + Ins	467 ± 28	258 ± 24	2.16	2.68
Glucose + NA + Ins	122 ± 6	155 ± 7	1.37	1.85
NA + Ins	41.7 ± 11	44.2 ± 16	1.56	2.13
NA	102 ± 8	104 ± 18	1.54	2.09

Table 4.13(b) The response of fat cells incubated under various conditions and the degree to which the TG-FFA substrate cycle has increased the sensitivity of the response

Incubation Conditions	Rate of FFA release = pathway flux =J	Rate of FFA reesterification = cycling flux =C	$\frac{\% \Delta F}{\% \Delta C}$ =Q	Sensitivity = $1 + \frac{C}{J}\left(\frac{Q-1}{Q}\right)$
Control	2.26 ± 1.06	7.07 ± 6.31	0.98	0.88
Glucose	7.91 ± 3.39	40.9 ± 5.3		
ADA	80.3 ± 12.1	44.0 ± 15.0	20.4	5.92
Glucose + ADA	328 ± 29	197 ± 20	2.56	4.15
Glucose + Insulin	6.22 ± 2.26	41.4 ± 3.7	-1.99	8.76
Glucose + NA	222 ± 12	173 ± 11	2.20	3.82
Glucose + ADA + Ins	467 ± 28	258 ± 24	2.61	4.19
Glucose + NA + Ins	122 ± 6	155 ± 7	1.68	3.09
NA + Ins	41.7 ± 11	44.2 ± 16	9.42	5.62
NA	102 ± 8	104 ± 18	2.09	3.69

For the glucose incubation, $1 + C/J = 6.17$. This is the degree of the increase in the sensitivity of the control of FFA release by TG-FFA cycling if an effector does not affect the rate of the back-reaction. The actual sensitivity is shown in the right hand column, $1 + \frac{C}{J}\left(\frac{Q-1}{Q}\right)$

1 + C/J has been calculated; this predicts the increase in sensitivity that the TG-FFA cycle will give to the control of FFA release if the back-reaction of the cycle is not affected by changes in the concentration of regulator. The actual sensitivity due to TG-FFA cycling that is observed in response to incubation with a regulator is shown in the right hand column of the tables.

It can be seen that in response to all but one of the incubation conditions shown in tables 4.12 and 4.13, the actual sensitivity realised by the TG-FFA cycle is not as great as that predicted using the expression 1 + C/J. Hence, in most cases studied here, when the rate of FFA release is stimulated by a regulator molecule, there is concomitant stimulation of reesterification, and hence the value of $\frac{Q-1}{Q}$ $\left(\text{or } \frac{n_f - n_b}{n_f}\right)$ is less than unity. The one exception to this statement can be seen in table 4.13(b). Here, the addition of insulin to the glucose incubation has decreased FFA release and increased FFA reesterification. Hence the sensitivity due to TG-FFA cycling as predicted from 1 + C/J for the glucose-only incubation has under-estimated the actual sensitivity achieved by cycling.

In most of the incubations shown, the value for the increase in sensitivity achieved by TG-FFA cycling is greater than unity. Hence, in most cases the cycle does increase the sensitivity of the control of FFA release. However, in several cases, the cycle actually leads to a decrease in the sensitivity of the control of FFA release. For example, in table 4.12, it can be seen that in response to insulin the TG-FFA cycle has actually decreased the sensitivity of control of FFA release. This illustrates that, as well as increasing the response, a substrate cycle can decrease the response to a regulator, thus providing insensitivity to the regulator.

4.4.3 Control of the rate of FFA reesterification

In all of the experiments shown in this chapter, it is noticeable that whenever the rate of lipolysis is increased, the rate of re-esterification is also increased. It might be that this reflects the equation used to determine the rate of FFA reesterification (i.e. FFA reesterification = 3x rate of glycerol release minus the rate of FFA accumulation). However, this possibility can be discounted since a similar correlation between the rates of lipolysis and cycling is seen when FFA reesterification is measured by using the synthesis method of measuring cycling (see chapter 3). Hence it appears that whenever the concentration of FFA is raised (by the action of any of the lipolytic hormones) the rate of the esterification pathway is increased. It is therefore relevant to consider how FFA may stimulate the rate of esterification, and hence the current ideas on the mechanism of control of the FFA esterification pathway will now be considered.

Investigations into the control of the rate of lipolysis have taken two main courses. The first was to measure the concentrations of the substrates used in triglyceride synthesis, and the second was to study the effects of hormones on the activity of the esterification pathway enzymes. The results obtained from these two approaches will be discussed in turn.

4.4.3.1 Control of esterification by substrate availability

For a long time it was considered that the concentration of glycerol phosphate (GOP) or fatty acyl CoA might be rate-limiting for FFA esterification. However, work by Denton & Halperin (1968) and Saggerson & Greenbaum (1970), which is summarised in tables 4.14 and 4.15, showed that there was no correlation between the concentrations of these precursors and the rate of triglyceride formation. For

TABLE 4.14 THE RELATION BETWEEN RATE OF TRIGLYCERIDE-GLYCEROL SYNTHESIS AND THE CONCENTRATIONS OF GLYCEROL PHOSTPHATE AND FATTY ACYL CoA (from Denton and Halperin, 1968)

TREATMENT		TRIGLYCERIDE-GLYCEROL SYNTHESIS (%)	GLYCEROL PHOSPHATE CONCENTRATION (%)	FATTY ACYL CoA CONCENTRATION (%)
CONTROL	EXPERIMENTAL			
None	+ Insulin	220	860	52
None	+ Adrenaline	570	135	77
Adrenaline	+ Insulin	240	100	110
Insulin	+ Adrenaline	530	20	152
Albumin	+ Adrenaline	590	57	110
Albumin + Insulin	+ Adrenaline	470	28	147
Albumin	+ Palmitate	210	99	97

All incubations contained glucose at 17mM.

Epididymal fat pads from Wistar rats (150-250g) fed ad libitum were preincubated for 30 minutes in medium containing glucose and then for 60 minutes in fresh medium containing indicated effectors.

Experimental results are shown as a percentage of their respective control rate or concentration. Concentrations used were; Albumin, 20mg/ml; Adrenaline, 5µg/ml; Palmitate, 1.3 mM; Fatty acyl CoA is measured as total acid-insoluble CoA.

TABLE 4.15 THE RELATIONSHIP BETWEEN THE RATE OF TRIGLYCERIDE-GLYCEROL SYNTHESIS AND THE CONCENTRATIONS OF GLYCEROL PHOSPHATE AND FATTY ACYL CoA (from Saggerson and Greenbaum, 1970)

TREATMENT	TRIGLYCERIDE-GLYCEROL SYNTHESIS (%)	GLYCEROL PHOSPHATE CONCENTRATION (%)	FATTY ACYL CoA CONCENTRATION (%)
Anti-insulin serum	100	100	100
Insulin	250	260	66
Insulin + Adrenaline + Albumin	1350	187	81
Insulin + Albumin + Oleate	630	156	70

All incubations contained 20mM glucose.

Epididymal fat pads from inbred albino rats (150-180g) fed ad libitum were preincubated for ten minutes under conditions similar to those in the main incubation, before being incubated for one hour in the main incubation.

Results are given as percentage of the rate or concentration seen with anti-insulin serum. Concentrations used were; Insulin, 200 mU/ml; Adrenaline, 5μg/ml; Anti-insulin serum, 5mU/ml; Albumin, 10mg/ml. Values are given as percentage of the anti-insulin incubation. Fatty acyl CoA was measured as total acid-insoluble CoA.

example, Denton & Halperin (see table 4.14) found that adrenaline increases the concentration of fatty acyl CoA by 10% and decreases the concentration of GOP (glycerol phosphate) by 40%, yet the rate of triglyceride synthesis was increased by 500%. Similarly, Saggerson & Greenbaum (1970) found a 1250% increase in the rate of triglyceride synthesis together with a 90% increase in GOP and a 20% decrease in fatty acyl CoA concentrations (see table 4.15). Hence changes in the (whole tissue) concentrations of GOP and fatty acyl CoA do not appear to be large enough to account for the changes in the rate of triglyceride synthesis. Only when insulin is added could the change in FFA esterification be due to a change in the concentration of glycerol phosphate.

If the rate of esterification is controlled by the availability of glycerol phosphate, then the intracellular concentrations of GOP must be within the K_m region for glycerol phosphate acyl transferase. Literature values for the K_m for glycerol phosphate acyl transferase (GOPAT) are summarised in table 4.16. As can be seen from this table, the variability in the reported values for the K_m is very large - a factor of 100. The differences presumably reflect the method of assaying the GOPAT, and hence the details of the assay are also outlined in this table. The problems involved in assaying GOPAT are well illustrated by the results of Dodds et al. (table 4.16); these workers find a factor of 6 difference in the K_m for glycerol phosphate depending on whether they use GOP or palmitate incorporation into lipid. (They ascribe this difference in the two results to the fatty acid specificities of the three enzymes responsible for phosphatidate synthesis together with the contribution from the endogenous fatty acids of the preparation).

Glycerol phosphate acyl transferase is the first enzyme which

Table 4.16 Literature values for K_m for glycerol phosphate of glycerol phosphate acyl transferase

Reference	K_m for L-GOP (mM)	Parameter measured in assay	Enzyme Preparation used	Added acyl CoA source (μM)			% albumin present in assay
				FFA	CoA	Acyl CoA	
Steinberg et al (1961)	approx 0.1	^{14}C-palmitate into neutral lipid	Defatted fat-pad homogenate	5	50		
Angel & Roncari (1967)	0.86	^{14}C-palmitate into neutral lipid	Defatted fat-pad homogenate	80	80		
Jamdar & Fallon (1973)	0.13	^{14}C-GOP into phospholipid	Microsomes from fat pad homogenate				1.3
Sooranna & Saggerson (1976)	0.15	^{14}C-GOP into lipid	Defatted fat-cell homogenate			65	0.18
Dodds et al (1976)	0.6	(1,3^{3}H)-GOP into total lipid	Microsomes from fat pad homogenate	800	50		1.2
	0.09	^{14}C-palmitate into total lipid	Microsomes from fat pad homogenate	800	50		1.2
Schlossman & Bell (1976)	0.008	^{14}C-GOP into total lipid	Microsomes from isolated fat cells			50	2

is specific to the esterification pathway and it is found predominantly in the microsomal fraction of the cell (Schlossman & Bell, 1976). It is possible that the differences in the reported K_m for GOPAT are due to the GOPAT being situated on the inside of the microsomes. Hence, a spuriously high concentration of glycerol phosphate would be required in order that the GOP may permeate through the microsome membrane and thus reach the GOPAT. Differences between the various workers' homogenisation precedures could produce microsomes with different permeability characteristics towards glycerol phosphate, and this could therefore explain why there is such large variability in the literature value for the K_m of GOPAT for glycerol phosphate. If the differences between the homogenisation procedures is the answer for the observed variability, then it is reasonable to use the minimum observed value as being the best indicator for the K_m. Thus it would appear that the K_m (reported by Schlossman & Bell, 1976) of 8 μM glycerol phosphate is the best estimate for the K_m of GOPAT for GOP.

The problem of enzymes being situated inside microsomes can often be overcome by procedures which disrupt the microsomes, such as sonication or the use of detergents. Of the results shown in table 4.16, only Schlossman & Bell tried using either of these techniques on their system. They found that low concentrations of detergent did not affect GOPAT activity, whereas higher concentrations caused inhibition. These results seem to imply that enzyme compartmentation within microsomes is not a problem with GOPAT. However, the fact that the K_m reported by Schlossman & Bell is by far the lowest of all the observations suggests that compartmentation is not a problem with their system anyway. Hence compartmentalisation within microsomes could explain the variability in the reported values for the K_m.

The literature values for the intracellular concentration of glycerol phosphate are shown in table 4.17. The lowest concentrations of glycerol phosphate reported, 0.28 mM, is at least twice the K_m reported by the majority of investigators shown in table 4.16, and is 30 times the K_m of 8 μM found by Schlossman & Bell (1976). Therefore, based on the literature values for the K_m of GOPAT for GOP, together with the reported concentrations of GOP in the cell, the weight of evidence implies that the level of GOP is not a limiting factor for FFA esterification in fat pads incubated *in vitro*.

In each of the investigations shown in table 4.17, glucose was present in the incubation medium at a concentration of at least 8 mM; under these conditions there does not appear to be any correlation between the concentration of glycerol phosphate and the rate of FFA esterification (see above). However, a number of lines of evidence suggest that the concentration of GOP may become rate-limiting for FFA esterification under certain conditions (see section 4.2.5). It is also possible that GOP could limit esterification *in vivo*. The level of all the glycolytic intermediates in incubated fat pads is much higher (by five to fifteen times) than that found *in vivo* (Saggerson & Greenbaum, 1970). As suggested by Denton et al. (1966) due to the high activity of glycerol phosphate dehydrogenase in adipose tissue the concentration of GOP does not appear to be related to the rate of glycolysis, but is apparently determined by a combination of the NAD/NADH ratio and the levels of the other sugar phosphates in the cytoplasm (see Denton & Halperin, 1968). Hence, the combination of raised (relative to the *in vivo* situation) concentrations of glycolytic intermediates in incubated fat pads, together with the probability of a lowered NAD/NADH ratio *in vitro* (due to poor oxygenation of the tissue in the centre of the fat pad; evidence for this can be seen in the results of Saggerson & Greenbaum,

Table 4.17 Literature values for intracellular concentrations of Glycerol phosphate

Reference	Glycerol phosphate concentration	Incubation Conditions	µg atoms glyceride glycerol /g wet wt /hr
Denton & Randle (1967)	0.23	Glucose (17 mM) + Albumin (2%)	1.9
	1.1	Albumin + Ins (10 mU/ml)	4.1
	0.16	Albumin + Ins + Adrenaline (27 µM)	20.1
*Halperin & Denton (1969)	0.26	Glucose (8 mM)	-
	0.95	Ins (10 mU/ ml)	-
	4.3	Anaerobic conditions	-
	11.4	Ins + Anaerobic conditions	-
	0.39	Adrenaline (27 µM)	-
	0.33	Adrenaline + Ins	-
Saggerson & Greenbaum (1970)	0.83	Glucose (20 mM) + anti-insulin serum (5 mU/ml)	-
	2.1	Ins (200 mU/ ml)	-

All results are obtained using fat pads incubated with glucose.

* These values are calculated using a value for adipocyte intracellular water of 1.4 ml per 100g wet weight tissue (Denton et al., 1966).

1970) means that the concentration of glycerol phosphate in incubated fat pads is much greater than that occuring *in vivo*. It is therefore possible that glycerol phosphate could be rate-limiting for FFA esterification *in vivo*. However, for fat pads incubated *in vitro* the concentration of glycerol phosphate does not correlate satisfactorily with the rate of esterification, and hence in incubated adipose tissue the control of esterification must reside in some other factor.

The other major substrate for FFA esterification is fatty acyl CoA and it is therefore possible that this could limit the rate of triglyceride synthesis. It can be seen from tables 4.14 and 4.15 that the concentration of fatty acyl CoA in incubated fat pads does not correlate with the rate of FFA esterification. However, the concentrations of acyl CoA given in these tables are whole-tissue concentrations; it is likely that a large part of the acyl CoA in the tissue is bound to various intracellular binding sites (see Hallestrap & Denton, 1974). If the amount of fatty acyl CoA that is bound to these sites varies under different conditions, then the concentration of unbound fatty acyl CoA could vary without there being any changes in the total-tissue acyl CoA levels. It is very striking that whenever the concentration of FFA is increased (either by adding FFA or by stimulating lipolysis), the rate of esterification also increases (see the experiments reported in this chapter; see also Saggerson & Tomassi, 1971; Saggerson, 1972) and it has been suggested that FFA may compete with fatty acyl CoA for the same binding site, thus changing the concentration of unbound fatty acyl CoA. The identity of the fatty acyl CoA binding protein is unknown; in adipose tissue it has been suggested that an enzyme of glyceride synthesis could have such a role (Sooranna & Saggerson, 1975).

Using albumin as a model for the fatty acyl CoA binding protein, Halestrap & Denton (1974) have demonstrated that FFA and long-chain acyl CoA can compete for the same site, and therefore it seems possible that tissue FFA levels could be important in determining the concentration of unbound acyl CoA in the fat cell and hence the rate of FFA esterification.

4.4.3.2 Control of FFA esterification by means other than substrate concentration

It can be seen from table 4.1 that the maximal activity of each of the esterification pathway enzymes is reduced in fat cells that have been incubated with catecholamines (Saggerson et al., 1979). With at least one of the enzymes (glycerol phosphate acyl transferase) this change in maximal activity could be due to covalent modification (phosphorylation) of the enzyme. However, catecholamines are known to increase the synthesis of triglyceride in adipose tissue (see for example tables 4.14 and 4.15). Hence, even though a persistent change in the maximal activity of the esterification enzymes is seen, this change is in the direction opposite to that of the change in FFA esterification. Evidence which suggests that the control of the activity of the esterification enzymes (other than by substrate concentration) is important can be seen from the fact that insulin can prevent the persistent changes in the maximal activity seen with catecholamines (see table 4.1). This protective effect of insulin on the activity of the esterification enzymes could explain the observation that the highest rates of esterification are observed in the presence of glucose plus catecholamines plus insulin (see tables 4.2 and 4.4).

In summary, the mechanism of the control of FFA esterification is still unknown. It seems likely that the control of esterification resides in a combination of substrate availability plus the modulation

of enzyme activity by some factor other than substrate. What this factor might be, together with the relative importances of each of these mechanisms to the control of FFA esterification, is still unknown.

CHAPTER FIVE

In vivo treatment of animals followed by in vitro incubation of adipose tissue

5.1 Introduction

In chapter 4 results were presented which showed that the TG-FFA cycling rate can vary independently of flux in response to a short term incubation with hormones. Most of the experiments reported in chapter 4 involved the use of fat tissue taken from animals which had been fed ad libitum and then incubated with a variety of hormones. In chapter 5, the effects of different in vivo treatments on the properties of the TG-FFA cycle are reported.

The experiments in this chapter therefore involve the exposure of the animals to various treatments (such as fasting, corticosteroid treatment), after which the animals are killed. Their fat pads are then removed and incubated in vitro under a set of standard conditions. Hence it is possible to investigate whether treatment of the animal in vivo affects the response of the TG-FFA cycle to various hormones.

For the investigation of the in vitro responses to the hormone treatment there is a choice between the use of either isolated fat cells or intact fat pads. For reasons outlined below, the latter system was chosen for these experiments. Chronic treatment of animals in vivo often leads to changes in the average size of the fat cell in the animal. It is known that many of the responses of fat cells to incubation with hormones can vary according to the size of the fat cell (Zinder, 1971; Hartman et al., 1971; DiGirolamo et al., 1974). Also, it has been shown that during the preparation of adipocytes lysis of large adipocytes occurs. This leads to a suspension of fat cells which is not representative of the original population of cells

in the fat pad (see Jamdar, 1978). Hence isolated fat cells cannot be used for the comparison *in vitro* of the metabolism of adipose tissue from animals which have adipose tissue containing a different population of cell sizes. The use of fat pads avoids the problem of the lysis of large fat cells.

For the investigation of the response of the fat pads from amimals which had been subjected to various treatments *in vivo*, the following incubation conditions *in vitro* were used. The pads were incubated with either no added hormones, or with insulin, noradrenaline, or with insulin plus noradrenaline. These conditions were used in order to see how the adipose tissue responded to the hormones which are thought to be the main anabolic and catabolic hormones in the rat (see Hales et al., 1977).

5.2 Introduction to the treatments used in this chapter

In this section, the current ideas concerning the effects on adipose tissue of the treatments used in this chapter are reviewed. The treatments are alterations in the dietary, thyroid and corticosteroid status of the rats.

5.2.1 Effect of thyroid status on adipose tissue metabolism

Hyperthyroidism shows many of the characteristics of a state of increased adrenergic activity (tachycardia, sweating, increased metabolic rate, weight loss, palpitations and nervousness) whereas hypothyroidism exhibits characteristics of a decrease in adrenergic activity (Landsberg, 1977; Ciaraldi & Marinetti, 1978). The similarities between hyperthyroidism and states of increased adrenergic activity suggests that there is some sort of interaction between catecholamines and thyroid hormones to produce the manifestations of hyperthyroidism. Investigations into the interactions between thyroid hormones and

catecholamines have taken three main approaches. Firstly, it is possible that catecholamines stimulate the rate of thyroid hormone secretion. Secondly, thyroid hormones might affect the activity of the sympathoadrenal system. Lastly, they could affect the sensitivity of the target tissues to the action of catecholamines.

The first possibility given above (that catecholamines increase thyroid hormone secretion) does not appear to play an important role in the secretion of thyroid hormones. It has been shown that catecholamines will stimulate thyroid hormone release, but it appears that the presence of TSH (thyroid stimulating hormone) has an overriding effect (see review by Landsberg, 1977). Landsberg concludes that there is little evidence in support of the idea that catecholamines have a physiologically significant role in the initiation or maintenance of increased thyroid hormone output in hyperthyroidism.

Similarly, there is no evidence for the second idea given above, namely that thyroid hormones affect the activity of the sympathoadrenal system. Experiments show that in hyperthyroidism the activity of the sympathetic nervous system is normal or slightly suppressed, while in hypothyroidism sympathetic activity is significantly enhanced (see Landsberg, 1977). The activity of the adrenal medulla is not thought to alter in hyper- or hypothyroidism. Thus the similarities between hyperthyroidism and the effects of catecholamines are not a result of an increased activity of the sympathetic nervous system.

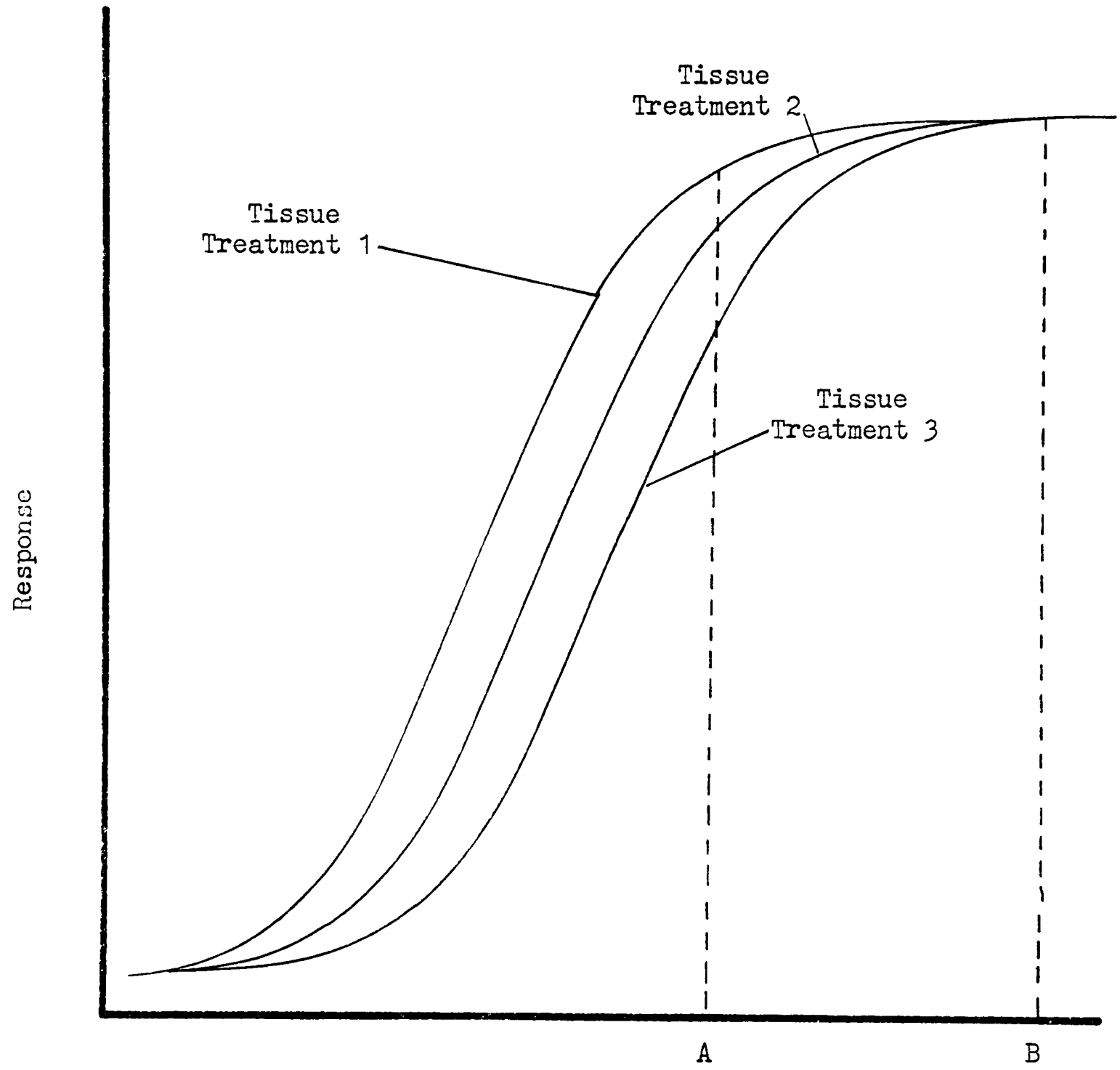
The third possibility, that thyroid hormones can alter the sensitivity of the target tissue to the action of catecholamines, does appear to be a mechanism of physiological significance. Experimental evidence is accumulating which suggests that thyroid hormones increase the sensitivity of the target tissue to the action of catecholamines. Because there is little or no change in the concentration

of catecholamines in the hyperthyroid state (see above), this increase in sensitivity to catecholamines leads to an increased response in the target tissue. The evidence which supports the idea that thyroid hormones affect the sensitivity of tissues to the action of catecholamines will now be briefly summarised.

As pointed out by Landsberg (1977), much of the work done on the action of thyroid hormones is very difficult to correlate. The problem is that if thyroid hormones change the sensitivity of a tissue to the action of catecholamines, then there will be a shift in the dose-response relationship. As shown in figure 5.1, if only one concentration of hormone is used in a comparison of tissues with different senstivities, then it is not possible to make any comparisons between the maximal response of the tissues (unless the concentration of hormone gives a maximal response in the least sensitive tissue). Hence, experiments using only one concentration of catecholamine do not enable the experiments to say whether thyroid hormones affect the rate of the process being measured unless, either fortuitously or by choice, the concentration of catecholamine used is such that it illicits a maximum response in the least sensitive tissue (see fig 5.1). Only the more recent studies on the effects of thyroid hormones have taken these considerations into account, and thus care must be taken in the interpretation of much of the earlier work. (The dangers in the interpretation of data from experiments using only one concentration of hormone are well illustrated by Kahn, 1978).

Thyroid hormones modulate the action of catecholamines in a number of tissues, including heart (Hornbrook & Conrad, 1972), liver (Malbon et al., 1978) and adipose tissue (Ichikawa et al., 1971; Armstrong et al., 1974). Adipose tissue taken from hyperthyroid animals is more sensitive to the actions of catecholamines, whereas hypothyroidism causes a decrease in the sensitivity of adipose tissue

Fig 5.1 Graph illustrating possible source of error in studying the maximal response of a tissue that changes in sensitivity (EC_{50}) to a hormone, but not in maximal response



If the experiment is done with hormone at a concentration of A, then the maximal response of the tissue will appear different in treatments 1,2 and 3. If the experiment is performed with hormone at a concentration of B, then the maximal responsiveness will be the same in all three treatments (see Kahn, 1978).

to catecholamines (Brodie et al., 1966; Fisher & Ball, 1967; Malbon et al., 1978). The increased responsiveness of adipose tissue is only seen after a lag period of at least three hours after administration of the thyroid hormone (Caldwell & Fain, 1971). Investigations into the response of adipose tissue to catecholamines have shown that the accumulation of cAMP in response to catecholamines is dependent on the thyroid status of the animal; the accumulation of cAMP is reduced in hypothyroid animals (Ichikawa et al., 1971; Van Inwagen et al., 1975) but does not appear to be affected in hyperthyroid animals (see Fain, 1973). A reduction in the rate of cAMP accumulation could be due to a decreased activity of adenylate cyclase or an increased activity of phosphodiesterase. It is debateable whether there is any change in the activity of phosphodiesterase in adipose tissue of hypothyroid animals (Armstrong et al., 1974; Correze et al., 1974; Van Inwegen et al., 1975; Malbon et al., 1978). Thus the reduced accumulation of cAMP in response to catecholamines does not appear to be due to increased phosphodiesterase activity. Similarly, hypothyroidism does not affect the maximal activity of adenylate cyclase in adipose tissue (Correze et al., 1974; Malbon et al., 1978) although there is a reduction in the maximal response of adenylate cyclase to catecholamines. This reduced response of adenylate cyclase could be responsible for a decreased rate of cAMP accumulation. Because the maximum (fluoride-induced) activity of adenylate cyclase is not changed by thyroid status, it is apparent that the decrease in the response to catecholamines must be due to changes occuring between the binding of the hormone and the activation of adenylate cyclase. The decrease in the response to catecholamines could be due to a change in the number of adrenergic receptors or it could be due to a decrease in the coupling between adenylate cyclase and the beta-receptor. Malbon et al.(1978) showed that there was no difference

in the number of beta receptors per cell in adipose tissue from hypo-, eu- and hyperthyroid animals, which suggests that a change in the number of beta-receptors is not responsible for the decreased maximal response to catecholamines. (In contrast to adipose tissue, thyroid hormones have been reported to modulate the number of beta-receptors in heart - see Williams et al., 1977; Ciaraldi & Marinetti, 1977.) However, it does seem that thyroid hormones can alter the interaction between the beta-receptors and the adenylate cyclase enzyme (see Malbon, 1980). The beta-receptor is believed to be coupled to the adenylate cyclase molecule by a protein which binds guanine-nucleotides (for a review on this coupling protein see Birnbaumer et al., 1978; Ross & Gilman, 1980; Limbird, 1981), and evidence suggests that thyroid hormones somehow affect this coupling protein such that hyperthyroidism increases (and hypothyroidism decreases) the affinity of the beta-receptor for agonist molecules (see Malbon, 1980). How thyroid hormones achieve this, whether by altering the amount of the coupling protein or by modification of some as yet unidentified component of the fat cell membrane, is not yet known.

A certain amount of evidence is accummulating which suggests that the ratio of alpha to beta adrenergic receptors in certain tissues is dependent on the thyroid status of the animal (see Landsberg, 1977). It is suggested that thyroid hormones could control an allosteric interconversion of the same protein molecule between an alpha and beta receptor (see Kunos et al., 1974). Speculation on this interconversion occuring has been made for rat atria, rat tail-artery and human adipose tissue (Landsberg, 1977) but does not appear to be important in rat adipose tissue.

Thyroid hormone-treated animals have an elevated plasma concentration

of FFA (Rich et al., 1959). This is probably a result of a combination of the increased basal rate of lipolysis seen in hyperthyroid adipose tissue (Fisher & Ball, 1967) together with the increase in the sensitivity of adipose tissue to catecholamines. Thyroid status does not appear to affect the maximal rate of lipolysis that is attainable in adipose tissue (see Fain, 1973; Malbon et al., 1978; Fisher & Ball, 1967); even though cAMP accummulation is decreased in hypothyroid adipose tissue, the concentration of cAMP attained in response to catecholamines is still much greater than that required to maximally stimulate lipolysis (see Correze et al., 1974; section 4.2.6.2). Comparisons between adipose tissue from animals of differing thyroid status have to be made with caution because of the problem outlined in fig 5.1. Another problem encountered with some of the earlier studies on the effects of thyroid hormones on adipose tissue metabolism is that results are sometimes expressed on a per wet weight of tissue basis (or even per weight of triglyceride). These are not ideal units for the comparison of hyperthyroid adipose tissue with normal tissue; hyperthyroidism causes a depletion of lipid from white adipose tissue, thus altering its composition (see for example Lewes & Hayes, 1978). It seems reasonable that the rate of a process in adipose tissue will be related more to the amount of fat-free tissue (since this is the fraction in which the enzymes responsible for catalysing reactions are found) than to the fat content. Hence, the expression of results as per wet weight of tissue or per weight of triglyceride will yield an artificially high result. Better units for comparison between the various thyroid states would be per weight of fat-free tissue, per weight of protein or per weight of DNA.

Thyroid hormones have a calorigenic action, not only on the

whole animal but also in adipose tissue. Various suggestions have been made for the mechanism of this increased calorigenesis; these include ion pumping using the Na^+-K^+ ATPase (Edelman & Ismail-Beigi, 1974), increased cardiovascular activity (Sestoft, 1980), uncoupling of oxidative phospharylation in mitochondria (see Edelman & Ismail-Beigi, 1974), and an increase in the rate of TG-FFA cycling (Fisher & Ball, 1967; Sestoft, 1980). The quantitative significances of each of these mechanisms have not been firmly established; perhaps the mechanisms by which thyroid hormones increase metabolic rate are the same as those stimulated by the catecholamines. Some insight into the calorigenic actions of thyroid hormones can perhaps be gained from the study of the calorigenic actions of catecholamines (no mean feat in itself).

There are several thyroid hormones found in the blood, the main ones of which are thyroxine (tetraiodothyronine), T-3 (triiodothyronine) and reverse T-3 (see for example Burman, 1978). There has been controversy over whether T-3, the more potent of the two hormones, is the only true hormone and thyroxine merely its pro-hormone. Extrathyroidal conversion of thyroxine to T-3 has been demonstrated (see Chiraseveenuprapund et al., 1978), and this conversion has been shown to be inhibited by the drug propyl thiouracil (Oppenheimer et al., 1972). Because rats treated with propyl thiouracil (PTU) become hypothyroid, it has been concluded that thyroxine must be converted to T-3 for maximum biological activity (see Frumess & Larsen, 1975). PTU is therefore a useful method of rendering an animal hypothyroid without operating to remove the thyroid gland.

5.2.2. Effect of corticosteroids on adipose tissue

The presence of glucocorticoids is essential for the normal functioning of many of the tissues of the body. The main action of

corticosteroids appears to be a 'permissive' one, since by themselves they have little effect on the target tissues, but their presence is required in order that certain other hormones may act. This is the case with many of the cAMP-dependent processes, and in this respect the permissive action of the corticosteroids is manifested in systems including gluconeogenesis and glycogenolysis in the liver (Friedman et al., 1967; Exton et al., 1970), glycogenolysis in heart and skeletal muscle (Miller et al., 1971; Shaeffer et al., 1969), and vassopressin action on water and sodium ion transport (Handler et al., 1969). It is not yet clear at which point of the cAMP system the corticosteroids exert their control (i.e. whether they affect the receptor, the coupling to adenylate cyclase, adenylate cyclase itself, phosphodiesterase or protein kinase), but it is known that protein synthesis is involved and the protein that is controlled is probably not the receptor which is responsible for the activation of the adenylate cyclase (see Steinberg, 1976).

Glucocorticoids have an important effect on the metabolism of both lipid and carbohydrate in adipose tissue. In general, glucocorticoids decrease the metabolism of glucose by adipose tissue and promote the release of fatty acids from the tissue.

The so-called 'permissive' action that glucocorticoids have on the lipolytic response of adipose tissue to catecholamines is well known. Both the basal and the hormone-stimulated rates of FFA release are increased in adipose tissue from glucocorticoid-treated animals; the increased release of FFA is seen with catecholamines, ACTH and glucagon (Shafir & Kerpel, 1964; Jeanrenaud, 1967; Exton et al., 1972; Fernandez & Saggerson, 1978). Conversely, adrenalectomy decreases the basal and hormone-stimulated rates of FFA release (see for example Shafrir & Kerpel, 1964). The alterations in the rate of release of FFA after adrenalectomy or corticosteroid

administration appear to be due to a combination of two factors. Firstly, treatment of animals with glucocorticoids decreases the uptake and utilisation of glucose by adipose tissue (Fain, 1962; Fain et al., 1963; Livingston & Lockwood, 1975) and this apparently leads to a decrease in the rate of FFA reesterification (Jeanrenaud, 1967; Shafrir & Kerpel, 1964). Conversely, adrenalectomy leads to increased rates of glucose uptake and utilisation (Fain, 1962; Shafrir & Kerpel, 1964). Secondly, the alterations in the basal and hormone-stimulated rates of FFA release in glucocorticoid-treated or adrenalectomised animals are al least partly due to changes in the rate of lipolysis (as measured by glycerol release - see Shafrir & Kerpel, 1964; Jeanrenaud, 1967; Exton et al., 1972; Skidmore et al., 1972; Fernandez & Saggerson, 1978).

The mechanism of the effect of glucocorticoids on lipolysis in adipose tissue is not known. It has been suggested that the lesion in lipolysis after adrenalectomy results from a decreased sensitivity of the lipolytic process to cAMP (Corbin & Park, 1969; Exton et al., 1972), since lipolysis is decreased without any change in the concentration of cAMP (Exton et al., 1972). However, these workers used fat pads; subsequent work with fat cells isolated from adrenalectomised animals has shown that the accummulation of cAMP in response to noradrenaline is decreased (Allen & Beck, 1972; Schönhöfer et al., 1972). Fernandez & Saggerson (1978) have shown that the impaired lipolytic response in fat cells from adrenalectomised animals can be corrected by addition of adenosine deaminase to the incubation medium. These results indicated that adenosine may have some role in the mechanism of the effects of glucocorticoids on adipose tissue. Further support for this ides can be found from the work of Green (1979). He found that adrenalectomy decreased the activity of adenosine kinase (one of the enzymes responsible for controlling

the concentration of adenosine - see section 4.2.6.4) in white adipose tissue, which suggests that the concentration of adenosine may be increased in adipose tissue from these animals. In this respect the known effects of adenosine fit very well with the observed changes due to adrenalectomy; increased glucose metabolism, decreased sensitivity to catecholamines and decreased lipolysis. The action of corticosteroids in restoring the lipolytic response of adipose tissue from adrenalectomised animals can be blocked by protein synthesis inhibitors (Moskowitz & Fain, 1970); it is tempting to speculate that these drugs act by preventing the synthesis of adenosine kinase in response to corticosteroids.

As mentioned above, it has been suggested that the lesion in lipolysis after adrenalectomy results from a decreased sensitivity of the process to cAMP. If this is the case, then the action of adenosine deaminase in restoring the lipolytic responsiveness to noradrenaline could simply be due to the removal of adenosine raising the concentration of cAMP to a level which overcomes this proposed desensitisation to cyclic AMP (Fernandez & Saggerson, 1978). Other workers have suggested that the lesion in responsiveness to lipolytic hormones after adrenalectomy lies in the coupling between the adrenoreceptor and adenylate cyclase (Schönhöfer et al., 1972; Skidmore et al., 1972). It is possible that adenosine could have a role in the modulation of this coupling process (Birnbaumer, 1978).

Studies on the action of corticosteroids on adipose tissue incubated <u>in vitro</u> showed that corticosteroids themselves do not increase lipolysis (except perhaps at unphysiologically high concentrations; Jeanrenaud, 1967). However, if growth hormone is simultaneously present, then lipolysis is activated after a lag period of 1-2 hours. This increase in lipolysis can be prevented

by inhibitors of RNA and protein synthesis (Fain et al., 1965). It therefore seems that these two hormones acting together may have an important role in increasing the mobilisation of lipid during starvation (see Newsholme & Start, 1973, for a discussion on the significance of growth hormone during starvation).

Metyrapone is a drug which inhibits the secretion of corticosterone from the adrenal glands. It is thought to do this by inhibiting the enzyme that hydroxylates deoxycorticosterone to corticosterone (see Nicola & Dahl, 1971). These workers found an 80% inhibition of corticosterone secretion in metyrapone-treated rat adrenal glands. The Eutheria class of vertebrates as a whole secretes cortisol as the major glucocorticoid, together with varying proportions of corticosterone and cortisone. However, rats and most strains of mice are exceptions to this rule in that corticosterone and not cortisol is the major glucocorticoid (see Sandor et al., 1976). In these species, therefore, treatment with metyrapone is a chemical means of adrenalectomising the animal.

5.2.3 Effect of dietary status on adipose tissue metabolism

During periods of starvation fat must be mobilised in order to provide fuel for the tissues. Thus adipose tissue must adapt in such a way as to increase the net mobilisation of FFA. This is achieved both by an increased basal rate of lipolysis (Saggerson & Greenbaum, 1970a) and a decreased rate of FFA reesterification (Jeanrenaud, 1967). Surprisingly, the changes due to starvation in the metabolism of fat by adipose tissue appear to have been poorly investigated. For example, it is not known if the increased basal rate of lipolysis in fasted adipose tissue is due to a coarse or fine control of the triglyceride lipase. However, the decrease in the proportion of FFA reesterified does not appear to be a

result of the changes in the activity of the esterification enzymes, since the maximal rate of triglyceride-glycerol synthesis varies little with dietary state (Saggerson & Greenbaum, 1970a). It has been suggested that the decreased rate of FFA esterification seen in fasted animals is a result of the lower glycerol phosphate levels in fasted adipose tissue (Ballard, 1972) but unfortunately the evidence in support of this idea is far from conclusive (see Saggerson & Greenbaum, 1970a; discussion in chapter 4).

In contrast to the control of triglyceride synthesis and breakdown, the mechanism of control of the changes due to starvation in the rate of fatty acid synthesis have been extensively investigated. Starvation decreases, whereas fasting-refeeding increases the rate of fatty acid synthesis in incubated adipose tissue (Saggerson & Greenbaum, 1970a; Saggerson & Tomassi, 1971). The main control points in the synthesis of fatty acids are believed to be at the level of glucose entry into the adipocyte (Crofford & Renold, 1965), pyruvate dehydrogenase (Saggerson & Greenbaum, 1970a; Coore et al., 1971) and acetyl CoA corboxylase (Halestrap & Denton, 1973, 1974). Insulin is known to activate each of these steps. Starvation slightly increases the sensitivity of adipose tissue to insulin, whereas refeeding restores the sensitivity to normal (Thomas et al., 1979). The significance of the increased sensitivity to insulin in the starved animal is not clear since it changes in the opposite direction to that expected on teleological grounds. However, there is little doubt that the decrease in plasma insulin levels is the major stimulus for the changes in adipose tissue seen on starvation (see Hales et al., 1978).

Lipoprotein lipase activity in adipose tissue decreases with fasting and increases again on refeeding (Spencer et al., 1978). Lipoprotein lipase is the rate-limiting enzyme for the uptake and

storage of plasma lipid (Robinson, 1970) and thus the changes seen during starvation direct the flow of plasma triglyceride away from fat cells. These changes in lipoprotein lipase are believed to be due to a combination of the actions of glucocorticoids and insulin (Ashby & Robinson, 1980).

5.2.4 Summary to introduction

In each of the three treatments reviewed above, it is possible that a change in the sensitivity of the TG-FFA cycle could play an important part in the response of adipose tissue to the treatment. During fasting and refeeding, the advantages of the increase in sensitivity of control of FFA flux to be gained by TG-FFA cycling are self-evident. In adipose tissue from hyperthyroid animals, it is possible that the change in the sensitivity of response to catecholamines could at least partly be due to an increased rate of TG-FFA cycling. Similarly, the permissive effects of the glucocorticoids on adipose tissue metabolism may be due to a decreased rate of TG-FFA cycling in the absence of glucocorticoids, thus reducing the response to lipolytic hormones. The experiments reported in this chapter are therefore an attempt to investigate the effects of the above treatments on the sensitivity of the TG-FFA cycle in adipose tissue. The experiments involve subjecting the animals to the treatment *in vivo* followed by removal and incubation of the peri-genital fat pads *in vitro*. Incubations were done under a variety of hormonal conditions to see if any given hormonal environment is necessary for a change in sensitivity. The results are presented in the next section.

5.3 Results

The results reported in table 5.1 show that the rate of TG-FFA cycling in incubated adipose tissue was markedly affected by the

TABLE 5.1 EFFECT OF FASTING AND FASTING-REFEEDING ON THE RESPONSE OF ADIPOSE TISSUE INCUBATED IN VITRO WITH HORMONES

a)

TG-FFA CYCLING (micromoles/g fat-free dry tissue/hour)				
HORMONES ADDED IN VITRO	NONE	INSULIN	NORADRENALINE	NORADRENALINE + INSULIN
TREATMENT IN VIVO				
Fasted	266 ± 36 ***	236 ± 25 ***	435 ± 39 **	526 ± 53 ***
Control	135 ± 10 *	43.0 ± 12.3 NS	976 ± 105 NS	526 ± 53 NS
Fasted-refed	496 ± 116 NS	33.1 ± 4.1 ***	1088 ± 113 ***	1347 ± 102 ***

Sprague-Dawley rats (average body weight 172g) were fed ad libitum (controls), or fasted for 48 hours, or fasted 48 hours and refed oxoid pellets ad libitum for 24 hours. After being killed by stunning and cervical dislocation, epididymal fat pads were removed, cut into two, and the four pieces of tissue randomly distributed between the four hormone treatments. They were preincubated for half an hour in Krebs-Ringer bicarbonate buffer containing 2% albumin plus hormones as for the main incubation. The main incubation was for one hour in buffer containing 4% albumin. Concentrations used were: glucose (present in all incubations), 10mM; insulin, 1mU/ml; noradrenaline, 10 micro molar. Values are shown as mean ± SEM (n=7). Symbols show results of t-tests between treatments adjacent to the symbols; bottom row of symbols show results of t-tests between fasted and fasted-refed animals. NS = $p > 0.05$,
* $P < 0.05$
** $p < 0.01$

TABLE 5.1 b) SENSITIVITY OF THE TG-FFA CYCLE IN ADIPOSE TISSUE FROM ANIMALS OF VARYING DIETARY STATUS

$$1 + \frac{\text{RATE TG-FFA CYCLING}}{\text{RATE FFA RELEASE}} = 1 + \frac{C}{\bar{J}}$$

HORMONES ADDED IN VITRO	NONE	INSULIN	NORADRENALINE	NORADRENALINE + INSULIN
TREATMENT IN VIVO				
Fasted	3.7 ± 0.3 NS	20.6 ± 5.4 *	2.2 ± 0.1 **	2.5 ± 0.2 ***
Control	5.2 ± 0. 7 NS	6.1 ± 1.4 NS	4.0 ± 0.4 NS	4.6 ± 0.3 NS
Fasted-refed	12.0 ± 3.9 NS	3.3 ± 0.5 *	3.8 ± 0.2 ***	4.9 ± 0.2 ***

Legend as for table 5.1 a

TABLE 5.1 c)

RATE OF GLYCEROL RELEASE umoles/ g fat-free dry weight/ hour				
HORMONES ADDED IN VITRO	NONE	INSULIN	NORADRENALINE	NORADRENALINE + INSULIN
TREATMENT IN VIVO				
Fasted	123 ± 14 **	85.3 ± 7.7 ***	273 ± 17 **	299 ± 28 ***
Control	56.9 ± 3.9 *	18.0 ± 4.2 NS	439 ± 42 NS	563 ± 32 NS
Fasted-refed	184 + 38	16.7 ± 1.8	490 ± 45	566 ± 43

Legend as for table 5.1 a

TABLE 5.1 d)

FFA RELEASE (micromoles/g fat-free dry weight/hour)				
HORMONES ADDED IN VITRO	NONE	INSULIN	NORADRENALINE	NORADRENALINE + INSULIN
TREATMENT IN VIVO				
Fasted	103 ± 11 ***	20.3 ± 6.1 NS	383 ± 22 NS	370 ± 43 NS
Control	35.8 ± 4.4 NS	11.0 ± 2.1 NS	340 ± 40 NS	376 ± 33 NS
Fasted-refed	53.9 ± 8.3	17.1 ± 2.3	381 ± 26	352 ± 30

Legend as for table 5.1 a

dietary status of the animal. Adipose tissue from fasted animals had a greater rate of cycling when incubated in the absence of hormones or with insulin, and a decreased rate in response to noradrenaline or noradrenaline plus insulin (table 5.1a). Similarly, fasting raised the basal rate of lipolysis and FFA release, thus increasing the efflux of FFA from the tissue under basal conditions, but reduced the lipolytic responsiveness to noradrenaline (table 5.1c,d). However, in spite of this lowered lipolytic responsiveness, the rate of FFA release from the tissue was unaffected by fasting (table 5.1d).

The increase in sensitivity that could be achieved by operation of the TG-FFA cycle (i.e. 1 + C/J) was also affected by dietary status. The sensitivity in the presence of noradrenaline or noradrenaline plus insulin was decreased by fasting (table 5.1b); this reflects the reduced rate of FFA reesterification in fasted adipose tissue. However, in the presence of insulin, the sensitivity of the cycle is increased due to a combination of a raised rate of lipolysis and no change in FFA release.

Refeeding the 48 hour-fasted rats was sufficient to return the rates of cycling in response to the various hormones to those found in the animals fed ad libitum (table 5.1a). Similarly, the sensitivity obtainable by TG-FFA cycling was also returned to the control level in the 24 hours-refed animals.

Alteration of the glucocorticoid status of the animal also affected the rate of TG-FFA cycling in incubated adipose tissue (table 5.2a). Cycling in the presence of noradrenaline or noradrenaline plus insulin was decreased by treating the rats with cortisol or with metyrapone. The decrease in the rate of cycling in adipose tissue from the cortisol-treated animals was due to an increased release of FFA from the tissue together with a decrease in the rate of lipolysis (tables 5.2c,d). The low rate of cycling

TABLE 5.2 EFFECT OF IN VIVO TREATMENT WITH CORTISOL AND METYRAPONE ON THE RESPONSE OF ADIPOSE TISSUE INCUBATED IN VITRO WITH HORMONES

a)

TG-FFA CYCLING (micromoles/g fat-free dry tissue/hour)				
HORMONES ADDED IN VITRO	NONE	INSULIN	NORADRENALINE	NORADRENALINE + INSULIN
TREATMENT IN VIVO				
Cortisol	131 ± 14 NS	174 ± 20 **	439 ± 30 ***	395 ± 36 **
Saline control	132 ± 20 NS	95.6 ± 12.3 NS	810 ± 64 *	930 ± 120 *
Metyrapone	137 ± 9 NS	80.1 ± 9.1 **	560 ± 64 NS	606 ± 61 *

Male Sprague-Dawley rats (average body weight 175g) were treated with either cortisol acetate (12.5mg/rat/day, 0.5ml i.p. for 3 days), or metyrapone (10mg/100g body weight/day, 0.5ml i.p. for 3 days). After being killed by stunning and cervical dislocation, epididymal fat pads were removed. Each pad was cut into two, and the four pieces were distributed randomly between the four hormone treatments. Incubations were for 1 hour with the following concentrations: glucose (present in all incubations), 10mM; insulin, 1mU/ml; noradrenaline, 10μM. Values are shown as mean +_ SEM (n=7 except for with metyranone when n=8). Bottom row of symbols show results of t-tests between cortisol and metyrapone-treated animals. NS = $p > 0.05$
* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

TABLE 5.2 b)

RATIO OF TG-FFA CYCLING TO FFA $\left(1 + \frac{C}{J}\right)$				
HORMONES ADDED IN VITRO	NONE	INSULIN	NORADRENALINE	NORADRENALINE + INSULIN
TREATMENT IN VIVO				
Cortisol	3.6 ± 0.9 *	4.7 ± 0.6 NS	2.0 ± 0.1 ***	2.5 ± 0.2 ***
Saline controls	24.1 ± 8.1 NS	5.4 ± 0.5 NS	4.0 ± 0.2 NS	4.8 ± 0.3 NS
Metyrapone	8.1 ± 1.5 *	6.8 ± 1.3 NS	4.0 ± 0.2 ***	4.1 ± 0.2 ***

Legend as for 5.2 a

TABLE 5.2 c)

GLYCEROL RELEASE INTO MEDIUM (micromoles/g fat-free dry tissue/hour)				
HORMONES ADDED IN VITRO	NONE	INSULIN	NORADRENALINE	NORADRENALINE + INSULIN
TREATMENT IN VIVO				
Cortisol	67.9 ± 4.7 *	76.4 ± 9.5 **	299 ± 17 NS	233 ± 24 *
Saline	47.6 ± 6.4 NS	39.2 ± 4.4 NS	364 ± 32 *	398 ± 54 *
Metyrapone	52.8 + 4.0 *	30.6 ± 2.6 **	249 ± 27 NS	267 ± 23 NS

Legend as for 5.2 a

TABLE 5.2 d)

FFA RELEASE INTO MEDIUM (micromoles/g fat-free dry tissue/hour)				
HORMONES ADDED IN VITRO	NONE	INSULIN	NORADRENALINE	NORADRENALINE + INSULIN
TREATMENT IN VIVO				
Cortisol	72.4 ± 13.3 **	55.2 ± 10.6 *	459 ± 39 **	304 ± 52 NS
Saline	10.6 ± 2.7 NS	22.1 ± 2.3 *	282 ± 34 *	263 ± 44 NS
Metyrapone	20.9 ± 4.6 **	11.8 ± 3.1 **	188 ± 19 ***	195 ± 11 NS

Legend as for 5.2 a

TABLE 5.3 EFFECT OF IN VIVO TREATMENT WITH TRIIODOTHYRONINE OR PROPYL THIOURACIL ON THE RESPONSE OF ADIPOSE TISSUE INCUBATED IN VITRO WITH HORMONES

a)

TG-FFA CYCLING (micromoles/g fat-free dry tissue/hour)				
HORMONES ADDED IN VITRO	NONE	INSULIN	NORADRENALINE	NORADRENALINE + INSULIN
TREATMENT IN VIVO				
Triiodothyronine	130 ± 17 NS	92.3 ± 10.1 NS	632 ± 56 NS	914 ± 127 NS
Saline control	156 ± 13 NS	86.5 ± 14.1 *	569 ± 77 NS	933 ± 60 NS
Propyl thiouracil	157 ± 14 NS	46.8 ± 10.7 **	652 ± 34 NS	925 ± 50 NS

Male Sprague-Dawley rats (average body weight 160g) were fed ad libitum on oxoid throughout the experiment. Rats were injected daily with either saline, triiodothyronine (65µg/100g body weight for 4 days, 0.5ml i.p.) or propyl thiouracil (1mg/100g body weight for 7 days, 0.5ml i.p.). After being killed by stunning and cervical dislocation, epididymal fat pads were removed, cut into two pieces and distributed randomly between the four hormone treatments. Tissue was incubated for an hour with 10mM glucose and 4% albumin. Concentrations of hormones used were: insulin, 1mU/ml; noradrenaline, 10µmolar. Values are shown as mean ± SEM (n=7 for controls, n=8 for other groups). Bottom row of symbols show results for t-tests between triiodothyronine and propyl thiouracil treated rats. NS = $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

TABLE 5.3 b)

RATIO OF CYCLING TO FFA RELEASE $\left(1 + \frac{C}{J}\right)$				
HORMONES ADDED IN VITRO	NONE	INSULIN	NORADRENALINE	NORADRENALINE + INSULIN
TREATMENT IN VIVO				
Triiodothyronine	3.4 ± 0.5 **	14.3 ± 3.2 *	3.3 ± 0.3 NS	5.3 ± 0.5 NS
Saline controls	7.7 ± 1.0 NS	5.1 ± 0.3 NS	3.1 ± 0.3 ***	5.8 ± 0.6 **
Propyl thiouracil	7.1 ± 2.0 NS	4.6 ± 0.4 *	5.4 ± 0.3 ***	9.2 ± 0.6 ***

Legend as for table 5.3 a

TABLE 5.3 c)

RATE OF GLYCEROL RELEASE (μ moles/g fat-free dry tissue/hour)				
HORMONES ADDED IN VITRO	NONE	INSULIN	NORADRENALINE	NORADRENALINE + INSULIN
TREATMENT IN VIVO				
Triiodothronine	67.3 ± 6.8 NS	33.5 ± 3.2 NS	309 ± 21.6 NS	377 ± 49 NS
Saline controls	62.4 ± 4.9 NS	35.7 ± 5.5 *	281 ± 31 NS	379 ± 18.8 NS
Propyl thiouracil	64.9 ± 5.2 NS	21.7 ± 3.5 *	268 ± 14.1 NS	349 ± 22.3 NS

Legend as for table 5.3 a

TABLE 5.3 d)

RATE OF FFA RELEASE (μ moles/g fat-free dry tissue/hour)				
HORMONES ADDED IN VITRO	NONE	INSULIN	NORADRENALINE	NORADRENALINE + INSULIN
TREATMENT IN VIVO				
Triiodothyronine	71.7 ± 14.6 ***	8.1 ± 2.1 **	294 ± 27.1 NS	218 ± 30 NS
Saline controls	31.5 ± 10.1 NS	20.7 ± 2.5 NS	274 ± 26 **	203 ± 16 **
Propyl thiouracil	37.4 ± 6.5 ***	18.3 ± 2.1 **	153 ± 11.9 **	121 ± 20 *

Legend as for table 5.3 a

in the adipose tissue from the metyrapone-treated animals was due to the rate of lipolysis in the noradrenaline and noradrenaline plus insulin incubations being decreased. A reduced lipolytic response to noradrenaline or noradrenaline plus insulin is therefore common to both cortisol- and metyrapone-treated tissues. However, in contrast to the cortisol-treated tissue (from which FFA release was increased), metyrapone treatment decreased the release of FFA from adipose tissue (table 5.2d). This difference between the effects of cortisol and metyrapone accounts for the observation that the TG-FFA cycle is decreased in sensitivity by cortisol treatment whereas metyrapone treatment does not change the value of 1 + C/J (table 5.2b).

Making rats hyper- or hypothyroid had little or no effect on the rate of TG-FFA cycling in incubated fat pads (table 5.3a). Similarly, alteration of thyroid status did not affect the rate of lipolysis in response to a variety of hormone treatments (table 5.3c). However, in contrast to the lack of effect on cycling and lipolysis, the rate of FFA release was changed by triiodothyronine- or PTU-treatment (table 5.3d). Even though these changes in the rate of FFA release did not appear to be at the expense of a significant alteration in the rate of FFA reesterification (table 5.3a), they were large enough to alter the ratio of C/J (table 5.3b). The ratio of C/J implies that hyperthyroid tissue under basal conditions has a lowered sensitivity of release of FFA, whereas hypothyroid tissue when stimulated by noradrenaline has an increased sensitivity of control of FFA release.

5.4 Discussion

The experiments reported in this chapter indicate that the rate of TG-FFA cycling in incubated adipose tissue can vary according

to the dietary and hormonal status of the animal. Because the role of adipose tissue is as a fuel store, it must be able to respond, both anabolically and catabolically, to the FFA requirements of the animal. Two mechanistic possibilities for changing FFA flux are available to adipose tissue; alterations may be made in the rate of lipolysis or esterification. Thus for example, in order to increase the stores of triglyceride, either the rate of lipolysis could be inhibited or esterification could be enhanced (or both). Conversely, mobilisation of FFA could be achieved by stimulation of lipolysis and/or inhibition of FFA reesterification. Three chronic in vivo treatments which are known to affect the metabolism of fat in adipose tissue were investigated in this chapter. The results from these experiments show that the animal uses modulation of both lipolysis and FFA reesterification in the chronic control of FFA metabolism in adipose tissue.

In adipose tissue from fasted animals, the enhanced rate of FFA release is achieved by an increase in the rate of lipolysis (table 5.4). The reesterification of FFA is also increased under these conditions, but the net balance between lipolysis and re-esterification gives an increased rate of FFA release. Thus an enhanced outflow of FFA from adipose tissue is achieved as a result of stimulation of lipolysis only. This mechanism is also found in tissue from cortisol-treated animals when incubated under basal conditions or with insulin (table 5.4). Similarly, inhibition of lipolysis appears to be the primary mechanism for decreasing FFA release in response to noradrenaline in fat pads from metyrapone-treated animals.

In contrast to the modulation of lipolysis being responsible for controlling the rate of FFA release from the tissue, the release of FFA from fat pads taken from cortisol-treated animals and incubated

TABLE 5.4 SUMMARY OF CHANGES IN FFA FLUX DUE TO ALTERATION OF DIETARY, GLUCOCORTICOID AND THYROID STATE

a) INCUBATIONS UNDER BASAL CONDITIONS

TREATMENT IN VIVO	FFA RELEASE	RATE CYCLING	GLYCEROL RELEASE	SENSITIVITY $1 + C/J$
Fasted	↑	↑	↑	-
Fasted-refed	-	↑	↑	-
Cortisol	↑	-	↑	↓
Metyrapone	-	-	-	-
Triiodothyronine	↑	-	-	↓
Propylthiouracil	-	-	-	-

The qualitative effects of each of the treatments used in vivo are summarized in this table. Results for adipose tissue incubated under basal conditions are summarized in table a); incubations with insulin are shown in table b) and with noradrenaline in table c).

Statistically significant results are shown as follows; ↑ indicates an increase due to the treatment (as compared to the control), ↓ indicates a decrease, and - indicates no change.

Results from tables 5.1 to 5.3

TABLE 5.4 (cont'd)

b) INCUBATIONS WITH INSULIN

TREATMENT IN VIVO	FFA RELEASE	RATE CYCLING	GLYCEROL RELEASE	SENSITIVITY $1 + C/J$
Fasted	-	↑	↑	↑
Fasted-refed	-	-	-	-
Cortisol	↑	↑	↑	-
Metyrapone	↓	-	-	-
Triiodothyronine	↓	-	-	↑
Propylthiouracil	-	↓	↓	-

c) INCUBATIONS WITH NORADRENALINE

TREATMENT IN VIVO	FFA RELEASE	RATE CYCLING	GLYCEROL RELEASE	SENSITIVITY $1 + C/J$
Fasted	-	↓	↓	↓
Fasted-refed	-	-	-	-
Cortisol	↑	↓	-	↓
Metyrapone	↓	↓	↓	-
Triiodothyronine		-	-	-
Propythiouracil	↓	-	-	↑

with noradrenaline is enhanced simply by a reduction in the rate of FFA reesterification (table 5.4). This is consistent with the results of Jeanrenaud(1967) who concluded that the increased rate of FFA release from adipose tissue from glucocorticoid-treated animals was solely due to a reduction in glucose metabolism decreasing the rate of FFA reesterification.

During fasting, FFA released from adipose tissue are, directly or indirectly via ketone bodies, the major fuel supply for the animal (see Newsholme & Start, 1973; Hales et al., 1978). At times of demand for FFA, it might be expected that the ratio of C/J would be increased in order to increase the sensitivity of control of FFA release. In fact, in most of the incubations of fasted adipose tissue there is a decrease in the ratio of C/J, a situation which at first sight may appear to be disadvantageous to the animal. However, the decrease in the ratio of C/J in fact means that the TG-FFA has already done its job. Thus it has enabled the tissue to give a greater release of FFA (for a given submaximal stimulus) than would have been possible if the cycle did not operate, and this increase in FFA release inevitably leads to a decrease in the ratio of C/J. The only incubation in which tissue from fasted animals exhibits an increase in the ratio of C/J is that in the presence of insulin. As may be seen from table 5.1c, fasting greatly diminishes the antilipolytic action of insulin on adipose tisssue, and thus insulin is less able to inhibit fatty acid mobilisation. However, the high sensitivity of the TG-FFA cycle under these conditions would seem to ensure that FFA mobilisation will be dependent on the cellular (and blood) FFA levels and thus the FFA requirements of the animal.

CHAPTER SIX

Measurement of the rate of TG-FFA cycling in vivo

6.1 Introduction

In chapter 3 it was shown that an estimate of the rate of TG-FFA cycling could be obtained by measuring the incorporation of tritium from tritiated water into both the glycerol and fatty acid moieties of triglyceride. The experiments reported in chapter 3 were carried out using pieces of epididymal fat pad that were incubated *in vitro*; this method can also be applied to the measurement of the cycle *in vivo*. It would be possible to estimate the rate of TG-FFA cycling by measuring the rates of release of glycerol and FFA from adipose tissue, but this would require cannulation of the blood supply to and from the fat depot and would probably require the animal to be anaesthetised. Similarly, use of ^{14}C-labelled glucose to measure the rate of cycling would require knowledge of the specific activity of the glucose in the plasma - this would involve frequent blood samples, a process which would require restraints on the animal. The advantage of the tritiated water method of measuring the rate of TG-FFA cycling is that the experiments can be performed on the intact, conscious, unrestrained animal.

In this chapter, the effects of a selection of treatments on the rate of TG-FFA cycling in white adipose tissue are reported. The treatments used include the variation of the dietary status of the animal, beta agonist and antagonist drugs, alteration of thyroid status, glucocorticoid treatment, diabetes, cold exposure, and investigations into the effects of various forms of obesity (genetic, cafeteria feeding and goldthioglucose-induced). In addition, the rate of TG-FFA cycling in brown adipose tissue is estimated under a selection of the above treatments. The chapter is formatted such that

related treatments are grouped together, each group forming a section of the chapter. Each of these sections is then sub-divided into an introduction, results and discussion.

6.2 Effect of dietary status on the rate of TG-FFA cycling in vivo

6.2.1 Introduction

The transition from the fasted to the fed state requires a reversal in the direction of flux of many of the metabolic processes in an animal. Adipose tissue, for example, changes from net triglyceride hydrolysis to net triglyceride synthesis. As discussed in chapter one, substrate cycles provide a mechanism by which the direction of flux can easily be reversed. In addition, they increase the sensitivity of control of non-equilibrium reactions, thus reducing the magnitude of the change in the concentration of regulator (e.g. hormone or substrate) required to stimulate the flux through a pathway (see chapter one). It is possible that the TG-FFA cycle could be stimulated during feeding in order to increase the sensitivity to changes in the concentration of pathway substrates.

Recently, much evidence has accumulated which shows that the activity of the sympathetic nervous system is increased by feeding (for a review see Young & Landsberg, 1977). This evidence includes the turnover of noradrenaline in the heart, pancreas and liver (Young & Landsberg, 1976, 1977a), increased urinary excretion of noradrenaline and its metabolites (Herrera et al., 1969) and haemodynamic changes (Vatner et al., 1970, 1970a; Young & Landsberg, 1977). As yet, however, it has not been demonstrated that sympathetic tone (i.e. the level of activity of the sympathetic nervous system) is altered in white adipose tissue of the fed animal. The experiments with fat cells and fat pads *in vitro* showed that noradrenaline stimulated the rate of TG-FFA cycling, and that this increase could be blocked using the

beta-blocking drug propranolol. Hence, in view of these results, the effect of administering a beta agonist to an intact animal, and also the effects of propranolol on fed animals, were studied. These experiments will show if an increase in adrenergic stimulation in adipose tissue has the same effect on the rate of the TG-FFA cycle as does noradrenaline on adipose tissue incubated *in vitro*, and also if feeding is able to alter the level of sympathetic tone in white adipose tissue *in vivo*.

As discussed in chapter 3, one method for estimating the rate of TG-FFA cycling involves the measurement of the rate of triglyceride synthesis. This method (the synthesis method) requires the assumption that for every triglyceride-glycerol moiety formed, three FFA are esterified. These FFA may be derived from one of three main sources; *de novo* synthesis by the adipocyte, lipolysis of endogenous triglyceride, or from lipolysis of exogenous triglyceride via the enzyme lipoprotein lipase. The first of these processes (fatty acid synthesis) can easily be measured, and the second represents the rate of the TG-FFA cycle. The rate of this cycle can only be estimated if the supply of fatty acids from the third potential source is known, and this requires a knowledge of the activity of lipoprotein lipase. Hence the metabolism of lipoproteins will be briefly discussed.

Lipoproteins in the blood are an extremely important means for the transport of large amounts of triglyceride between the various tissues of the body. Both the small intestine and the liver are able to release lipoproteins into the bloodstream. The triglyceride is then removed by the action of the enzymes lipoprotein lipase and phospholipase which are present in the target tissue. Lipoprotein lipase is known to reside extracellularly, probably in association with the endothelial cells of the capillary walls. The extracellular hydrolysis of the lipoprotein triglyceride probably results in a

high local concentration of FFA, which then diffuse through the capillary wall into the target cell for oxidation or esterification (see Robinson et al., 1975; Goldstein & Brown, 1976; Havel et al., 1980 for reviews on lipoprotein metabolism).

In the fed state, both the liver and the small intestine release lipoproteins into the blood, the substrate necessary for the synthesis of the triglyceride being supplied by the food ingested. However, in the fasted state, the inflow from the small intestine is no longer important and the liver is the major source of lipoprotein synthesis. Under these conditions, adipose tissue releases FFA into the blood and these are the main source of the fatty acids used by the liver for triglyceride synthesis (see Recknagel, 1967).

Once the lipoprotein is released into the blood, it can only be used by those tissues that possess an active lipoprotein lipase. The activity of lipoprotein lipase in various tissues is known to respond to the metabolic status of the animal. In the fed state, the activity of lipoprotein lipase is high in adipose tissue, but this activity is decreased by fasting (Spencer et al., 1978; Ashby & Robinson, 1980). In contrast, fasting increases the activity of lipoprotein lipase in muscle (Hollenberg, 1960; Borensztajn et al., 1970; Kotlar & Borensztajn, 1977). These alterations ensure that storage of food in fat depots occurs during conditions of plenty, and that during fasting the flux of triglyceride is directed towards other tissues. A good illustration of the action of lipoprotein lipase in directing lipoproteins towards a target tissue is the increased activity of the enzyme in the lactating mammary gland (Otway & Robinson 1968; Hamosh et al., 1970). However, as yet the quantitative importance of lipoproteins in providing fuel to the tissues of the body (as compared to say FFA, glucose and ketone bodies) is not yet clearly established.

As mentioned above, the influx of FFA into the adipocyte due to

the action of lipoprotein lipase must be known if the rate of TG-FFA cycling is to be investigated. In adipose tissue, the activity of lipoprotein lipase is decreased by fasting (see above). Hence, it was decided to investigate the fraction of the total FFA esterified in adipose tissue of fed animals that was derived from blood triglyceride. The direct measurement of the influx of FFA from the hydrolysis of plasma lipoprotein would be technically difficult since it would require the radiolabelling of blood triglycerides and knowledge of their specific activity. However, if the lipoprotein lipase enzyme was inactivated, then this influx of FFA would by negligible. The activity of lipoprotein lipase may be lowered by administration of either heparin or Triton WR-1339, and the relative merits of the two methods will now be compared.

The injection of heparin into the blood stream causes the dissociation of the lipoprotein lipase molecule from its point of attachment to the capillary endothelial cell. Thus adipose tissue from a heparin-treated animal has a lower lipoprotein lipase activity than tissue from a non-treated animal. However, the disadvantage of this method is that the enzyme is still present in the blood and is still able to hydrolyse the lipoprotein triglyceride, thus releasing FFA and raising the plasma FFA concentration; injection of heparin in fact lowers the blood triglyceride concentration (Kjekshus et al., 1980; see also Olivecrona et al., 1977, for a review on heparin-lipoprotein lipase interactions). Thus with use of heparin it is still possible that considerable rates of FFA uptake into the adipocyte will occur.

The second method of preventing the action of lipoprotein lipase is by using Triton WR-1339, which is a non-ionic surface-active agent. The intravenous injection of this compound leads to a dramatic increase in the concentration of triglyceride in the blood, due to the action

of Triton in modifying the physical state of the lipoprotein substrate, thus inhibiting the action of lipoprotein lipase. Triton does not have any effect on the rate of release of lipoproteins into the blood, and does not cause any histological damage to the liver, lungs, intestines, heart, aorta and adrenal glands. Thus, administration of triton to the live animal is apparently a method which inhibits the uptake of lipoprotein from the blood but does not lead to tissue damage (see Recknagel, 1967, for a discussion on triton WR-1339).

6.2.2 Outline of method

The basis of the method was to inject tritiated water into the animal and then kill it an hour later. The fat pad under investigation was dissected out and the triglyceride extracted from it. After saponification of the triglyceride, the radioactivity in the fatty acid and glycerol moieties was determined (see chapter 2 for full details). Hence, provided the specific activity of the tritiated water inside the adipocyte is known, it is possible to calculate the rate of TG-FFA cycling using the synthesis method of estimating cycling (see chapter 3).

Measurement of the specific activity of tritiated water inside the fat cell would be very difficult because of the problems of actually obtaining a sample of intracellular water. Hence it was assumed to be the same as that of the plasma. This assumption requires the rapid equilibration of the injected tritiated water with the total body water of the animal. Steinberg et al. (1976) found that after intraperitoneal injection of tritiated water, the specific activity of the plasma water rapidly increased to reach a peak by 6 minutes; thereafter the specific radioactivity declined by about 20% in the next 10 minutes and subsequently remained constant. Hence in the equilibration of intraperitoneally-injected water with the body water (and thus

presumably with adipocyte intracellular water) should be complete. This fairly rapid mixing of the tritiated water implies that the error in assuming that the specific activity of tritium in the adipocyte is constant over an hour will be small.

In the experiments investigating the effect of dietary status on the rate of TG-FFA cycling, the mice used were meal-feed trained. This was done in order to be sure that all the mice would be in the same dietary state. If the animals were not meal-feed trained, a large variation in dietary state could be observed since some animals might have just eaten whereas others may have eaten several hours earlier. Another reason for meal-feed training is that it ensures that the mice will feed at a known time and in daylight (usually they eat most of their food at night). Thus problems such as the mice not eating during the experimental period, and the need to set up a reverse-light cycle environment, are avoided.

An hour after the start of meal-feeding, the mice were injected with either saline (or Triton/saline) containing tritiated water. The injections were given an hour after the start of feeding (as opposed to at the start of feeding) so as to allow the digestion of the meal to get under-way. The dosing of Triton has to be as an intravenous injection since it must reach the bloodstram quickly in order to have effects within the duration of the experiment (1 hr).

6.2.3 Results and discussion

Triton WR-1339 did not have a statistically significant effect on the rate of triglyceride or fatty acid synthesis in the parametrial fat pads of fed mice (table 6.1). However, it did raise the serum lipoprotein concentration by 400%, which indicates that it inhibited the uptake of triglyceride(see later). From the data in table 6.1, the source of the fatty acids required to support the synthesis of

Table 6.1 Effect of triton on triglyceride-glycerol synthesis as measured by tritiated water incorporation into mouse parametrial fat pad

In vivo treatment	Rates micromoles/g fat-free dry tissue/hour		
	TG-FFA cycling	Fatty acid synthesis	Triglyceride -glycerol synthesis
CONTROL Meal fed Saline injected	470 ± 21	577 ± 27	349 ± 13
	NS	NS	NS
TEST Meal fed Triton injected	410 ± 23	570 ± 22	327 ± 10

Female CFLP mice (average body weight 27g on day of experiment) were trained to meal-feed between 10:00 and 14:00 hours each day. At 11:00 hours on the day of the experiment they were injected through the tail vein with 0.1 ml of either saline or saline/triton WR-1339 (15 mg/mouse). Each injection contained 2 mCi tritiated water. An hour after injection the animals were killed by stunning and neck-bleeding. Blood was centrifuged and serum triglyceride and tritium levels were determined. Serum triglyceride levels were 80 ± 4 and 382 ± 24 mg triglyceride/ 100 ml serum for the control and test treatments respectively. Parametrial fat pads were excised for triglyceride extraction. Calculations assume 3.3 tritium atoms incorporated per triglyceride-glycerol. Results are given as mean ± SEM (N=10). Results for t-tests between the two treatments are indicated by the following symbols: NS = $p > 0.05$

*** = $p < 0.001$

triglyceride can be calculated by using the observed rate of fatty acid synthesis and the Triton-inhibited change in triglyceride synthesis. Thus 55% of the fatty acids which are esterified in the meal-fed animal are synthesised de novo and only about 6% come from blood lipoprotein. Hence about 40% of the FFA required for triglyceride synthesis is apparently derived from the hydrolysis of endogenous triglyceride, and this represents a rate of TG-FFA cycling of approximately 37 micromoles FFA reesterified per gram fresh tissue per hour.

The dramatic increase in the serum triglyceride concentration that occurs on Triton treatment is due to inhibition of the uptake of lipoproteins from the blood (see above). Since in the fed animal a large proportion of the lipoprotein triglyceride is taken up and esterified in adipose tissue, the effect of treatment with Triton on the adipose tissue of a fed animal would be expected to be a decrease in the rate of triglyceride synthesis. However, as mentioned above, there was no statistically significant decrease in the synthesis of triglyceride in adipose tissue; the mean rate of triglyceride synthesis was only decreased by about 22 micromoles TG-glycerol/g fat-free dry tissue/hour (see table 6.1). To determine whether these results are compatable, it can be calculated whether the decrease in the mean rate of FFA esterification can account for the accumulation of triglyceride in the blood. If certain assumptions are made, then it is possible to calculate the decrease in the synthesis of triglyceride that would be expected on the basis of the observed accumulation of lipid in the blood.

It is assumed that the average 30 gram mouse containes 3 ml of blood and 3g of adipose tissue (Rogers & Webb, 1980), and that the average triglyceride molecule has a molecular weight of about 800 (tripalmitate). It can then be calculated that the increase of 300 mg triglyceride/100 ml blood observed in this experiment

corresponds to the build-up of about 11 micromoles of triglyceride in the blood of the Triton-treated mouse. Assuming that this triglycer de was stored solely in adipose tissue then the increase in blood lipid corresponds to a decreased uptake by adipose tissue of about 3 micromoles of triglyceride/g fresh tissue/hour (equivalent to 30-40 micromoles triglyceride/g fat-free dry tissue/hour). Hence the effect of Triton on the synthesis of triglyceride-glycerol (i.e. 22 micromoles triglyceride/g fat-free dry tissue/hour) compares very well with the change expected (30-40 micromoles TG/g fat-free dry tissue/hour) on the basis of the increase in blood triglyceride.

In this experiment the effect of Triton on triglyceride-glycerol synthesis in adipose tissue was not statistically significant. In spite of this, Triton treatment was used in some of the experiments comparing meal-fed and fasted animals.

An experiment investigating the effect of feeding showed that meal-feeding lead to a doubling in the rate of TG-FFA cycling in white adipose tissue (table 6.2). Over 95% of the FFA required to support this synthesis of triglyceride was derived from the lipolysis of endogenous triglyceride. The increase in the rate of cycling was reduced to the fasting level by administration of propranolol (table 6.3). The simplest interpretation of these results is that there is a level of adrenergic 'tone' in the adipose tissue of the fed animal, this tone being responsible for the increased rate of TG-FFA cycling observed under these conditions. Certainly, the activity of the sympathetic nervous system is known to be decreased by fasting (Young & Landsberg, 1981). However, it is of interest to note that propranolol treatment has also reduced the rate of fatty acid synthesis in the fed animal by 95% (table 6.3). This result is difficult to reconcile with the idea that propranolol is acting solely in adipose tissue to reduce the rate of TG-FFA cycling, since catecholamines are

Table 6.2 Effect of feeding on rate of TG-FFA cycle in mouse parametrial fat pad

In vivo treatment	Rates micromoles/g fat-free dry tissue/hour		
	TG-FFA cycling	Fatty acid synthesis	Triglyceride -glycerol synthesis
Meal fed Triton injected	427 ± 42	335 ± 56	254 ±20
	***	***	***
44 hour fasted Triton injected	210 ± 25	5.2 ± 0.8	72 ± 9
	NS	NS	NS
44 hour fasted Saline injected	201 ± 26	4.3 ± 0.5	68 ± 9
	***	***	***

Female CFLP mice were trained to meal-feed between 10:00 and 14:00 hours each day. At 11:00 on the day of the experiment they were injected through the tail vein with 0.1 ml of either saline or saline/triton WR-1339. Each injection contained 2 mCi tritiated water. Average body weight of animals was 34g measured before the meal on day of experiment; triton was dosed at 15 mg/mouse. An hour after injection the animals were killed by stunning and neck-bleeding. Blood was centrifuged and serum triglyceride levels and tritium specific activity were determined (see methods). Parametrial fat pads were excised for triglyceride extraction. Serum triglyceride levels were 354 ± 56, 243 ± 19 and 55 ± 2 mg triglyceride /100 ml serum for meal-fed triton injected, fasted triton injected and fasted saline injected treatments respectively. Results for t-tests between treatments immediately above and below each symbol (except for the bottom row which are for tests between fed triton-injected and fasted saline-injected groups) are indicated by the following symbols: NS = $p > 0.05$
Results are given as mean ± SEM (N=9). *** = $p < 0.001$

Table 6.3 Effect of propranolol on the rate of TG-FFA cycling in the parametrial fat pad of fed mice

In vivo treatment	Rates micromoles/g fat-free dry tissue/hour		
	TG-FFA cycling	Fatty acid synthesis	Triglyceride -glycerol synthesis
CONTROL Fed Saline injected	459 ± 62	146 ± 31	201 ± 30
	**	**	**
TEST Fed Propranolol injected	210 ± 12	8.1 ± 2.3	73 ± 4
	**	*	NS
CONTROL 24 hour fasted Saline injected	245 ± 21	2.7 ± 0.2	83 ± 7
	**	**	**

Female CFLP mice (average body weight 29g before feeding on day of experiment) were fed ad libitum on oxoid pellets. They were deprived of food for 24 hours before the experiment. At 10:00 on the day of the experiment they were fed oxoid, and forty minutes after the start of feeding they were injected with either saline or dl-propranolol (0.1 ml, 20 mg/kg i.p.). After one hour of feeding they were injected with a triton WR-1339/saline/tritiated water mixture (2 mCi tritiated water and 15 mg triton/mouse), and the animals were killed by stunning and neck bleeding 60 minutes later. Parametrial fat pads were excised for triglyceride extraction. Calculations assume 3.3 tritium atoms incorporated per triglyceride-glycerol. Results are given as mean ± SEM (N=10). Results for t-tests between treatments immediately above and below each symbol (except for the bottom row which is for tests between fed and fasted controls) are indicated by the following symbols: NS = $p > 0.05$

* = $p < 0.05$

** = $p < 0.01$

known to inhibit fatty acid synthesis rather than stimulate it (see for example Halperin & Denton, 1969). It seems likely that the effects of propranolol on adipose tissue of the fed mouse are due to an indirect effect as well as a direct beta-blocking effect on the tissue. This is probably because propranolol is known to inhibit the secretion of insulin from the pancreas (Ashmore, 1970; Wright et al., 1979; there is some doubt, however, as to whether this is a specific beta-blocking effect since the effects of d- and dl-propranolol on insulin release are the same, suggesting that propranolol might be acting as an anaesthetic - see Myers & Hope-Gill, 1979; Furman & Tayo, 1974); a decrease in insulin secretion could explain the low rates of fatty acid synthesis in the propranolol-treated fed mice. It is possible that the decreased concentration of insulin could also limit the availability of carbon precursor within the adipocyte, thus making the concentration of glycerol phosphate a limiting factor for the esterification of FFA. This would therefore explain the decreased rate of TG-FFA cycling. The effects of adrenergic drugs in the intact animal, and the difficulty in interpreting them due to their interactions with the secretion on insulin, are discussed further in section 6.7.

In order to investigate the effects of adrenergic stimulation in white adipose tissue (thus mimicking an increase in sympathetic tone in the tissue), mice were treated with the beta agonist fenoterol. Since the experiments using tritiated water require that the animal is subjected to the treatment under investigation for an hour, it was not possible to use an injection of noradrenaline itself to investigate the action of catecholamines. This is because noradrenaline in the blood is very quickly metabolised, thus making it difficult to study a long term <u>in vivo</u> effect. The problem can be circumvented by the use of drugs which are metabolised much more slowly, and fenoterol

Table 6.4 Effect of fenoterol (a beta agonist) and propranolol (a beta blocker) on the rate of TG-FFA cycling in parametrial fat pads of fasted mice

In vivo treatment	Rates micromoles/g fat-free dry tissue/hour		
	TG-FFA cycling	Fatty acid synthesis	Triglyceride -glycerol synthesis
TEST Fenoterol injected	1238 ± 53	85 ± 12	441 ± 20
	***	***	***
CONTROL Saline injected	243 ± 25	6.4 ± 0.4	83 ± 8
	**	*	*
TEST Propranolol injected	371 ± 43	27 ± 8	133 ± 17
	***	*	***

Female CFLP mice (average body weight of 23g on day of experiment) were trained to meal-feed between 11:00 and 15:00 hours each day. On day of experiment, mice were not fed and were injected at about 11:00 with either saline, fenoterol (50 mg/kg, i.p.) or dl-propranolol (20 mg/kg, i.p.) each injection containing 2 mCi tritiated water. An hour later they were killed by stunning and neck-bleeding. Parametrial fat pads were excised for triglyceride extraction. Calculations assume 3.3 tritium atoms per triglyceride-glycerol moiety. Results are given as mean ± SEM. Results for t-tests between treatments immediately above and below each symbol (except for bottom row which are for tests between fenoterol and propranolol treated animals) are indicated by the following symbols:

* = $p < 0.05$

** = $p < 0.01$

*** = $p < 0.001$

was chosen as a beta-agonist for this investigation. The mice were not fed on the day of the experiment since it was reasoned that any increase in the rate of TG-FFA cycling that was due to fenoterol would be more easily detectable if the rate of cycling in the animal was not already elevated.

Fenoterol treatment increased the rate of TG-FFA cycling to five times the control level in 20-hour fasted mice (table 6.4). The experiment therefore suggests similarities between the response of adipose tissue to adrenergic stimulation *in vivo* and *in vitro*. However, the complexity of the response to changes in the level of 'adrenergic tone' is again suggested by the observation that the rate of fatty acid synthesis in adipose tissue is also increased by the action of fenoterol (table 6.4), though the rate of fatty acid synthesis is still less than half of that seen in the fed mouse (tables 6.2 and 6.3). The effect of propranolol in the fasted animal appears anolamous in this experiment; it has increased the rates of triglyceride and fatty acid synthesis, an action which is opposite to that found in the fed animal. This might be due to an inhibitory action of propranolol on the release of insulin, thus lowering further the insulin levels and de-inhibiting lipolysis.

6.3 Effect of thyroid hormones and glucocorticoids on TG-FFA cycling *in vivo*

6.3.1 Introduction

Because of the known effects of thyroid hormones and glucocorticoids on the metabolism of adipose tissue (see chapter 5), the effect of alteration of thyroid and glucocorticoid status on the rate of TG-FFA cycling in white adipose tissue *in vivo* was investigated.

Table 6.5 Effect of thyroid status on the rate of TG-FFA cycling in parametrial fat pads from mice (5 days hormone injection, 20 hour fasted)

In vivo treatment	Rate micromoles/g fat-free dry tissue/hour		
	TG-FFA cycling	Fatty acid synthesis	Triglyceride -glycerol synthesis
HYPERTHYROID T-3 injected	271 ± 40	4.9 ± 0.4	92 ± 13
	*	NS	*
EUTHYROID Saline injected	477 ± 59	6.0 ± 0.4	161 ± 20
	NS	NS	NS
HYPOTHYROID PTU injected	466 ± 61	6.0 ± 0.4	157 ± 21
	*	NS	*

Female CFLP mice (average body weight 32.2g at start of hormone injections) were trained to meal feed between 10:00 and 14:00 hours, and this was maintained throughout the experiment. For five days before the experiment, they were injected (0.2 ml, i.p.) with either saline, triiodothyronine (50 µg/100g body weight) or propyl thiouracil (PTU, 3 mg/100g). On the sixth day, they were not allowed to eat, and were injected with tritiated water (0.1 ml, i.p., 2 mCi/mouse). An hour later the mice were killed by stunning and neck bleeding, and the parametrial fat pads were removed for fat extraction. Calculations assume 3.3 tritium atoms per triglyceride-glycerol moiety. Results are shown as mean ± SEM (N=10). Results for t-tests between treatments immediately above and below the symbols (except for the bottom row which is for tests between hyper- and hypothyroid animals) are indicated by the following symbols:

NS = $p > 0.05$

* = $p < 0.05$

Table 6.6 Effect of triiodothyronine injection on the rate of TG-FFA cycling in parametrial fat pads in mice (2 days hormone injection, meal fed animals)

In vivo treatment	Rates micromoles/g fat-free dry tissue/hour		
	TG-FFA cycling	Fatty acid synthesis	Triglyceride -glycerol synthesis
EUTHYROID Saline injected	777 ± 35	549 ± 52	440 ± 21
	NS	NS	NS
HYPERTHYROID T-3 injected	708 ± 68	472 ± 61	393 ± 37

Female CFLP mice (average body weight 31g at start of hormone injections) were trained to meal-feed between 10:00 and 14:00 hours each day, and this was maintained throughout the experiment. For two days before the experiment, they were injected (0.2 m l, i.p.) with either saline or triiodothyronine (50 µg/100g body weight). At the start of the third day they were allowed to feed as usual, and were injected with tritiated water (2 mCi, 0.1 ml i.p.) at 11:00 hours. An hour later they were killed by stunning and neck bleeding and the parametrial fat pad was excised for fat extraction. Calculations assume 3.3 tritium atoms per triglyceride-glycerol moiety synthesised. Results are shown as mean ± SEM (N=9 for euthyroid animals, N = 10 for hyperthyroid animals). Results for t-tests between the two treatments are indicated by the following symbols: NS = $p > 0.05$

Table 6.7 Effect of corticosteroid treatment on rate of TG-FFA cycling in parametrial fat pads in mice

In vivo treatment	Rates micromoles/g fat-free dry tissue/hour		
	TG-FFA cycling	Fatty acid synthesis	Triglyceride -glycerol synthesis
CONTROL Saline injected	171 ± 13	6.0 ± 0.9	59 ± 4
	NS	NS	NS
TEST Cortisol injected	194 ± 12	6.4 ± 0.5	67 ± 4

Female CFLP mice (average body weight 28.5g at start of cortisol treatment) were trained to meal-feed between 11:00 and 15:00 hours each day, and this feeding pattern was maintained throughout the experiment. Mice were injected daily at about 09:30 with either saline or cortisol-21-Na-succinate (0.1 ml i.p., 5 mg/100g) for two days. On the morning of the third day, mice were injected with the hormones as usual, but they were not allowed any feed. At 09:45 they were injected with tritiated water (0.1 ml i.p., 2 mCi/mouse) and an hour later they were killed by stunning and neck bleeding. Parametrial fat pads were removed for extraction of triglyceride. Calculations assume 3.3 tritium atoms per triglyceride-glycerol moiety. Results are shown as mean ± SEM (N = 10). Results for t-tests between the two treatments are indicated by the following symbols: NS = $p > 0.05$

6.3.2 Results

Treatment of mice with triiodothyronine for five days reduced the rate of TG-FFA cycling in adipose tissue (table 6.5); two days of triiodothyronine threatment did not have a statistically significant effect (table 6.6). Similarly, the rate of cycling was not changed in hypothyroid mice (table 6.5), or in mice treated with glucocorticoids (table 6.7).

6.3.3 Discussion

The parametrial fat pad from mice treated with triiodothyronine for five days was small and very red in colour due to the depletion of the triglyceride droplet (Lewis & Hayes, 1978). It is possible that the decreased rate of TG-FFA cycling observed in these mice could have been due to the lack of triglyceride for use as substrate for the cycle. Treatment of mice with triiodothyronine for only two days did not affect the rate of TG-FFA cycling (table 6.6). Hence it appears that the TG-FFA cycle in white adipose tissue does not contribute to the increased metabolic rate in hyperthyroid animals (see chapter 5). Perhaps the decrease in the rate of FFA reesterification reflects the outward flux of FFA from the fat pad in order to provide fuel for the tissues that are responsible for the increased metabolic rate in these animals.

Adipose tissue taken from animals treated with glucocorticoids has an increased rate of FFA release (see chapter 5). It has been suggested that this is because of a reduced rate of FFA reesterification (Shafrir & Kerpel, 1964; Jeanrenaud, 1967; Chernick et al., 1972). However, the data in table 6.7 show that glucocorticoid treatment did not affect the rate of TG-FFA cycling in mice. However, there is an objection to this experiment, since the corticosteroid administered, cortisol-Na-21-succinate, is a very water soluble

form of the hormone, and it is possible that the injection was quickly metabolised by the animal. Corticosteroids need several hours in which to act and it is possible that the cortisol levels were not raised for long enough for them to exert their effects. The experiment requires repeating using a less soluble form of the hormone such as cortisol acetate or a drug such as dexamethasone, together with the measurement of another factor known to be affected by glucocorticoid treatment (for example the increase in activity of hepatic phosphoenolpyruvate carboxykinase).

6.4 Effect of cold treatment on the rate of TG-FFA cycling in vivo

6.4.1 Introduction

In view of the thermogenic properties of substrate cycles, there has been much speculation on the possible role of the TG-FFA cycle in adipose tissue as a mechanism for producing heat (Ball & Jungas, 1961, 1964; Ikemoto et al., 1969; Sestoft, 1980). Hence it was of interest to use the tritiated water technique to measure the rate of TG-FFA cycling in cold-treated animals. The mice used in these experiments were fed ad libitum since they need to have a constant supply of food in order to maintain body temperature. Food was removed ten hours before tritiated water administration so as to have all the animals in the same dietary state.

6.4.2 Results and discussion

Neither long nor short term cold exposure had any effect on the rate of TG-FFA cycling in mouse parametrial fat tissue (table 6.8). There is perhaps a slight increase in the rate of cycling in the four day cold-exposed animals, but this is not statistically significant. It is clear that the TG-FFA cycle in white adipose tissue is not an important thermogenic mechanism during cold exposure.

Table 6.8 Effect of short and long-term cold exposure on rate of TG-FFA cycling in parametrial fat pads

In vivo treatment	Rates micromoles/g fat-free dry tissue/hour		
	TG-FFA cycling	Fatty acid synthesis	Triglyceride -glycerol synthesis
TEST 4 hour cold exposed	289 ± 16	3.6 ± 0.3	97 ± 5
	NS	NS	NS
CONTROL Room temperature	288 ± 20	4.5 ± 0.4	97 ± 7
	NS	NS	NS
TEST 4 day cold exposed	334 ± 20	5.0 ± 0.4	133 ± 7

Female CFLP mice (average body weight 30.9g four days before death) were fed *ad libitum* on powdered oxoid. Control mice were kept at room temperature (20°C) and the test mice were kept at 12-15°C for either four hours or four days before tritiated water injection. Food was removed 10 hours before administration of tritiated water, which was injected i.p. (2 mCi/mouse). An hour after tritium injection the mice were killed by stunning and neck bleeding, and the parametrial fat pads were removed for triglyceride extraction. Calculations assume 3.3 tritium atoms incorporated per triglyceride-glycerol moiety. Results are given as mean ± SEM (N = 10). Results for t-tests between treatments immediately above and below the symbol are indicated by the following symbols: NS = $p > 0.05$

6.5 Obesity and TG-FFA cycling in vivo

6.5.1 Introduction

One of the challenges in bioenergetics is to determine how it is that apparently similar individuals can maintain body weight balance in spite of widely differing food intakes. There are several reports of pairs of individuals of the same weight and with similar levels of physical activity for which one individual eats twice as much as the other (see Miller, 1979; Sims et al., 1973). This variability in individual food requirement whilst maintaining a constant body weight must be due to differences in the efficiency of food utilisation. The challenge mentioned above is to identify the factors and tissues which control the overall efficiency of energy utilisation in the intact animal.

Over the last few years, it has become increasingly apparent that the activity of the sympathetic nervous system plays a major role in the control of the metabolic rate of an animal (see for example Young & Landsberg, 1977, 1981; Rothwell & Stock, 1979; Girardier & Seydoux, 1981). A difference in the degree of sympathetic activity (sympathetic 'tone') in two animals would therefore lead to one animal being more efficient and thus being able to store more food as fat than the other animal. However, as yet it has not been shown that a low level of sympathetic tone is responsible for a propensity to obesity. Indeed, even if a low level of sympathetic tone is responsible for causing obesity, it is still not known which tissues and/or mechanisms are responsible for the differences in metabolic rate between lean and obese animals.

It has been suggested that differences in the rate of substrate cycling could be responsible for the differences in metabolic rate between lean and obese animals (Newsholme & Crabtree, 1976). It was shown in section 6.2 that injection of a beta-agonist into mice

(thus mimicking an increase in sympathetic activity) increased the rate of TG-FFA cycling. Although the TG-FFA cycle accounts for only a small part of the metabolic rate of a mouse (see chapter 7), it is possible that the sympathetic nervous system could stimulate several substrate cycles in a variety of tissue, thus causing a large change in metabolic rate.

Another suggestion for the metabolic lesion which leads to obesity is a lack of responsivemess of brown adipose tissue (Rothwell & Stock, 1979; Girardier & Seydoux, 1981). This tissue is so called because of its high lipid content and brown colour, but it is structurally, metabolically and functionally distinct from white adipose tissue. The role of this tissue is as a heat-generating system - it is particularly prevalent in hibernating mammals. The production of heat in brown adipose tissue is thought to be controlled by the activity of the sympathetic nervous system (Heim & Hull, 1966, 1966a; Hull & Segall, 1965; Himms-Hagen, 1976; Foster & Frydman, 1977; Young & Landsberg, 1981; Shimazu, 1981). Release of noradrenaline at the nerve ending leads to activation of the triglyceride lipase via the cAMP-dependent protein kinase system (see Nicholls, 1977). Surprisingly, and in contrast to white adipose tissue, this does not increase the concentration of FFA within the brown adipocyte due to the high concentrations of FFA seen in the absence of noradrenaline (Bieber et al., 1975).

Various suggestions have been put forward for the heat-producing process in brown adipose tissue, including substrate cycles and ion-pumping across the cell membrane, but it is now generally accepted that heat production is achieved by the uncoupling of oxidative phosphorylation (see Newsholme & Start, 1973; Nicholls, 1977). This is due to the brown adipose tissue mitochondrion having a unique uncoupling mechanism in the form of a high-conductance ion uniport

through which protons can cross the inner membrane. This uniport in the mitochondral membrane thus dissipates the proton gradient across the membrane without any concomitant synthesis of ATP, the energy instead being released as heat (for a review see Nicholls, 1977). The conductance of this pathway is thought to be inhibited by the binding of purine nucleotides to a site on the outer face of the inner mitochondrial membrane which has a high affinity for purine nucleotides (see Heaton et al., 1978). However, it is not yet known what the stimulus is that causes the removal of the purine nucleotides from this binding site or indeed if such removal is necessary for the activation of heat production in brown adipose tissue.

It was decided to use the tritiated water method to investigate the TG-FFA in different models of obesity. Thus it was hoped to see if the rate of TG-FFA cycling was different in lean and obese animals. At the same time, it was hoped to obtain some indication of the level of sympathetic tone in both brown and white adipose tissue of obese and lean animals by determining the amount of propranolol-sensitive TG-FFA cycling. The use of the tritiated water technique for measuring the TG-FFA cycle in brown adipose involves several assumptions, and these are discussed in the next section. However, before these assumptions are given, the models of obesity that were used in this thesis will be introduced.

There are several models that are commonly in use for investigations into the problem of obesity. These include the genetically obese strains of rats and mice (Bray & York, 1971) or mice which are obese due to lesioning of the ventro-medial hypothalamus (either physically or chemically using goldthioglucose - see Bray & York, 1979; Liebelt & Perry, 1967), or the so-called 'cafeteria-fed' animal. The latter involves giving the animal a wide variety of palatable foods, which leads to over-eating and obesity (Rothwell & Stock, 1979). Cafeteria

feeding is perhaps the best model for the study of the increase in obesity in 'Western Man', since obesity in both cases involves over-eating (Rothwell & Stock, 1979). However, as mentioned above, certain individuals are able to store unusually large quantities of fat even when they are on a comparatively low food intake, and an insight into the reasons for this effect is more likely to be gained from the study of the various strains of genetically obese animals that are available (see Bray & York, 1971).

Treatment of mice with goldthioglucose (GTG) is known to destroy the ventro-medial hypothalamus (VMH) - see Liebelt & Perry, 1967. This region of the brain has connections with sympathetic nervous system centres in the brain stem and it is known to participate in the control of feeding behaviour (Ban, 1975; Lytle, 1977). The VMH is also known to be a regulatory centre for lipolysis in brown and white adipose tissues, these effects being mediated by the sympathetic innervation of the tissues (Shimazu & Takahashi, 1980; Shimazu, 1981; Seydoux et al., 1981).

It is possible that the obesity caused by GTG treatment is a result of the damage to the VMH leading to a decrease in sympathetic nervous activity in the animal, thus decreasing energy expenditure. Even though GTG is known to destroy the VMH, it is not clear as to what effect it has on the activity of the sympathetic nervous system. Based on studies into the lipolytic response of adipose tissue after a variety of treatments, Bray & York (1979) have concluded that sympathetic activity is decreased by destruction of the VMH. On the other hand, by following the turnover of tritiated noradrenaline in the heart, Young & Landsberg (1980) have shown that the decrease in sympathetic activity normally associated with fasting is not seen in GTG-treated animals; thus sympathetic activity is raised in both the fed and the fasted states. It therefore appears that destruction of

the VMH can increase or decrease sympathetic activity. However, Young & Landsberg's work was done in the heart, and they accept that it is conceivable that sympathetic outflow to adipose tissue and other areas may be affected differently from the heart. Hence the effects of feeding on the activity of the TG-FFA cycle in brown and white adipose tissue were investigated in order to see if there were any changes in the propranolol-sensitive rate of cycling in these tissues.

6.5.2 Use of $^{3}H_2O$ to measure TG-FFA cycling in brown adipose tissue

There is evidence which suggests that the rate of the TG-FFA cycle is increased in noradrenaline-stimulated brown adipose tissue. In scapular brown fat, both *in vivo* and *in vitro* the response to noradrenaline is an increase in the incorporation of ^{14}C-glucose into triglyceride-glycerol, this change not being accompanied by any increase in fatty acid synthesis (Steiner & Cahill, 1964; Himms-Hagen, 1965; Steiner et al., 1968; Knight & Myant, 1970). It has been estimated that the re-synthesis of triglyceride could only account for at most 1% of the oxygen consumption of brown fat incubated *in vitro* (Lindberg et al., 1967; Knight & Myant, 1970). However, due to the problem of having to know the specific activity of the glycerol phosphate used for triglyceride synthesis inside the brown fat cell, it has not been possible to measure the rate of TG-FFA cycling by using the ^{14}C-glucose method *in vivo*. Hence using ^{14}C-glucose it has only been possible to look at the qualitative effects of cold treatment on the synthesis of triglyceride. It is possible that the increased incorporation of ^{14}C-glucose into triglyceride-glycerol that was observed *in vivo* in the cold-treated animals does not in fact represent an increased rate of triglyceride synthesis, but rather an increase in the proportion of glycerol

phosphate that is derived from blood glucose (and thus radiolabelled) as opposed to being derived from endogenous glycogen or from glycerol released in lipolysis. Hence it was of interest to use tritiated water to follow the incorporation of tritium into the triglyceride-glycerol moiety in the brown adipose tissue of mice. From this it should be possible to observe (qualitatively if not quantitatively) any changes in triglyceride synthesis in response to _in vivo_ treatments. Also, it should be possible to compare tritium incorporation in obese and lean animals, and to compare the levels of propranolol-sensitive tritium incorporation. Thus it may be possible to gain an insight into the level of sympathetic activity in this tissue in the live, unharmed animal, and to do this both in obese and lean animals. Therefore in the experiments with the obese animals, both white and brown adipose tissue depots were removed from the tritiated-water treated mice.

In the experiments in which the incorporation of tritium into the glyceride-glycerol moiety in brown adipose tissue was measured, the results have been presented as a rate of triglyceride synthesis (rather than a rate of tritium incorporation) from which a rate of TG-FFA cycling has been derived. However, it should be stressed that no studies on the validity of using the tritiated water method of measuring triglyceride synthesis have been done in brown adipose tissue. This was largely because one of the major problems confronting the study of the metabolism of brown adipose tissue is the lack of a viable _in vitro_ preparation. Hence it was not possible to compare the incorporation of tritiated water and ^{14}C-glucose in the way that was done in white adipose tissue. Therefore, in the experiments with brown adipose tissue it has been assumed that 3.3 tritium atoms are incorporated per triglyceride-glycerol moiety synthesised; this

value is that found for triglyceride synthesis in white adipose tissue. As discussed in chapter 3, for 3.3 tritium atoms to be incorporated per triglyceride-glycerol, the aldolase and triose phosphate isomerase reactions need to be close to equilibrium. The author is not aware of any reports on the equilibrium nature of these enzymes in brown adipose tissue, and these results assume that the enzymes are at equilibrium.

Brown adipose tissue possesses an active glycerol kinase (see Knight & Myant, 1970). Thus glycerol phosphate needed for the synthesis of triglyceride might be derived from the glycerol released by triglyceride hydrolysis within the brown fat cell. Any glycerol phsophate derived from this source will only contain 3.3 tritium atoms if the glycerol phosphate dehydrogenase, triose phosphate isomerase, aldolase, fructose-1,6-diphosphate to fructose-6-phosphate conversion and phosphoglucosisomerase reactions are all close to equilibrium (see chapter 4, section 4.3). The penultimate condition is very unlikely, which means that glycerol will probably contain a maximum of three tritium atoms. Nevertheless, in the calculations for cycling in brown fat, it has been assumed that each glycerol phosphate used in triglyceride synthesis contains 3.3 tritium atoms. The rate of triglyceride-glycerol synthesis shown will therefore represent an estimate of the minimum possible rate, and thus also a minimum rate of cycling in this tissue.

As mentioned above, it was proposed to use the degree of propranolol-sensitive TG-FFA cycling in brown adipose tissue (as estimated by tritium incorporation) as an indicator of adrenergic tone in brown adipose tissue. A useful test of this method would be to study the rate of cycling in brown adipose tissue under conditions in which adrenergic tone in brown adipose tissue is known to be increased; it was decided to use cold treatment of the animals.

There is little doubt that brown adipose tissue has a major role as a thermogenic tissue in the cold-stressed animal (Heim & Hull, 1966, 1966a; Lindberg, 1970; Foster & Frydman, 1977; Cannon & Johansson, 1980). The increased heat production in brown adipose tissue is as a result of stimulation by the sympathetic nerves in the tissue (Heim & Hull, 1966a); denervation of the tissue or injection with propranolol prevents the stimulation of brown adipose tissue by cold (Hull & Segall, 1965; Heim & Hull, 1966a). It is known that both during incubation of brown adipose tissue with noradrenaline *in vitro* and during cold exposure *in vivo* there is an increase in the rate of triglyceride synthesis (without an increase in fatty acid synthesis) in brown adipose tissue (Steiner & Cahill, 1964; Himms-Hagen, 1965; Steiner et al., 1968; Knight & Myant, 1970). These reports indicate that the rate of TG-FFA cycling in brown fat is increased in response to cold treatment, which suggests that this treatment is a good test for the use of the tritiated water technique in brown adipose tissue. The results are shown in table 6.9.

The experiment confirms the observation made earlier that cold treatment has little or no effect on the rate of TG-FFA cycling in parametrial (white) adipose tissue (table 6.9a; section 6.4). However, the rate of TG-FFA cycling in brown adipose tissue was doubled by cold exposure (table 6.9b); this increased rate of cycling was returned to the control level by administration of propranolol. Treatment of mice (at room temperature) with fenoterol nearly tripled the rate of TG-FFA cycling in brown adipose tissue (table 6.9b). These observations support the idea that the rate of TG-FFA cycling in brown adipose tissue is stimulated by the action of catecholamines. Thus it appears that the degree of propranolol-sensitive TG-FFA cycling in brown and white adipose tissue can be used as a qualitative indicator of the adrenergic tone in these two tissues.

Table 6.9a Rates of TG-FFA cycling in white adipose tissue in response to cold exposure and treatment with fenoterol

In vivo treatment	Rates micromoles/g wet tissue/hour		
	TG-FFA cycling	Fatty acid synthesis	Triglyceride -glycerol synthesis
CONTROL	4.6 ± 1.2	0.18 ± 0.08	1.6 ± 0.4
	NS	NS	NS
COLD	6.4 ± 0.5	0.17 ± 0.01	2.2 ± 0.2
	*	NS	*
COLD + PROPRANOLOL	4.2 ± 0.6	0.15 ± 0.04	1.45 ± 0.2
FENOTEROL	31.7 ± 3.6	0.79 ± 0.12	10.8 ± 1.2
	***	**	***

Female CD1 mice (27-33g) fed ad libitum were fasted from 08:00 hours on the day of the experiment. At 09:30 they were injected with tritiated water (2 mCi i.p.) containing, where necessary, dl-propranolol (20 mg/kg) or fenoterol (50 mg/kg). The cold-treated animals were then put into a cold room (4°C). An hour after tritium injection the mice were killed by stunning and neck bleeding. The parametrial white adipose tissue and the inter-scapular brown adipose tissue were removed for triglyceride extraction. Calculations assume 3.3 tritium atoms incorporated per triglyceride-glycerol moiety. Results are given as mean ± SEM (N =7 for controls, N = 6 for fenoterol and N = 8 for the cold treated). Results for t-tests between treatments immediately above and below the symbol (except in the bottom row which are between control and fenoterol treated groups) are indicated by the following symbols: NS = $p > 0.05$

* = $p < 0.05$

** = $p < 0.01$

*** = $p < 0.001$

Table 9.6b Rates of TG-FFA cycling in brown adipose tissue in response to cold exposure and treatment with fenoterol

In vivo treatment	Rates micromoles/g wet tissue/hour		
	TG-FFA cycling	Fatty acid synthesis	Triglyceride -glycerol synthesis
CONTROL	46.2 ± 5.4	2.31 ± 0.33	16.2 ± 1.9
	*	NS	*
COLD	96.2 ± 19.3	1.63 ± 0.23	32.6 ± 6.4
	*	NS	*
COLD + FENOTEROL	42.9 ± 9.1	1.11 ± 0.2	14.7 ± 3.1
FENOTEROL	131 ± 26	2.60 ± 0.29	44.4 ± 8.6
	*	NS	*

For legend see table 9.6a

6.5.3 Results and discussion

ob/ob mice

The first model of obesity studied was the genetically-obese (ob/ob) mouse. The results presented in table 6.10b show that there is no difference in the incorporation of tritium into triglyceride-glycerol in brown fat of lean and obese (ob/ob) mice. Also, in both lean and obese animals, there is no effect of propranolol on the rate of tritium incorporation. Triglyceride synthesis is known to be increased both *in vivo* and *in vitro* in response to noradrenaline (see above). It would therefore seem that there is no appreciable sympathetic tone in the brown adipose tissue of fed lean and ob/ob mice. This therefore suggests that an increase in sympathetic tone in brown adipose tissue is not important in producing the thermic effect of food (since these animals were fed).

Obese mice white adipose tissue showed a much higher (seven-fold) rate of fatty acid synthesis than did their lean littermates (see table 6.10a). The results expressed here are per gram of fat-free dry tissue; if the results are expressed as per gram of wet tissue, then the obese mice still have a four-fold greater rate of fatty acid synthesis in parametrial fat. This high rate of fatty acid synthesis is not due to the obese mice eating more, since the food eaten by each group of mice was similar (the lean mice ate 7 grams of oxoid on the day of the experiment and the obese ate 6.8 grams). In view of the greater quantity of fat present in the obese mouse, when the whole animal is considered it is clear that the obese mouse has a much enhanced rate of fatty acid synthesis in white adipose tissue.

The rate of triglyceride-glycerol synthesis in parametrial adipose tissue was twice as high in obese mice than in lean mice (table 6.10a). This enhanced rate of triglyceride synthesis is not

Table 6.10a Effect of propranolol on the rate of TG-FFA cycling in parametrial adipose tissue of fed lean and genetically obese mice

In vivo treatment	Rates micromoles/g fat-free dry tissue/hour		
	TG-FFA cycling	Fatty acid synthesis	Triglyceride -glycerol synthesis
LEAN MICE Saline injected	257 ± 21	35 ± 12	97 ± 10
	NS	*	*
LEAN MICE Propranolol injected	202 ± 16	11 ± 1	71 ± 5
	**	***	***
OBESE MICE Propranolol injected	301 ± 22	192 ± 27	165 ± 16
	NS	NS	NS
OBESE MICE Saline injected	312 ± 21	245 ± 22	186 ± 10
	NS	***	***

The obese mice used in this experiment were the ob/ob strain set on the C57 Black background. Their lean littermates(ob/+) were used as controls. For a week before the experiment, they were fed powdered (as opposed to caked) oxoid in order to measure food intake. At 17:00 hours on the day before the experiment, the mice were deprived of food. On the day of the experiment (before feeding) the mice were weighed; average body weight of lean mice was 15.4g; obese mice, 19.1g. At 09:30 hours they were fed powdered oxoid, and half an hour later they were injected with either saline or dl-propranolol (0.1 ml i.p., 20 mg/kg of average body weight). Forty minutes later, mice were tail-injected with triton WR-1339 in saline (0.1 ml i.v., 7.7 mg triton per mouse) containing 2 mCi tritiated water. An hour later they were killed by stunning and neck bleeding. The parametrial white fat and

(cont'd over)

Table 6.10b Effect of propranolol on the rate of TG-FFA cycling‡ in scapular brown adipose tissue of fed lean and genetically obese mice

In vivo treatment	Rates micromoles/g fat-free dry tissue/hour		
	TG-FFA cycling	Fatty acid synthesis	Triglyceride -glycerol synthesis
LEAN Saline injected	230 ± 28	63 ± 10	98 ± 11
	NS	NS	NS
LEAN Propranolol injected	210 ± 31	42 ± 6	84 ± 11
	NS	*	NS
OBESE Propranolol injected	181 ± 13	24 ± 3	68 ± 5
	NS	*	NS
OBESE Saline injected	187 ± 20	38 ± 5	75 ± 7

(cont'd from previous table - 6.10a)
scapular brown fat depots were removed for extraction of triglyceride. Calculations assume 3.3 tritium atoms incorporated per triglyceride-glycerol moiety. Results are shown as mean ± SEM (N = 8). Bottom row of symbols are the results of t-tests between saline-injected lean and obese mice, and are indicated by: NS = $p > 0.05$
* = $p < 0.05$
*** = $p < 0.001$

‡ See section 6.5.2 for assumptions necessary in measuring cycling in brown adipose tissue.

accompanied by a statistically significant increase in the TG-FFA cycle, because the rate of fatty acid synthesis is also increased in the obese mice. Nevertheless, the rate of the TG-FFA cycle is, if anything, greater in the obese mice than in the lean mic. However, the level of adrenergic tone in the obese mice is apparently lower than in the lean animals, since there is a significant difference between the two groups of propranolol-treated animals. In this experiment the effect of propranolol on the rate of cycling is not as marked as in the experiment shown in table 6.3; neither in the lean nor the obese animals did propranolol have a statistically significant effect. Why this should be is not clear; certainly the rates of fatty acid synthesis seen in this experiment are lower than those usually observed, and these differences may have something to do with the fact that the mice used in this experiment are younger than those used in the other experiments. Another difference between this experiment and earlier ones is that in this experiment the animals were not meal-feed trained. It is possible that such training produces a greater response of the sympathetic nervous system to food than is seen in animals fed ad libitum.

Cafeteria animals

The second model used to investigate the effect of obesity on the rate of TG-FFA cycling was the cafeteria-fed animal. A large number of mice were fed either oxoid pellets or a cafeteria diet. When the latter animals were significantly heavier than their oxoid-fed controls, the highest and lowest weight gain animals from each diet were selected out. The level of sympathetic tone in each group of animals was then estimated by observing the effect of propranolol treatment on the rate of TG-FFA cycling in white fat tissue, and the rate of tritium incorporation into triglyceride-glycerol in

brown adipose tissue.

The changes in body weight in the cafeteria and oxoid-fed animals are shown in table 6.11. The highest weight-gain cafeteria mice gained nearly twice as much weight as the highest weight-gain oxoid animals. In contrast, the lowest weight-gain mice in the cafeteria group put on exactly the same amount of weight as did the low weight-gain controls. Hence the phenomenon reported by Rothwell & Stock (1979), which they claim is not simply due to the low weight gain cafeteria animals not eating the cafeteria food, is also seen here. (However, it should be stressed that in this experiment no attempt was made to measure the food intake of high and low-weight gain-groups. Hence the possibility that the differences in weight-gain represents a difference in food intake cannot be excluded).

The results from the experiment are divided into two tables; table 6.12 shows the results observed in white adipose tissue, and table 6.13 the results for brown adipose tissue. Only in the white adipose tissue of the high weight-gain cafeteria animals does propranolol cause a statistically significant decrease in the rate of TG-FFA cycling. In the low weight-gain mice's parametrial fat, propranolol actually causes an increase in the rate of cycling - this is therefore similar to the efect seen in table 6.4. Tritium incorporation into triglyceride-glycerol in brown adipose tissue is not affected by propranolol in any of the animals (table 6.13).

Rothwell & Stock (1979) report that when their cafeteria-fed animals are taken off the cafeteria diet and put back onto an ordinary oxoid diet, they lose weight down to the levels of the control animals. They suggest that this is due to sympathetic activity causing the brown adipose tissue to produce heat. However, the experiments reported here do not support the idea the sympathetic activity in brown adipose tissue is maintained in the ex-cafeteria

Table 6.11 Starting body weights and changes in body weight over the three months of cafeteria or oxoid feeding

GROUP OF ANIMALS	Body weights at start of treatment (g)	Change in body weight over three months of feeding (g)
OXOID FED High weight-gain	26.3 ± 0.6	12.4 ± 0.4
	NS	***
OXOID FED Low weight-gain	25.1 ± 0.6	8.4 ± 0.4
	NS	NS
CAFETERIA FED Low weight-gain	26.0 ± 0.4	8.3 ± 0.4
	NS	***
CAFETERIA FED High weight-gain	26.4 ± 0.5	21.8 ± 1.0
	NS	***

See tables 6.12 and 6.13 for legend. Results shown here are mean ± SEM (N = 14).

Table 6.12a Effect of propranolol on rate of TG-FFA cycling in parametrial adipose tissue of overnight-fasted high and low body weight- gain oxoid-fed mice

In vivo treatment	Rates micromoles/g fat-free dry tissue/hour		
	TG-FFA cycling	Fatty acid synthesis	Triglyceride -glycerol synthesis
OXOID FED High weight-gain Saline injected	160 ± 15 (NS)	5.7 ± 1.7	55 ± 5
	NS	NS	NS
OXOID FED High weight-gain Propranolol injected	204 ± 24 (NS)	4.1 ± 0.4	69 ± 8
	NS	NS	NS
OXOID FED Low weight-gain Propranolol injected	254 ± 24 (*)	5.2 ± 2.3	86 ± 8
	*	NS	NS
OXOID FED Low weight-gain Saline injected	182 ± 22 (NS)	4.4 ± 1.0	62 ± 8
	NS	NS	NS

For three months female CFLP mice were fed ad libitum on either oxoid or a cafeteria diet consisting of, for example, chocolate, cakes, biscuits and meat plus access to oxoid. On the day before the experiment the animals that had gained the greatest and least body weight over the period of cafeteria or oxoid-feeding were selected out (14 high weight-gain and 14 low weight-gain selected from 50 animals

(cont'd over)

Table 6.12b Effect of propranolol on the rate of TG-FFA cycling in parametrial adipose tissue of overnight fasted high and low body weight-gain cafeteria-fed mice

In vivo treatment	Rates micromoles/g fat-free dry tissue/hour		
	TG-FFA cycling	Fatty acid synthesis	Triglyceride -glycerol synthesis
CAFETERIA FED High weight-gain Saline injected	197 ± 14	7.5 ± 1.0	68 ± 5
	*	NS	*
CAFETERIA FED High weight-gain Propranolol injected	148 ± 16	7.5 ± 0.9	52 ± 5
	NS	**	NS
CAFETERIA FED Low weight gain Propranolol injected	190 ± 12	3.4 ± 0.6	64 ± 4
	NS	NS	NS
CAFETERIA FED Low weight-gain Saline injected	183 ± 10	3.5 ± 0.4	62 ± 3
	NS	**	NS

(cont'd from previous table - 6.12a)

on each of the two diets. Average body weights and changes in body weight over the three months of feeding are shown in table 6.11. The mice were fasted from 17:00 hours on the day before the experiment. At 10:30 on the day of the experiment, half of each group of 14 animals were dosed with tritiated water (2 mCi in 0.1 ml saline, i.p.) or with tritiated water containing dl-propranolol (20 mg/kg). An hour after injection,

(cont'd over)

Table 6.13a Effect of propranolol on TG-FFA cycling± in brown fat of overnight-fasted high and low body weight-gain in oxoid-fed mice

In vivo treatment	Rates micromoles/g fat-free dry tissue/hour		
	TG-FFA cycling	Fatty acid synthesis	Triglyceride -glycerol synthesis
OXOID FED High weight-gain Saline injected	231 ± 25 (NS)	14.8 ± 1.0	82 ± 8
	NS	NS	NS
OXOID FED High weight-gain Propranolol injected	173 ± 22 (NS)	17.5 ± 2.8	63 ± 8
	NS	NS	NS
OXOID FED Low weight-gain Propranolol injected	152 ± 12 (NS)	15.0 ± 1.6	56 ± 4
	NS	NS	NS
OXOID FED Low weight-gain Saline injected	313 ± 80 (NS)	15.9 ± 3.0	110 ± 28
	NS	NS	NS

(cont'd from tables 6.12a and 6.12b)
animals were killed by stunning and neck-bleeding, and parametrial white fat and scapular brown fat dipots were excised for triglyceride extraction. Calculations assume 3.3 tritium atoms per triglyceride-glycerol moiety. Results are shown as mean ± SEM (N = 7). Symbols at side of cycling rates indicate results of t-tests between oxoid and cafeteria fed animals.

± See section 6.5.2 for assumptions made in measuring this parameter in brown adipose tissue.

(cont'd over)

Table 6.13b Effect of propranolol on TG-FFA cyling‡ in brown fat of overnight-fasted high and low body weight-gain cafeteria-fed mice

In vivo treatment	Rates micromoles/g fat-free dry tissue/hour		
	TG-FFA cycling	Fatty acid synthesis	Triglyceride -glycerol synthesis
CAFETERIA FED High weight-gain Saline injected	172 ± 11	14.8 ± 1.1	62 ± 4
	NS	NS	NS
CAFETERIA FED High weight-gain Propranolol injected	198 ± 23	17.1 ± 1.3	72 ± 7
	NS	***	NS
CAFETERIA FED Low weight-gain Propranolol injected	182 ± 18	9.9 ± 0.7	64 ± 6
	NS	NS	NS
CAFETERIA FED Low weight-gain Saline injected	204 ± 17	13.7 ± 1.6	73 ± 6
	NS	NS	NS

(cont'd from tables 6.12a, 6.12b and 6.13a)

Results for t-tests between treatments immediately above and below symbols (except for the bottom row which are for tests between saline-injected high and low weight-gain animals) are indicated by the following symbols:

NS = $p > 0.05$

* = $p < 0.05$

** = $p < 0.01$

*** = $p < 0.001$

‡ See section 6.5.2 for assumptions made in measuring this parameter in brown adipose tissue.

animals since propranolol did not decrease the rate of TG-FFA cycling in this tissue in ex-cafeteria animals (table 6.13). It is possible that the period of nearly a day of fasting before the injection of tritiated water could have lead to the reduction of sympathetic tone to basal levels. Recent work by Rothwell and Stock (1981) and Rowe et al., (1981), has suggested that insulin must be present for the maintenance of diet-induced thermogenesis. Perhaps a raised level of sympathetic activity would be detectable if the animals were in a fed state. If fed animals were to be used, they would have to be fed by using oral dosing - in this way it is known that both the cafeteria and control animals get the same amount of the same food-stuff. (The animals cannot be left to just feed ad libitum, because it is difficult to get the cafeteria animals to eat the same amount of the same food as the control animals; the cafeteria diet 'spoils' the mouse into expecting its more palatable food.)

Goldthioglucose mice

The third model of obesity that was investigated in this thesis was the use of VMH-lesioned animals; this was done using gold thioglucose. The rate of TG-FFA cycling in white adipose tissue was increased in fasted GTG-treated animals (table 6.14a). There was no effect of feeding on the rate of TG-FFA cycling in white adipose tissue, which suggests that there was no change in sympathetic activity in white adipose tissue when the GTG animals were fed. Unfortunately, the effect of propranolol on white adipose tissue is masked by a large variability in results; hence from these results it is not possible to say whether the level of sympathetic tone in white adipose tissue was high in the fasted state or was not stimulated by feeding.

Feeding increased the rate of TG-FFA cycling in brown adipose tissue in the control animals by a factor of two, but had no effect

Table 6.14a Effect of goldthioglucose treatment on the rate of TG-FFA cycling in white adipose tissue in response to feeding

In vivo treatment	Rates micromoles/g fat-free dry tissue/hour		
	TG-FFA cycling	Fatty acid synthesis	Triglyceride -glycerol synthesis
CONTROL fasted	234 ± 67	6.5 ± 1.3	80.1 ± 22.1
	NS	NS	NS
CONTROL fed	306 ± 41	9.3 ± 3.1	105 ± 14
	NS	NS	NS
CONTROL fed Propranolol injected	222 ± 29	12.8 ± 3.8	78.3 ± 9.4
	NS	NS	NS
GTG TREATED fasted	432 ± 37 (*)	7.1 ± 2.2 (NS)	146 ± 12 (*)
	NS	NS	NS
GTG TREATED fed	435 ± 47 (NS)	11.4 ± 4.9 (NS)	149 ± 17 (NS)
	NS	NS	NS
GTG TREATED fed Propranolol injected	1054 ± 543 (NS)	29.3 ± 10.1 (NS)	361 ± 184 (NS)
	NS	NS	NS

A batch of 100 female CD1 mice (20-22g) were divided into two lots, 27 mice for controls and 73 for GTG treatment. The latter were dosed with goldthioglucose (0.9g/kg i.p. in saline) and for the next five days were fed two small meals per day (if they have access ad libitum to food

(cont'd over)

Table 6.14b Effect of goldthioglucose treatment on the rate of TG-FFA cycling in brown adipose tissue in response to feeding

In vivo treatment	Rates micromoles/g fat-free dry tissue/hour		
	TG-FFA cycling	Fatty acid synthesis	Triglyceride -glycerol synthesis
CONTROL fasted	378 ± 107	15.7 ± 1.5	131 ± 36
	**	***	**
CONTROL fed	803 ± 87	57.6 ± 6.6	287 ± 29
	NS	NS	NS
CONTROL fed Propranolol injected	789 ± 142	61.6 ± 14	283 ± 50
	*	*	*
GTG TREATED fasted	414 ± 20 (NS)	15.4 ± 1.5 (NS)	143 ± 7 (NS)
	NS	*	NS
GTG TREATED fed	420 ± 58 (**)	40.7 ± 7.2 (NS)	153 ± 21 (**)
	NS	NS	NS
GTG TREATED fed Propranolol injected	324 ± 47 (*)	42.0 ± 7.8 (NS)	122 ± 16 (*)
	NS	*	NS

(cont'd from table 6.14a)
they eat massively and this appears to increase the mortality rate). After 5 days they were fed ad libitum. Three weeks after dosing, the GTG-treated animals that were obviously becoming obese were selected out and used for

(cont'd over)

Continuation of legend for tables 6.14a and b

(cont'd from table 6.14b)

the experiment. Mice were fasted from 17:00 hours on the day before the experiment. At 10:00 on the day of the experiment, the relevant cages were fed with oxoid pellets. Half an hour after the start of feeding, they were injected with tritiated water (1 mCi, i.p.) containing, where necessary, dl-propranolol (20 mg/kg). An hour later they were killed by stunning and neck bleeding, and parametrial white adipose tissue and interscapular brown adipose tissue were removed for triglyceride extraction. Calculations assume 3.3 tritium atoms per triglyceride-glycerol; results are given as mean ± SEM (N = 7). Symbols between the lines give results of t-tests between treatments immediately above and below the symbol (except for symbols below the fed + propranolol treatments which are for comparisons between fasted and fed + propranolol groups of the same batch of animals). Symbols beside the SEM values give results of t-tests between control and GTG-treated animals. Statistical significance is indicated by NS = $p > 0.05$

* = $p < 0.05$

** = $p < 0.01$

*** = $p < 0.001$

on cycling in the GTG-treated animals (table 6.14b). These results suggest that feeding increased sympathetic activity in brown adipose tissue of control animals but not in GTG-treated animals. As mentioned in the introduction, Young & Landsberg (1980) showed that the decrease in sympathetic activity that is usually seen when fed animals are fasted is no longer seen in GTG-treated animals. Hence the sympathetic tone in the fasted state was the same as in the fed state, and they concluded that the suppression of sympathetic tone during fasting was impaired by VMH-lesioning. However, the TG-FFA cycling results shown here suggest that the reason that GTG-treated animals have the same sympathetic tone in the fasted and fed state is that there is no increase in tone when these animals are fed (rather than there being no decrease when they are fasted). These results therefore support the conclusions of Bray & York, (1979) who concluded that sympathetic tone was decreased in VMH- lesioned animals. A reduction in sympathetic tone would lead to a lower metabolic rate; this decrease in tone could therefore be responsible for the obesity that is observed in VMH-lesioned animals.

6.6 The TG-FFA cycle in diabetic mice

6.6.1 Introduction

In juvenile-onset diabetes there is a deficiency of insulin in the plasma. This leads to an increase in the concentration of glucose in the blood, since the rate of gluconeogenesis is increased and the rate of glucose uptake by muscle and adipose tissue is decreased. Decreased insulin levels lead to an increase in lipolysis in adipose tissue, which in turn leads to an increase in plasma FFA. In view of the profound effects that a lack of insulin has on fat metabolism in the animal, it was of interest to determine whether diabetes had any effect on the TG-FFA cycle. Hence a group of mice was alloxan-

Table 6.15 Effect of diabetes on rate of TG-FFA cycling in mouse white and brown‡ adipose tissue

In vivo treatment	Rates micromoles/g fat-free dry tissue/hour		
	TG-FFA cycling‡	Fatty acid synthesis	Triglyceride -glycerol synthesis
PARAMETRIAL FAT			
Control	207 ± 19	5.4 ± 1.4	71 ± 6
	***	**	***
Diabetic	75 ±12	1.0 ± 0.1	25 ± 4
BROWN FAT			
Control	272 ± 42	15.3 ± 1.5	96 ± 14
	*	***	*
Diabetic	167 ± 16	3.1 ± 0.3	57 ± 6

Female CFLP mice were fed ad libitum. Six days before the experiment, mice were injected with either saline or alloxan (100mg/kg i.v.); average body weight at this time was 36.1g. Mice were fasted from 17:00 hours on the day before the experiment. On the day of the experiment, mice were injected with tritiated water (2 mCi in saline, 0.1 ml i.p.), and they were killed an hour later by stunning and neck bleeding. Parametrial white fat and scapular brown fat depots were excised for triglyceride extraction. Calculations assume 3.3 tritium atoms per triglyceride-glycerol moiety synthesised. Results are shown as mean ± SEM (N =14 for controls, N = 10 for diabetics). Results of t-tests between treatments immediately above and below symbols are indicated by the following symbols: * = $p < 0.05$

** = $p < 0.01$

*** = $p < 0.001$

‡ See section 6.5.2 for assumptions made for measuring this parameter in brown adipose tissue.

treated; this drug damages the islet cells of the pancreas, thus stopping insulin secretion and causing diabetes.

6.6.2 Results and discussion

Table 6.15 shows that the rate of TG-FFA cycling was decreased in both white and brown adipose tissue of the diabetic animal. It might be expected that the rate of cycling would increase in the diabetic animal, due to the mass-action effect of the increased concentrations of FFA on the esterification pathway (see chapter 4). However, it appears to be possible that, due to the decreased glucose uptake that is seen in the absence of insulin, the availability of glycerol phosphate inside the adipocyte is reduced to an extent such that reesterification of FFA is limited.

6.7 Discussion of treatments used in vivo

Of the seventeen treatments to which mice were subjected, eleven had no statistically significant effect on the rate of TG-FFA cycling in white adipose tissue *in vivo*, three increased the rate of cycling, and three decreased it (see table 6.16a). The treatments which increased cycling were feeding, injection with fenoterol, and goldthioglucose treatment. The treatments which decreased cycling in white adipose tissue were diabetes, hyperthyroidism, and propranolol in fed animals. In twelve of the seventeen treatments, the rate of TG-FFA cycling in brown adipose tissue was also estimated (table 6.16b). In this tissue, cold exposure, feeding and fenoterol injection all increased the rate of TG-FFA cycling, whereas goldthioglucose treatment in fed animals, diabetes, and propranolol injection into cold-exposed animals all decreased cycling. Thus it appears that the rate of TG-FFA cycling can vary in both brown and white adipose tissue *in vivo*.

From the results reported in this chapter, it is not possible to

Table 6.16a Summary of effects of treatments on the rate of TG-FFA cycling in parametrial (white) adipose tissue in vivo

TREATMENT IN VIVO AND DIETARY STATUS OF ANIMAL		EFFECT ON RATE OF TG-FFA CYCLING
Triton injection	-fasted	0
	-fed	0
Meal feeding	-fed	↑
Propranolol	-fasted	0
	-fed	↓
Fenoterol	-fasted	↑
Hyperthyroidism	-fasted	↓
Hypothyroidism	-fasted	0
Glucocorticoids	-fasted	0
4 hr cold exposure	-fasted	0
4 day cold exposure	-fasted	0
ob/ob	-fed	0
Cafeteria feeding	-fasted	0
Goldthioglucose	-fasted	↑
	-fed	0
(propranolol +)	-fed	0
Diabetes	-fasted	↓

Results are taken from tables 6.1 to 6.15. 0 indicates no effect due to treatment, ↑ an increase and ↓ a decrease in the rate of TG-FFA cycling.

Table 6.16b Summary of effects of treatments on the rate of TG-FFA cycling[‡] in interscapular (brown) adipose tissue in vivo

TREATMENT IN VIVO AND DIETARY STATUS OF ANIMAL		EFFECT ON RATE OF TG-FFA CYCLING[‡]
Feeding	-	↑
Propranolol	-fasted	0
	-fed	0
Short term cold	-fasted	↑
(propranolol +)	-fasted	↓
Fenoterol	-fasted	↑
ob/ob	-fed	0
Cafeteria feeding	-fasted	0
Goldthioglucose	-fasted	0
	-fed	↓
(propranolol +)	-fed	↓
Diabetes	-fasted	↓

‡ See section 6.5.2 for assumptions made in estimating the TG-FFA in brown adipose tissue by using the tritium incorporation method.

make any assessment of the effect of the treatments on the sensitivity of the control of FFA flux by TG-FFA cycling in adipose tissue. This is because the release or uptake of FFA into the adipose tissues was not measured, and hence it is not possible to quantify the ratio of cycling to flux. There seems little doubt that the rate of TG-FFA cycling in both white and brown adipose tissue is increased by feeding, and, at least in white adipose tissue, this increase can be blocked by propranolol (in the one experiment where cycling in brown adipose tissue was measured, propranolol failed to have a statistically significant effect - see table 6.14b; unfortunately this experiment was not repeated). The action of propranolol in reducing the rate of cycling suggests that adipose tissue in the fed animal is stimulated by catecholamines. As mentioned in chapter 4 section 2, fatty acid synthesis in adipose tissue incubated <u>in vitro</u> is inhibited by the action of catecholamines. Teleologically therefore, an increase in adrenergic stimulation of adipose tissue during feeding might not be expected since it would tend to inhibit the synthesis of fatty acids. However, it is possible that an increase in sympathetic tone in adipose tissue is required by the tissue in order to provide the necessary sensitivity of control for the esterification of fatty acids. There is no doubt that an increased rate of TG-FFA cycling in adipose tissue would increase the sensitivity of control of the FFA esterification process. The results obtained for adipose tissue incubated <u>in vitro</u> (chapter 4) show that insulin by itself decreases the rate of TG-FFA cycling; a combination of noradrenaline and insulin greatly stimulates cycling. Hence it is possible that the sympathetic activity in adipose tissue of fed animals has the function of increasing the sensitivity of control of FFA esterification. In addition, other substrate cycles (such as those involved in the metabolism of glucose to fatty acids; for example, glucose/ glucose-6-phosphate,

fructose-1,6-diphosphate/ fructose-6-phosphate) may be stimulated by the action of noradrenaline. In this respect it is of interest that not only did propranolol decrease TG-FFA cycling in adipose tissue, but it also inhibited the rate of fatty acid synthesis (table 6.3). However, as mentioned in section 6.3.3 and discussed further below, the action of propranolol on adipose tissue in fed animals has to be interpreted in the light of the effects of propranolol on insulin secretion. It would be of interest to repeat these experiments in animals in which the changes in the concentration of insulin were prevented - this could be done by the injection of insulin or by using an inhibitor of insulin secretion such as mannoheptulose.

The secretion of insulin from the pancreas is a process which is controlled, at least in part, by catecholamines (Ashmore, 1970; Wright et al., 1979). Alpha-adrenergic stimulation will inhibit insulin release, whilst beta-adrenergic stimulation increases insulin release from the mammalian islet. Thus administration of either a beta agonist or an alpha-blocker to the intact animal will increase plasma insulin (Ashmore, 1970). Hence the increased fatty acid synthesis observed when fenoterol was given to the mouse can be explained by the secretion of insulin from the pancreas. The increased levels of insulin may also raise the intracellular concentration of glycerol phosphate (by stimulating glucose transport into the adipocyte - see chapter 4) and thus increase the rate of TG-FFA cycling by enhancing the esterification of FFA. However, it is difficult to explain *why* propranolol should have an effect similar to that seen with fenoterol.

Propranolol treatment in fact had two effects; it increased the rate of TG-FFA cycling in the fasted animal, but decreased it in the fed animal (compare tables 6.3 and 6.4). Until recently, it was thought likely that the mobilisation of triglyceride and glycogen in

the fasted animal was due to the action of catecholamines (see Young & Landsberg, 1977). However, experiments in a number of tissues (though not in adipose tissue) have suggested that sympathetic activity is decreased by fasting and increased by feeding (Landsberg & Young, 1978; Young & Landsberg, 1979, 1980, 1981; Landsberg et al., 1980). The effect of propranolol in inhibiting the rate of TG-FFA cycling in the fed mouse can be explained on the basis of its antagonism of an increased level of sympathetic tone in this tissue. The fact that in the fasted mice propranolol did not decrease but instead increased the rate of cycling, suggests that sympathetic tone is low in adipose tissue of the fasted mouse.

In several of the experiments reported in this chapter, a comparison of the rate of TG-FFA cycling in control and propranolol-treated mice was used as a means of estimating 'sympathetic tone' in adipose tissue. For two reasons, this comparison can be taken as no more than an indicator of sympathetic tone. Firstly, the administration of propranolol to the intact animal will decrease the rate of insulin secretion. Hence the effects seen on adipose tissue are due to a combination of an injibition of any adrenergic activity, and a decrease in insulin concentration. Secondly, it is likely that there will not be a direct relationship between sympathetic tone in adipose tissue and the rate of the TG-FFA cycle in this tissue. This is because the rate of cycling will to some extent be dependent on the concentration of FFA in the tissue. This latter will vary not only in response to changes in sympathetic tone (and hence lipolysis), but also in response to changes in the blood flow through the adipose tissue depot and in the whole animal's demand for FFA. However, it does appear that determination of the degree of propranolol-sensitive rate of cycling gives some indication of the level of adrenergic stimulation in brown and white adipose tissue.

(An appraisal of the tritiated water method of measuring the rate of TG-FFA cycling in adipose tissue *in vivo* is given in appendix V).

CHAPTER SEVEN

General Discussion

7.1 Introduction

In chapter one the theoretical properties of substrate cycles were discussed. In subsequent chapters, the response of the TG-FFA cycle to a variety of treatments was investigated in a number of systems; incubated fat pads and isolated fat cells, and also *in vivo*. This chapter will attempt to integrate the various pieces of experimental work, with the aim of answering the questions raised by the theoretical considerations of the TG-FFA cycle in chapter one.

7.2 Contribution of the TG-FFA cycle to the metabolic rate

Much interest has surrounded the rate of FFA reesterification in view of speculation on the possibility of the cycle being responsible for such phenomena as the thermic effect of food, or as a contributor to the increase in metabolic rate seen in hyperthyroidism or cold-induced thermogenesis (see Ball & Jungas, 1961, 1964: Stirling & Stock, 1968; Prusiner & Poe, 1968; Sestoft, 1980). Since the TG-FFA cycle was first reported by Lebœuf et al. (1959), there have been several investigations into the rate at which the cycle operates. Most of these investigations were on fat pads or isolated fat cells incubated with different hormones (see for example Vaughan & Steinberg, 1963; Jungas & Ball, 1963; Hubbard & Matthew, 1971; Evans & Garratt, 1977). Ball (1965) calculated that if the maximum rate of TG-FFA cycling observed in fat pads *in vitro* were to occur *in vivo*, it would account for 15% of a rat's basal metabolic rate (BMR). He also calculated that if human adipose were capable of the same rate of reesterification as in rat adipose tissue, then the TG-FFA cycle would account for 100% of BMR in humans. Ball's calculations were extended

to other species by Baldwin (1970) who estimated that on average a value of about 12% of BMR could be accounted for by the TG-FFA cycle if maximally stimulated. From the results that were obtained in this thesis for the rate of FFA reesterification in fat pads incubated in vitro, it is possible to estimate the contribution of the TG-FFA cycle to the BMR of a rat and to compare this with Ball's estimate.

In table 7.1 the energy metabolism of the reactions involved in the TG-FFA cycle are considered. The complete breakdown and resynthesis of one triglyceride molecule costs the animal the hydrolysis of eight ATP molecules. Each micromole of FFA that is reesterified leads to a heat output of 0.2 Joules. Using this figure, together with the knowledge of the BMR of a rat and the maximum rate of TG-FFA cycling observed in fat pads incubated in vitro, it can be calculated that the TG-FFA cycle in adipose tissue could account for 4% of the basal metabolic rate of a rat (see table 7.2). This value is a quarter of that estimated by Ball (1965). Part of this difference is due to a difference in the maximum rate of TG-FFA cycling observed in incubated adipose tissue; Ball uses 68 whereas the author only observed 33 micromoles FFA reesterified/g fresh tissue/hour. This difference is probably due to differences in the incubation conditions - for example, the size of tissue incubated or the concentration of FFA in the incubation medium. The rest of this difference arises from the value assumed for the percentage contribution of adipose tissue to the body weight of the rat; Ball assumes 20% body fat whereas a value of 10% is probably closer to reality (see for example Harris et al., 1981).

It should be noted that the above calculations are based on the maximum rate of TG-FFA cycling observed in incubated adipose tissue

Table 7.1 Estimation of energy used in TG-FFA cycling (Baldwin, 1970)

Reaction	Tissue
Triglyceride $\longrightarrow$ 3FFA + Glycerol	Adipose
Glycerol + ATP + NAD^+ $\longrightarrow$ $\frac{1}{2}$ Glucose + ADP + NADH	Liver
$\frac{1}{2}$ Glucose + ATP + NADH $\longrightarrow$ Glycerol phosphate + ADP + NAD	Adipose
3FFA + 3ATP $\longrightarrow$ 3Fatty acyl CoA + 3AMP	Adipose
3AMP + 3ATP $\longrightarrow$ 6ADP	Adipose
Fatty acyl CoA + Glycerol phosphate $\longrightarrow$ Triglyceride	Adipose
OVERALL: 8ATP $\longrightarrow$ 8ADP + $8P_i$	

Therefore the release and reesterification of 3 FFA costs the animal the hydrolysis of eight ATP molecules.

The heat released by the hydrolysis of ATP in the cell is about 75 kJ/mole ATP hydrolysed (Newsholme & Start, 1973; Newsholme & Crabtree, 1976).

Therefore, for each micromole of FFA that is reesterified there is an enthalpy change of

$$\frac{8 \times -75 \times 1000}{3 \times 10^6}$$

$$= \underline{-\ 0.2 \text{ joules}}$$

Table 7.2 Maximal rate of TG-FFA cycling observed in vitro as a percentage of the basal metabolic rate (BMR) of a rat

BMR of a rat is 16 kJ/hour/kg body weight (Passmore & Robson, 1971)
Therefore the BMR of a 160g rat is 2600 J/hour

The maximum rate of TG-FFA cycling observed in incubated fat pads was 33 micromoles FFA reesterified/g fresh tissue/hour. (This was in the presence of glucose + noradrenaline + insulin).
The heat produced by this rate of cycling (table 7.1) = 33 x 0.2
= 6.6 joules/g fresh tissue /hour

Assuming that 10% of a rat is adipose tissue (i.e. 16g adipose tissue per 160g rat - see Harris et al., 1981) and that the metabolism of the epididymal fat pad used in the incubation is representative of all the adipose tissue of the rat, then the maximum rate of heat production that the TG-FFA cycle could account for is

$$\frac{10 \times 160 \times 6.6}{100}$$

= 106 J/rat/hour

As a percentage of the basal metabolic rate; this is

$$\frac{106 \times 100}{2600}$$

= 4.0% of the Basal Metabolic Rate of the rat

and the basal metabolic rate of a rat. It is unreasonable to suppose that the maximum rate of TG-FFA cycling in adipose tissue incubated *in vitro* will be representative of the rate of cycling occurring in the live animal under the conditions at which the BMR is determined. A better estimate of the contribution of the TG-FFA cycle to the BMR can be obtained using the results of experiments *in vivo* (see table 7.3). The calculations in this table are based on the rate of cycling observed in overnight-fasted animals, because out of the treatments used *in vivo* this most nearly approximates to the conditions under which the BMR are measured (i.e. resting in the post-absorptive state). Two methods of estimating the contribution of the cycle to the basal metabolic rate are used. The first, based of the heat produced by the hydrolysis of ATP, shows that the TG-FFA cycle in white adipose tissue can account for about 0.7% of the basal metabolic rate (table 7.3a). The second method, based on the oxygen consumption of an overnight-fasted mouse, shows that the cycle in white adipose tissue would account for about 0.5% of the oxygen consumption. This latter calculation is repeated for brown adipose tissue in table 7.3c; the TG-FFA cycle in this tissue can account for about 0.08% of the basal oxygen consumption of the animal. Thus in the overnight fasted mouse the TG-FFA cycle in brown and white adipose tissue can account for perhaps 1% of the oxygen consumption.

The author is anaware of any studies done *in vivo* in rats or mice with which the *in vivo* results given in this thesis may be compared. However, some work has been done on FFA and glycerol output using arteriovenous differences across the inguinal fat pad in anaesthetised dogs. Using this preparation, Vik-Mo & Mjøs (1978) found a rate of FFA reesterification in the overnight-fasted dog of 1.4 micromoles/g fresh tissue/hour. Assuming that dogs have 10%

Table 7.3 Percentage contribution of the TG-FFA cycle (as measured in vivo in fasted mice) to the basal metabolic rate

a) Calculations based on the energy released by ATP hydrolysis

BMR of a mouse is 31 kJ/kg body weight/hour (Passmore & Robson, 1971).
Therefore the BMR of a 30g mouse is 940 J/hour

Rate of TG-FFA cycling observed in fasted mice was 11 micromoles/g fresh tissue/hour (see section 6.3).
The heat produced by this rate of cycling (table 7.1) = 11 x 0.2

= 2.2 joules/g fresh tissue/hour

Assuming that a 30g mouse has 3g of white adipose tissue (Rogers & Webb, 1980) which behaves in a similar manner to that of the parametrial fat pad, then the rate of heat production in the adipose tissue of a mouse that the TG-FFA cycle could account for is

2.3 x 3

= 6.6 J/mouse/hour

Therefore in the fasted mouse the percentage of the basal metabolic rate accounted for by the TG-FFA cycle in white adipose tissue is

$$\frac{6.6 \times 100}{940}$$

= 0.7% of the Basal Metabolic Rate of the mouse

Table 7.3 Percentage contribution of the TG-FFA cycle (as measured in vivo in fasted mice) to the basal metabolic rate

b) Calculations based on the oxygen consumption of a mouse

Rate of oxygen consumption of an overnight-fasted mouse is 63 ml/hour (Arch, 1981) = 2.8 mmole O_2/hour

Assuming that 6 ATP molecules are produced per O_2 used, this represents a phosphorylation of 6 x 2.8

= 16.8 mmoles ATP/hour

The reesterification of one FFA molecule requires the hydrolysis of 8/3 ATP molecules (table 7.1).

The rate of TG-FFA cycling observed in fasted mice was 11 micromoles FFA reesterified/g fresh tissue/hour (section 6.3)

Therefore the TG-FFA cycle is responsible for the hydrolysis of

$$\frac{11 \times 8}{3}$$

= 29 micromoles ATP/g fresh tissue/hour

Assuming 3g white adipose tissue in a 30g mouse (Rogers & Webb, 1980) the TG-FFA cycle in white adipose tissue will account for

$$\frac{29 \times 3 \times 100}{16.8 \times 1000}$$

= 0.52% of the basal oxygen consumption of the mouse

Table 7.3 Percentage contribution of the TG-FFA cycle (as measured in vivo in fasted mice) to the basal metabolic rate

c) Calculations for brown fat

Rate of TG-FFA cycling in brown adipose tissue of overnight-fasted mouse is 46 micromoles FFA reesterified/g fresh tissue/hour (table 6.9b).

Assuming that brown adipose tissue accounts for 1% of the body weight of a mouse (this value for mice is assumed to be similar to that of the rat - see Foster & Frydman, 1977; Smith & Roberts, 1964) i.e. 0.3g brown adipose tissue in a 30g mouse, then the total rate of FFA reesterification in brown adipose tissue is

$$46 \times 0.3$$

$$= \underline{14 \text{ micromoles/mouse/hour}}$$

The TG-FFA cycle in brown adipose tissue will account for

$$\frac{14 \times 100}{16.8 \times 1000}$$

$$= \underline{0.08\% \text{ of the oxygen consumption of the mouse}}$$

(see table 7.3b)

of their body weight as adipose tissue, and a basal metabolic rate of 6.3 kJ/kg body weight/hour (Passmore & Robson, 1971), then the TG-FFA cycle could account for about 0.12% of the BMR of the dog. This value, obtained using the breakdown method of measuring the TG-FFA cycle, is similar to the value of 0.7% found by using the synthesis method in mice (table 7.3). The figure of 15% of the BMR that Ball (1965) estimated as due to the reesterification of FFA therefore appears to be too high by as much as 20-fold.

During feeding the oxygen consumption of a meal-feed trained mouse increases from about 85 to 135 ml O_2/30g mouse/hour (peak value, measured $1\frac{1}{2}$ hours after start of feeding - Arch, 1981). Feeding increases the rate of TG-FFA cycling in white adipose tissue by about 8 μmoles FFA reesterified/g fresh tissue/hour (see section 6.3) and in brown adipose tissue by about 50 μmoles FFA reesterified/g fresh tissue/hour (see table 6.14b). Using the assumptions given in table 7.3 for the amount of fat in a mouse, feeding therefore increases the rate of FFA reesterification in the brown plus white adipose tissue of a 30g mouse by 40 μmoles/hour. This increase in cycling therefore accounts for about 0.9% of the increase in oxygen consumption that is seen when a mouse is fed.

Fenoterol dosed at 50 mg/kg increased the rate of FFA reesterification in white adipose tissue by 65 and in brown adipose tissue by 85 μmoles/g fresh tissue/hour (section 6.4 and table 6.9b), an increase of about 220 μmoles FFA reesterified/mouse/hour. This dose of fenoterol will increase oxygen consumption by about 50 ml O_2/mouse/hour (Arch, 1981). The increased TG-FFA cycling in the brown and white adipose tissue of the mouse therefore accounts for about 3.5% of the increased oxygen consumption of the fenoterol-treated mouse.

The above calculations show that the TG-FFA cycle in adipose tissue accounts for only a very small part of the metabolic rate of a mouse. However, as mentioned in chapter one, this cycle is only one of many possible substrate cycles. The summation of each of these could add up to a significant fraction of the metabolic rate of the animal.

The specific dynamic action (SDA) of food is the name given to the phenomenon of the increase in metabolic rate that is observed when an animal is fed. There have been many investigations into the SDA of food but no satisfactory biochemical explanation has yet been offered. However, Newsholme & Crabtree (1976) have suggested that the SDA could be explained by an increase in the rate of substrate cycling during the metabolism of the digested products of the food. The results reported in this thesis which show that the rate of the TG-FFA cycle is doubled when an animal is fed support this hypothesis. If the rate of various other sybstrate cycles is increased to an extent similar to that of the TG-FFA cycle, then substrate cycles could indeed account for a significant amount of the SDA of food.

7.3 Energy balance during fat synthesis

During the synthesis of fatty acids, there is a requirement for two moles of reducing power (in the form of NADPH) per mole of acetyl CoA incorporated into fatty acid. Two possible sources of this reducing power are recognised; the pentose phosphate pathway and the malate cycle. In theory, the malate cycle could provide between 0 and 50% of the NADPH required for acetyl CoA synthesis, with the pentose phosphate pathway providing the rest. In fact, experimental studies indicate that in adipose tissue during active lipogenesis, the pentose phosphate pathway provides about 60% of the NADPH and the malate cycle completes the balance (see Flatt, 1970, for a more

detailed account of these pathways).

In a paper in which the energy metabolism associated with fatty acid synthesis was carefully accounted, Flatt (1970) showed that the conversion of glucose to fat in adipose tissue should give a net increase in cellular ATP. The amount of ATP yield per acetyl CoA formed varied depending on the relative amounts of reducing power produced in the malate cycle and the pentose phosphate pathway. If the malate cycle produced 50% of the NADPH, then for every acetyl CoA converted to fat there would be a yield of approximately one ATP. If, however, the malate cycle was not active and the pentose phosphate pathway was responsible for producing all of the NADPH, then five ATP molecules would be produced per acetyl CoA converted to fat. (The pathways and calculations for the derivation of these figures are quite involved and will not be considered in detail here. However, a summary of the reactions involved in fatty acid synthesis from glucose is given in appendix IV; this summary gives a simplified example which illustrates how net ATP production occurs during fatty acid synthesis. The reader is referred to Flatt (1970) and Flatt & Ball (1964) for further information.) The magnitude of these yields of ATP for the metabolism of adipose cells can be appreciated if it is recalled that during anaerobic glycolysis in muscle, a single ATP molecule is gained for each molecule of lactate that is formed from glucose. As mentioned above, the malate cycle in fact provides about 40% of the NADPH, which leads to a production of 1.8 ATP molecules per acetyl CoA synthesised.

Flatt (1970) suggested that the maximum rate of conversion of glucose to fat should be limited by the tissue's ability to use the ATP which is generated in excess during triglyceride synthesis. He proposed that the energy-generating nature of the lipogenic process could provide the means for regulating fatty acid synthesis.

Experimental evidence in support of this idea is available. Saggerson (1972a) showed that the synthesis of fatty acids in fat cells was increased by uncoupling agents or electron-acceptors. Similarly, it was found that the addition of small amounts of FFA to incubations stimulated the rate of fatty acid synthesis (Saggerson, 1972). Saggerson concluded that this action of FFA was due to the utilisation of ATP and NADH for the esterification of the FFA. Thus it is possible that one of the functions of the TG-FFA cycle in adipose tissue is that of utilising the ATP and NADH produced during the synthesis of fatty acids from glucose. Saggerson's results suggest that the TG-FFA cycle can stimulate the rate of fatty acid synthesis, but the rate of FFA esterification in the absence of exogenous FFA is not sufficient to relieve the increase in ATP. In contrast, however, a study by Evans & Garratt (1977), showed that the energy required to support the observed rate of cycling would be sufficient to hydrolyse the ATP produced by the lipogenic pathways. In the experiments in this thesis in which both the rate of cycling and the rate of fatty acid synthesis were measured, it is possible to determine whether the rate of FFA reesterification is sufficient to prevent an increase in cellular ATP levels.

In table 7.4 the derivation of the rate of TG-FFA cycling that is necessary to prevent an increase in cellular ATP during fatty acid synthesis is shown. The relationship between the rates of fatty acid synthesis and TG-FFA cycling in adipose tissue both *in vivo* and *in vitro* is shown in figs 7.2 and 7.1 respectively. It can be seen that in incubated fat pads the rate of TG-FFA cycling is sufficient to prevent an increase in the concentration of ATP even at the highest rates of fatty acid synthesis observed (fig 7.1). These results therefore agree with those of Evans & Garratt (1977). However, at

Table 7.4 Derivation of the minimum rate of TG-FFA cycling necessary to prevent a build-up of ATP during fatty acid synthesis

Flatt (1970) calculates 1.8 ATP molecules to be produced per acetyl CoA incorporated into fatty acid.

Assuming the average fatty acid synthesised in adipose tissue to have 16 carbon atoms, then the amount of ATP produced by the synthesis of one micromole of fatty acid

$$= 8 \times 1.8$$

$$= \underline{14.4 \text{ micromoles ATP}}$$

For every micromole of FFA that is reesterified in the TG-FFA cycle, 10/3 micromoles of ATP are used. This figure is derived from table 7.1 by omitting the conversion of glycerol back to glucose, a process which occurs in the liver and which is accompanied by the production of 2ATP molecules (1 ATP used to phosphorylate the glycerol and one NADH produced in gluconeogenesis). The reactions occuring in the liver are omitted because at present only the reactions which occur inside the adipocyte are being considered, so as to follow ATP metabolism inside the cell. (In fig 7.1 the energy metabolism of the whole animal is being considered).

Therefore the rate of cycling necessary to prevent a build-up of ATP during fatty acid synthesis

$$= \frac{\text{ATP surplus} \times 3}{10}$$

$$= \frac{\text{Rate fatty acid synthesis} \times 14.4 \times 3}{10}$$

$$= \underline{\text{Rate fatty acid synthesis} \times 4.3}$$

Table 7.4 Derivation of the minimum rate of TG-FFA cycling necessary to prevent a build-up of ATP during fatty acid synthesis

A graph of the rate of cycling against the rate of fatty acid synthesis will show when the rate of cycling is sufficient to remove the excess ATP produced during fatty acid synthesis from glucose. Any points above the line of gradient 4.3 will have a high enough rate of cycling, any points below the line will indicate that cycling is not sufficient to remove the excess ATP produced.

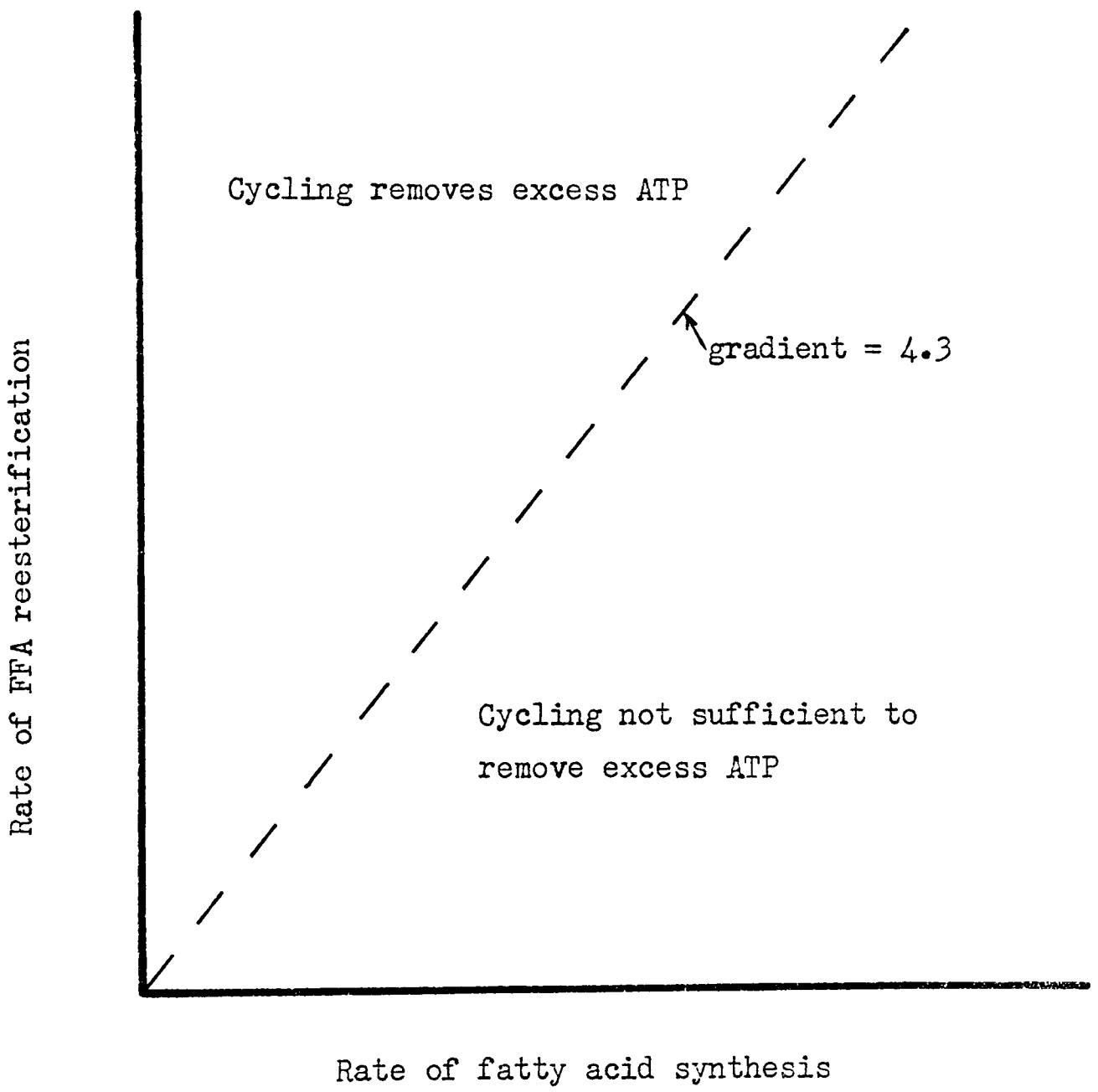


Fig 7.1 Rate of TG-FFA cyling against fatty acid synthesis in white adipose tissue incubated *in vitro* under various conditions

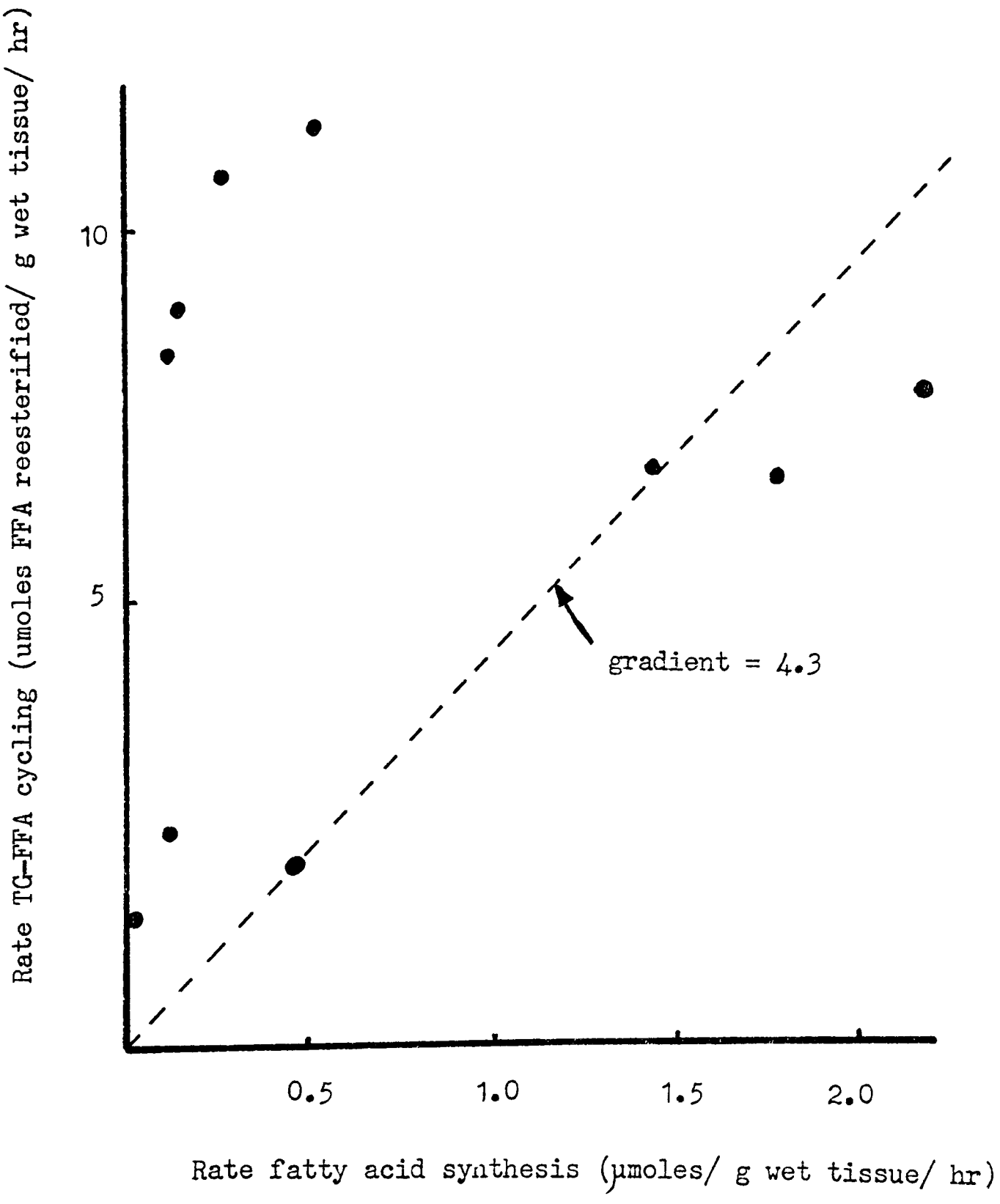
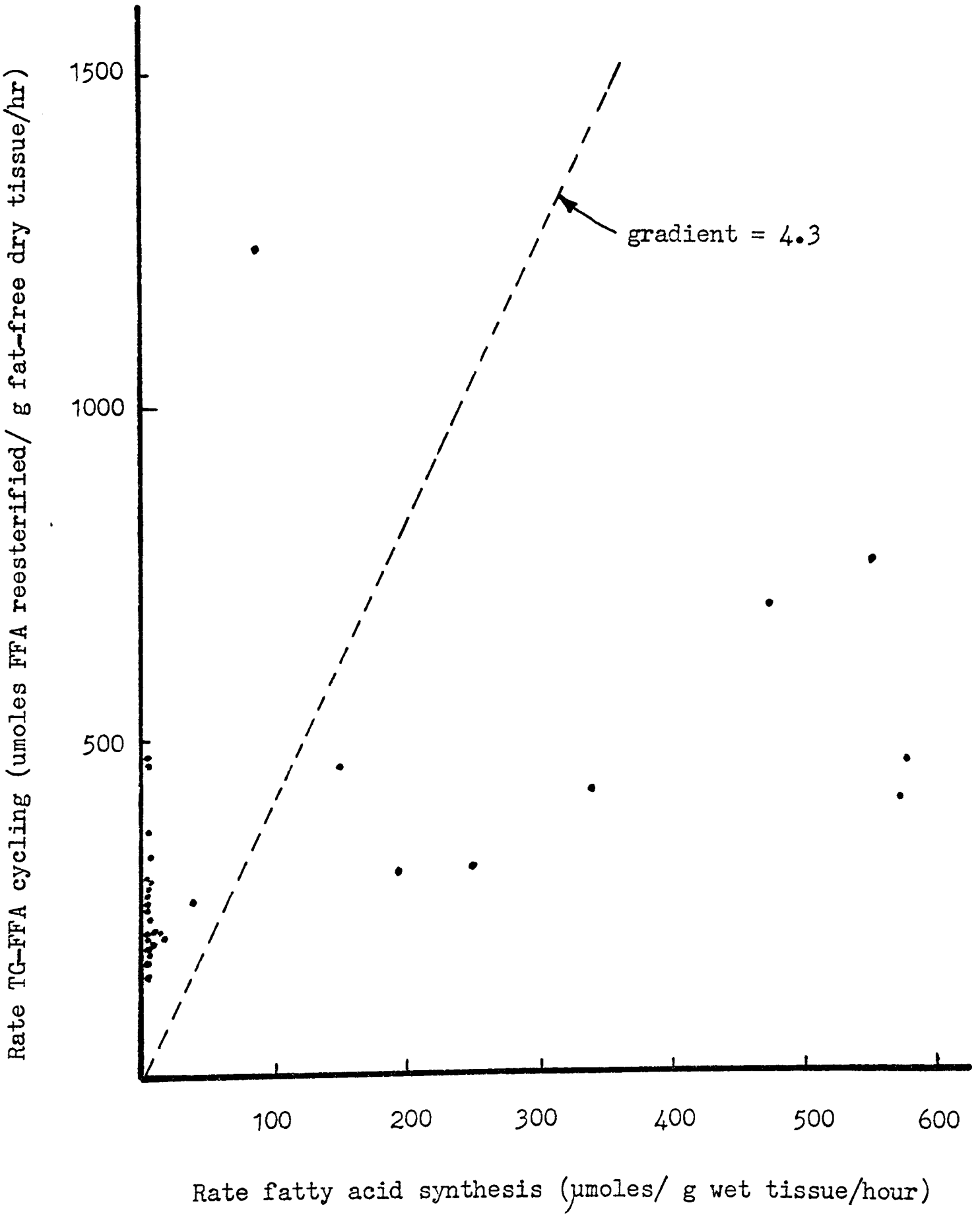


Fig 7.2 Rate of TG-FFA cycling against fatty acid synthesis in white adipose tissue *in vivo* under various conditions



high rates of fatty acid synthesis in adipose tissue *in vivo*, it appears that the rate of FFA reesterification is not sufficient by itself to prevent the expected increase in cellular ATP (fig 7.2), suggesting that the concentration of this nucleotide could limit the rate of fatty acid synthesis *in vivo*. Two arguments might be used against these experimental results to show that the rate of cycling is sufficient to prevent an increase in ATP, but they both seem unlikely. The first is that only a small part of the rate of fatty acid synthesis observed in these experiments is due to synthesis from glucose. This is possible because the tritium incorporation method of measuring fatty acid synthesis measures the total fatty acid synthesis. If any substrate other than glucose were to have a significant contribution to the rate of fatty acid synthesis *in vivo*, then this would lead to an overestimate of the amount of ATP being produced by fatty acid synthesis. However, there is good evidence that (at least during the storage of a meal) glucose is the major carbon source used for fatty acid synthesis in white adipose tissue (Hems et al., 1975), and hence this criticism does not apply to these results.

The other criticism that could invalidate the assumption that fatty acid synthesis is solely from glucose is that there may be an influx of tritium-labelled fatty acids which had been synthesised in some other tissue (for example, the liver). This possibility can also be discounted since the mice used in the experiments were treated with Triton WR-1339 (see chapter 6). Therefore it appears that at high rates of fatty acid synthesis from glucose *in vivo* the rate of TG-FFA cycling would not be sufficient by itself to prevent the increase in ATP that is predicted to accompany the synthesis of fatty acids. Experiments involving freeze-clamping of adipose tissue

have shown that the ATP concentration in white adipose tissue changes very little between fed, fasted and fasted-refed animals (Ballard, 1972), suggesting that the control of fatty acid synthesis by a raised concentration of ATP as suggested by Flatt (1970) does not occur. Some process in adipose tissue in addition to the TG-FFA cycle (for example, other substrate cycles) could therefore be responsible for the hydrolysis of the ATP produced during fatty acid synthesis from glucose.

7.4 The TG-FFA cycle and sensitivity

In the early work on the reesterification of FFA in adipose tissue, the TG-FFA cycle was regarded as at best a physiologically important thermogenic mechanism, and at worst as a waste of ATP. It was not until over ten years after the first evidence for the TG-FFA cycle was obtained that Newsholme & Crabtree (1970) suggested a new reason for substrate cycling. They showed that substrate cycles could provide a mechanism of variable sensitivity for controlling the flux through a metabolic pathway. Operation of the TG-FFA cycle should therefore increase the sensitivity of the triglyceride metabolism.

As discussed in chapter one, there are a number of ways in which the flux through an enzyme reaction can be controlled. These include the variation of the concentrations of substrates and/or cofactors for the reaction and, if the enzyme reaction is non-equilibrium, the variation of the concentration of allosteric regulator or of the concentration of the enzyme protein itself. Each of these means has a certain sensitivity, and this senstivity determines the maximum change in flux through a metabolic pathway that can be obtained in response to a given stimulaus. The maximum sensitivity that each of the above mechanisms can impart to the response of the pathway flux

to changes in the regulator concentration can be increased by having an enzyme which opposes the action of the rate-limiting enzyme and which is simultaneously active, thus forming a substrate cycle. (Note the distinction between 'flux through a metaboic pathway', which is the flow of metabolites through the whole pathway and is not the same as the 'flow through an enzyme reaction', which considers the flow through a single enzyme reaction. The sensitivity of the latter is fixed as one of the mechanisms given above, whereas the former can gain sensitivity by operating a substrate cycle. Substrate cycles can be considered as metabolic control mechanisms that are 'kinetically primed' to respond to a change in stimulus; in contrast, mechanisms such as cooperativity can be regarded as 'structurally primed' - see Newsholme & Crabtree, 1978.)

What criteria must be met to establish that the TG-FFA cycle is used in adipose tissue as a mechanism for increasing the sensitivity of control of FFA metabolism? Newsholme & Crabtree (1976) demonstrated that the sensitivity of control of pathway flux that is attainable by substrate cycling is given by the expression

$$\text{Sensitivity} = \frac{\%\text{ change in pathway flux}}{\%\text{ change in regulator}} = 1 + \frac{C}{J}$$

where C is the rate of cycling and J is the pathway flux. However, this equation is limited in so far as it only predicts the sensitivity that is attainable by substrate cycling under conditions when the rate of the back reaction of the cycle is not affected by the regulator. If the rate of the back reaction of the cycle is changed by the action of the regulator molecule, then this will affect the sensitivity to be gained by operating the sybstrate cycle. In appendix III it is shown that if the sensitivities of the forward and back reactions of the cycle are denoted by n_f and n_b respectively, then the sensitivity

of control of pathway flux is given by

$$\text{Sensitivity} = n_f \quad 1 + \frac{C}{J}\left(\frac{n_f - n_b}{n_f}\right)$$

If, in response to a stimulus, n_f and n_b are equal, then the above equation reduces to

$$\text{Sensitivity} = n_f$$

and the substrate cycle will not affect the sensitivity of response of the pathway. If it is known that in response to the addition of regulator, n_f will be greater than n_b, then it is possible to say that the substrate cycle will increase the sensitivity of response to the regulator. Obviously, prediction of the values of n_f and n_b in response to any given regulator would require a very detailed knowledge of the properties of the molecular mechanisms involved in the action of the regulator on the tissue, and at present such detailed knowledge is not available for the TG-FFA cycle. Hence, on the basis of a single determination of the rate of substrate cycling under any one set of conditions it is not possible to predict whether the substrate cycle will actually affect the sensitivity of control of pathway flux. However, if the rate of cycling and the pathway flux are measured under two different sets of conditions, then it is possible to determine whether the cycle has a role in increasing the sensitivity of response.

In appendix III and chapter 4 it is shown that if the ratio of the % change in the rate of lipolysis to the % change in the rate of esterification in response to a regulator is denoted by Q, then the sensitivity gained by cycling

$$= 1 + \frac{C}{J}\left(\frac{Q-1}{Q}\right)$$

Since the value of Q can be determined by experiment, this equation provides an easy way of determining whether a substrate cycle is used as a sensitising mechanism.

Consideration of the equations above will show how important the ratio of C/J is to the sensitivity properties of substrate cycles. The determination of this ratio, together with the investigation of whether it varies under different conditions, is therefore a major aim of a study of substrate cycles. The experiments reported in this thesis indicate that the ratio of C/J in incubated adipose tissue does vary in response to both acute hormone administration *in vitro* and chronic hormone administration *in vivo* (see chapters 4 and 5). However, it is worth stressing that there are two ways in which a regulator can affect the sensitivity properties of substrate cycles. Firstly, they can vary the 'potential' sensitivity by changing the ratio of C/J (as mentioned above, this is seen to vary from hormone to hormone). Secondly, at any given value for C/J, the sensitivity exhibited by a substrate cycle in response to a regulator can vary according to how the regulator affects the rates of the forwards and back reactions of the cycle (i.e. n_f and n_b in the equations above). This second possibility has also been demonstrated for adipose tissue incubated *in vitro* (see chapter 4). Thus there seems little doubt that the TG-FFA cycle increases the sensitivity of control of FFA release in adipose tissue incubated *in vitro*.

In the experiments using the tritiated water method to measure the rate of TG-FFA cycling *in vivo*, the flux of FFA into or out of adipose tissue was not measured. Hence, when using this method it is not possible to investigate the sensitivity properties of the TG-FFA cycle. The study of these properties in the intact animal would require the cannulation of blood vessels leading to and from

the adipose tissue depot under investigation; this was not attempted in this thesis. However, an indication that the TG-FFA is a mechanism of variable sensitivity *in vivo* can be gained from the results of Vik-Mo & Mjøs (1978). These workers perfused subcutaneous adipose tissue depots in anaesthetised dogs and measured the rates of glycerol and FFA release (see table 7.5). Their results indicate that the ratio of cycling to flux is variable *in vivo*, and that the TG-FFA cycle can increase the sensitivity of control of FFA release.

As discussed above, the TG-FFA cycle imparts sensitivity to the control of FFA release. It is pertinent to ask to what extent the rate of release of FFA varies *in vivo*, and to compare this degree of variation with the magnitude of the change in stimulus that is thought to initiate these changes. This should give some idea of the sensitivity of control of FFA release that is required *in vivo*. During starvation, for example, the turnover of FFA is increased by a factor of three, which suggests that a three-fold increase in the rate of lipolysis would be sufficient to satisfy the demand for FFA (Hales et al., 1978). The major stimulus to increase lipolysis during starvation is thought to be the drop in the concentration of insulin in the blood, and this concentration varies by a factor of 3-5 in the fed and fasted states (Newsholme & Start, 1973; Owen et al., 1979). Here the percentage change in the rate of lipolysis is, if anything, less than the percentage change in the concentration of insulin, and thus the sensitivity of the response to the stimulus is unity or less. Hence it appears that the sensitivity properties of the TG-FFA cycle need only play a minor role in the response to starvation. Another example in which the release of FFA from adipose tissue is changed is during exercise. This is perhaps the condition which demands the greatest change in the release of FFA from adipose tissue - during strenuous exercise, the consumption of oxygen can increase

Table 7.5 Rates of lipolysis, FFA release and TG-FFA cycling together with the sensitivity due to the TG-FFA cycle for inguinal subcutaneous adipose tissue perfused in situ in the anaesthetised dog (results of Vik-Mo & Mjøs, 1978)

Treatment	Rates (μmoles/100g tissue/min)			Ratio $\frac{C}{J}$	Sensitivity increase by $1 + \frac{C}{J}\left(\frac{Q-1}{Q}\right)$
	TG-FFA cycling C	Glycerol release	FFA release J		
Control	2.01	0.82	0.45	4.5	
Nicotinic acid	1.89	0.66	0.09	21.0	4.1
Isoprenaline	4.32	1.79	1.05	4.1	1.13
Isoprenaline + Nicotinic acid	3.33	1.19	0.24	13.9	-1.03

For the control incubation, $1 + C/J = 5.5$. This is the degree of the increase in sensitivity of control of FFA release by TG-FFA cycling if a regulator does not affect the rate of the back reaction of the cycle. The actual sensitivity afforded by the TG-FFA cycle in response to the treatments given in the table is shown in the right-hand column. Q is the

$$\frac{\%\text{ increase in the forwards reaction of the cycle}}{\%\text{ increase in the back reaction of the cycle}}$$

$$= \frac{n_f}{n_b}$$

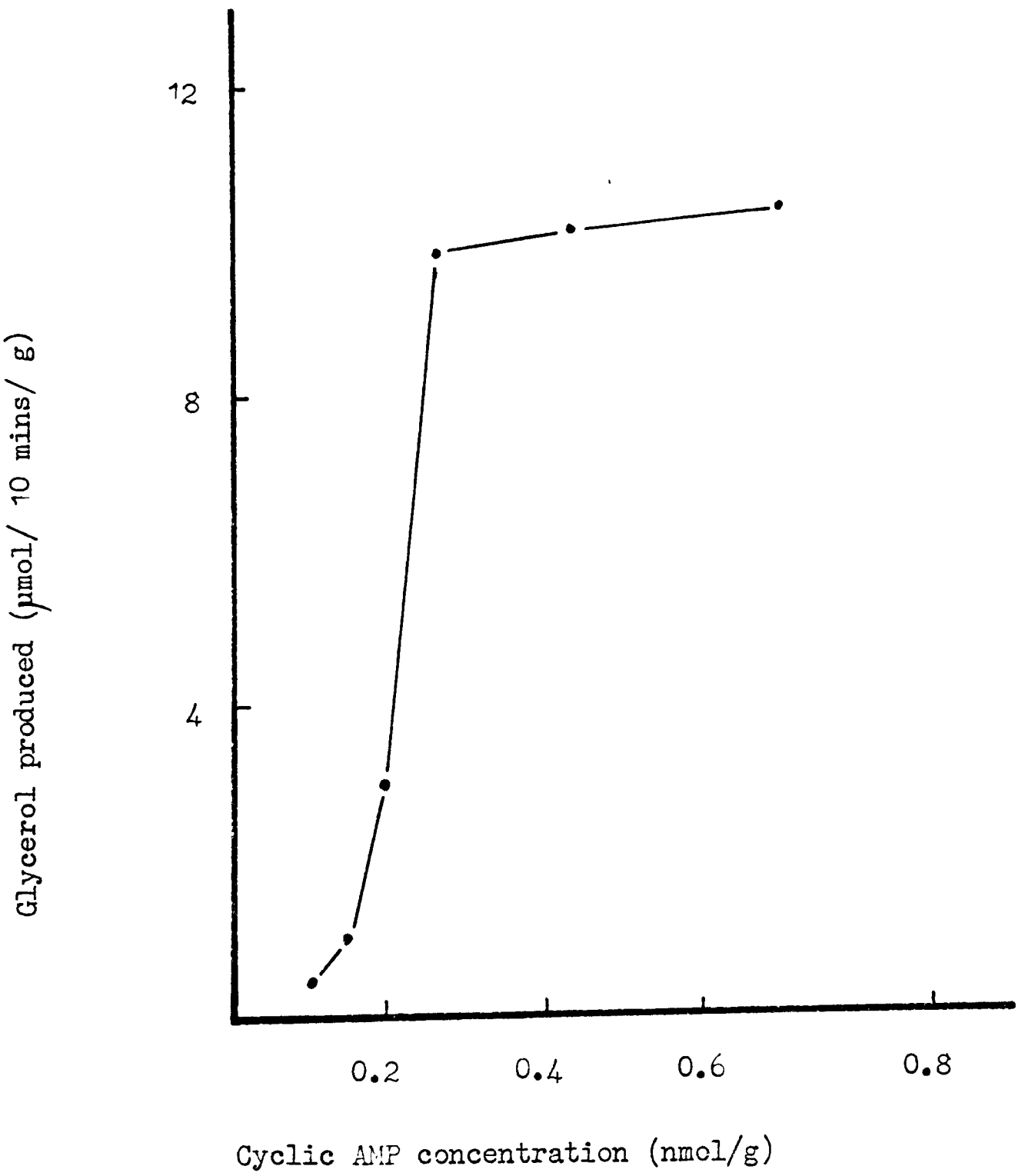
$$= \frac{\%\text{ increase in lipolysis}}{\%\text{ increase in esterification}}$$

by 20-fold or more (Passmore & Robson, 1971; Wahren, 1979). If the assumption is made that FFA supply all the fuel required for this increase in oxygen consumption, then a 20-fold increase in the rate of lipolysis should be sufficient to meet this energy requirement, and this must by approximately the maximum increase in the release of FFA from adipose tissue that is required in the live animal. How does the magnitude of this change compare with the changes in the stimuli which activate lipolysis during exercise?

The acute stimulation of lipolysis during exercise and stress is probably mediated by increased activity of the sympathetic nervous system (Hales et al., 1978). Noradrenaline released at the nerve-endings in adipose tissue binds to receptors on the fat cell membrane, thus stimulating adenylate cyclase to produce cAMP. This in turn activates cAMP-dependent protein kinase, which phosphorylates and activates the triglyceride lipase (see chapter 4). The primary stimulus to the adipocyte is therefore the change in the concentration of noradrenaline increasing the binding to the adrenergic receptor on the cell surface. Unfortunately, the magnitude of the change in the concentration of noradrenaline outside the fat cell is not known; it might be five-fold, ten-fold or a hundred-fold. However, as mentioned above, the binding of noradrenaline to a receptor increases the concentration of cAMP inside the adipocyte. It is possible to measure the concentration of cAMP in isolated fat cells in response to different concentrations of catecholamine, and to determine the corresponding rate of lipolysis. The results of such an experiment are shown in fig 7.3 (these results are from Hales et al., 1978; similar results have been obtained by Sengupta et al., 1981). From this graph, it can be seen that a change in the concentration of cAMP from 0.1 to 0.3 nmol/g cells was sufficient to increase the rate of glycerol release from about 0.5 to about 10 umoles/10 mins/g.

Fig 7.3 Relationship between cAMP and lipolysis in adipocytes stimulated with adrenaline (from Hales et al., 1978)

(This is the same figure as fig 4.3)



Thus a tripling of the concentration of cAMP caused a 20-fold increase in the rate of lipolysis, a sensitivity of about 7 (these results illustrate the great degree of sensitivity of a phosphorylation/dephosphorylation interconversion cycle). It can be seen from fig 7.3 that the adipocyte is easily able to produce sufficient cAMP for the lipolytic process to be maximally stimulated - increasing the concentration of cAMP above 0.3 nmol/g cells does not cause further stimulation of lipolysis. Hence a change in the concentration of cAMP from 0.1 to 0.3 nmol/g cells would be sufficient to satisfy the demands of the animal for FFA during the heaviest exercise. If it is assumed that the production of cAMP is directly proportional to the concentration of noradrenaline at the adrenoreceptor on the adipocyte's cell membrane (i.e. a sensitivity of unity), then a three-fold increase in the concentration of cAMP would only require a three-fold change in the concentration of noradrenaline. It is not unreasonable to assume that the concentration of noradrenaline at the end-foot of sympathetic nerves can vary by a factor of three. Hence, assuming that the cAMP concnetration in fat cells <u>in vivo</u> varies in the range 0.1 - 0.3 nmol/g cells, it appears that the phosphorylation/dephosphorylation mechanism of control of lipolysis has sufficient sensitivity to cope with the maximum demands of the animal for FFA.

The above discussions suggest that the TG-FFA cycle in white adipose tissue need only play a minor role in the provision of sensitivity of control of FFA release. However, the fact that it does provide senstivity in response to various treatments (shown using the equations derived in appendix III - see above) shows that the cycle must improve the sensitivity of control of FFA release. Perhaps it has an important sensitising role in cells which are a relatively long way from a sympathetic nreve ending or a capillary from which hormones may diffuse. The changes in the concentration of hormones at

these cells may be very much less than those used in the study of isolated fat cells incubated *in vitro* and hence the sensitivity afforded by the TG-FFA cycle would enhance the response of these cells.

In addition to sensitising the control of FFA release, the TG-FFA cycle will increase the sensitivity of control of FFA esterification. As discussed in chapter one, the FFA esterification reactions are non-equilibrium. However, they are not the flux-generating step in the overall process of storage of FFA in adipose tissue; lipoprotein lipase, which hydrolyses blood triglyceride thus releasing FFA for esterification inside the cell, is thought to be the flux-generating step for this process. Operation of the TG-FFA cycle will therefore not increase the sensitivity of control of triglyceride removal from the blood (because the esterification pathway is not the flux-generating step for FFA storage in adipose tissue), but it will increase the sensitivity of control of the flux through the esterification pathway. Hence, in order to achieve a certain flux through the esterification reactions, a smaller change in stimulus is needed than would be required in the absence of the TG-FFA cycle. The increase in sympathetic tone that is seen when animals are fed (see chapter 6) might have a very important role in increasing the sensitivity of the TG-FFA cycle in order to facilitate the storage of food in adipose tissue.

7.5 Control of the TG-FFA cycle

Katz & Rognstad (1975) have pointed out the possibility that some substrate cycles may have no specific function but are simply due to imperfections in the control of metabolism. Thus the TG-FFA cycle may operate simply because nature has been unable to develop a control mechanism that is capable of maintaining the rate of esterification at a low rate at the same time as lipolysis is stimulated (and vice versa). When lipolysis is stimulated in order to increase the release of FFA

for utilisation by other tissues, the rate of esterification would be expected to be inhibited. From a teleological point of view, it is difficult to see why the rate of esterification is increased when lipolysis is stimulated. The control of the lipolytic enzymes has been shown to be via cAMP-dependent protein kinase (see section 4.4), but investigations into the control of the esterification process have not been able to elucidate the method of control. The in vitro rate of esterification does not appear to correlate with changes in the concentration of either of the esterification pathway's substrates, long chain acyl CoA and glycerol phosphate. It has been shown that several of the esterification enzymes are subject to regulation by catecholamines, but there is no correlation between the changes in enzyme activity and the rate of esterification (see chapter 4). It seems possible that FFA act as activators of the esterification process since the rate of triglyceride synthesis correlates with the FFA levels (see Saggerson, 1972; Denton & Halperin, 1968; Saggerson & Greenbaum, 1970). The effect of FFA is not a mass action effect on the pathway, because FFA (which are one of the substrates for the long chain acyl CoA synthetase reaction) increase the rate of triglyceride synthesis without changing the concentration of acyl CoA (see table 3.16). The problem with investigations into the control of lipid metabolism is that lipid exists as a different phase from water. Thus the various intermediates in the formation and breakdown of triglyceride do not have a concentration in water and the reactions are therefore not amenable to the usual methods of determining equilibrium and non-equilibrium reactions by determining mass-action ratios. Even the comparison of enzyme activities is difficult due to problems with handling the substrates and the enzymes.

Considering lipolysis from a teleological point of view, it seems illogical that an increase in the concentration of FFA should stimulate

esterification, since the role of the lipolytic process is to provide an increased level of FFA for oxidation by the animal. On the other hand, considering the storage of lipid in the same way, it is reasonable that an increased concentration of FFA (due to the activity of lipoprotein lipase on blood lipoproteins) should stimulate the synthesis of triglyceride within the adipocyte. Perhaps the action of catecholamines in lowering the activity of several of the esterification enzymes is to reduce the rate of esterification that would otherwise be excessive in the presence of the high concentrations of FFA found during lipolysis.

If FFA act as activators of esterification, then why do they have this role? Why does esterification increase when lipolysis (and thus FFA levels) is increased? Presumably nature allows this to happen because it is in some way advantageous to the animal. The toxic effects of high concentrations of FFA on such things as cellular integrity and heart rhythm are well known (see for example Rodbell, 1965; Spector & Fletcher, 1978; Oliver, 1968, 1981). A stimulation of esterification at increased concentrations of FFA will certainly have a protective effect and prevent the concentration of FFA reaching too high a level. Experiments have shown that during flow restriction in perfused aipose tissue, the rate of lipoysis due to sympathetic stimulation is unaffected by the decreased blood flow (during which there is a build-up in FFA) whereas the rate of FFA release is decreased (Belfrage et al., 1978; Bülow & Madsen 1981). These results show that esterification is increased to prevent FFA accumulating to levels which would cause tissue damage; the FFA concentration does not reach a level where lipolysis is inhibited. However, the question is raised; why should there be increased FFA esterification at high FFA levels - why not simply have FFA feedback inhibit the triglyceride lipase?

It is possible that by chance the TG-FFA cycle's property of increased esterification at raised concentrations of FFA simply evolved before any mechanism of FFA inhibiting lipolysis at physiological FFA levels. Indeed, Newsholme & Start (1973) point out the advantage that the TG-FFA cycle would have had for the primitive organism. The organism would have a continual rate of lipolysis and this would be opposed by the esterification process. When the FFA concentration fell, the rate of esterification would fall and the FFA level would then rise again. When the FFA concentration rose to too high a level, the rate of esterification would increase. Thus the concentration of FFA could be maintained at a relatively constant level by the simple mass-action effect of FFA on the rate of esterification. The TG-FFA cycle's balancing or the rates of lipolysis and esterification is probably as accurate a method of maintaining blood FFA at a given level as a mechanism involving the allosteric regulation of lipolysis would be. In addition, the cycle improves the sensitivity of control of FFA flux and, due to the increased sympathetic nervous activity after a meal and the energy-consuming properties of substrate cycles, it has the advantage of being able to minimise any ATP build-up in adipose tissue during fatty acid synthesis from glucose.

7.6 The TG-FFA cycle in other tissues

Two tissues (other than adipose tissue) that have the ability to TG-FFA cycle are liver and muscle. In this thesis the cycle in these tissues was not investigated; however, it was thought to be appropriate to summarise the possible role of the TG-FFA cycle in these tissues and the problems involved in measuring the cycle in them.

7.6.1 TG-FFA cycle in muscle

FFA taken up from the blood by muscle can either be oxidised or esterified to give intracellular triglyceride (see for example Eaton & Steinberg, 1961; Jesmok et al., 1976). Under certain conditions, this triglyceride can be hydrolysed to FFA to provide energy for contraction (Denton & Randle, 1967; Olson & Hoeschen, 1967; Lech et al., 1977). Thus the reactions of esterification and lipolysis exist in the same cell and, if they occur simultaneously in the same compartment, they would form a TG-FFA substrate cycle.

It has been suggested that a TG-FFA cycle in muscle would buffer the intracellular concentration of FFA and prevent them from reaching toxic levels (Newsholme & Crabtree, 1976). The uptake of FFA by muscle is a passive diffusion process and is dependent on the blood concentration of FFA (Eaton & Steinberg, 1961). The rate of oxidation of FFA by muscle is, however, dependent both on the intracellular concentration of FFA and the rate of ATP utilisation by the muscle. Thus if the blood FFA concentration is elevated and some muscles are not mechanically active, the intracellular FFA could be prevented from reaching such levels by increasing the rate of esterification, and the TG-FFA cycle would provide sensitivity to changes in the concentration of FFA (see Newsholme & Crabtree, 1976). Similarly, the endogenous triglyceride could buffer intracellular FFA levels under conditons where the supply of FFA from the blood is insufficient to meet the requirements of the muscle (see Jesmok et al., 1976; Lech et al., 1977). It is possible that the rate of TG-FFA cycling is stimulated under certain conditions in order to improve the sensitivity of control of the intracellular concentration of FFA.

Evidence for the existence of the TG-FFA cycle in muscle is inconclusive. Kreisberg (1966) found that both glycerol release and

triglyceride synthesis was increased in perfused rat heart in response to adrenaline, suggesting that the TG-FFA cycle was stimulated by this hormone. In contrast, Jesmok et al., (1976) found that the release of glycerol from the perfused rat heart in response to adrenaline was exactly the same as the decrease in tissue triglyceride, which suggests that there is no reesterification of FFA released by lipolysis of endogenous triglyceride. Thus the existence of this cycle in muscle has not been conclusively demonstrated.

Measurement of the rate of TG-FFA cycling in muscle would be more difficult than in adipose tissue. As with adipose tissue, certain conditions have to be satisfied in order to use the synthesis or breakdown methods of measuring the cycle (see chapter 3); experiments would need to be done to demonstrate that each of these conditions do not affect the method being used to estimate cycling.

7.6.2 TG-FFA cycling in liver

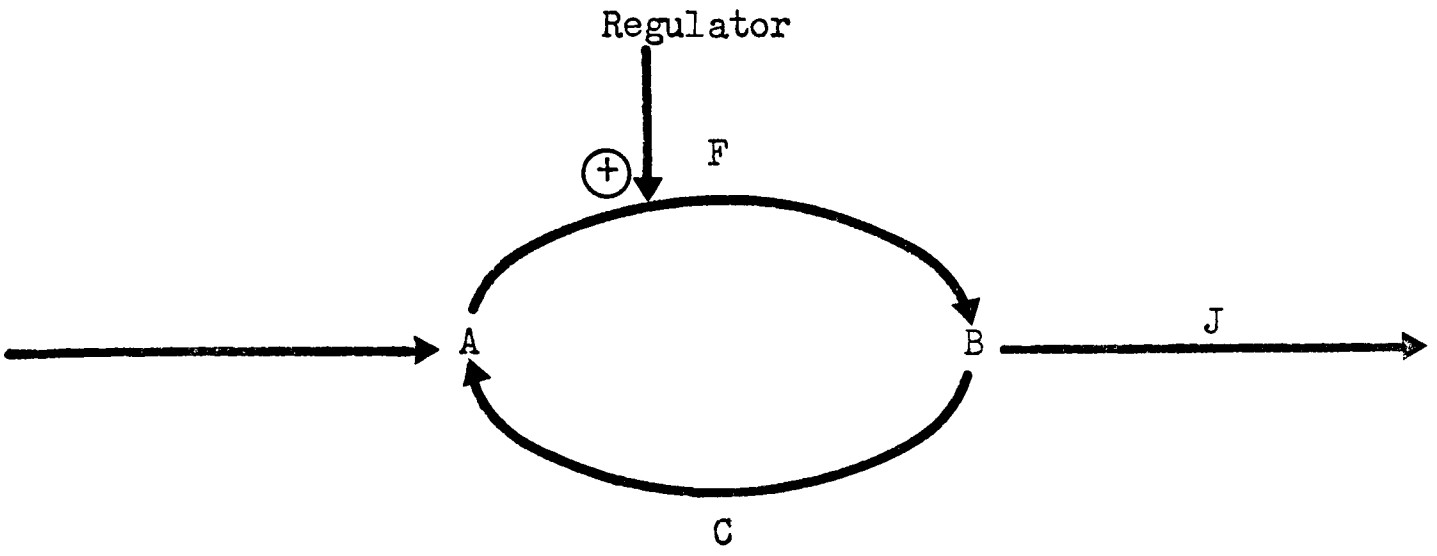
Liver has the enzymes necessary to both esterify FFA and hydrolyse triglyceride (see for example Hems, 1975; Sturton et al., 1978; Bates & Saggerson, 1979; Knox et al., 1979) and therefore it is possible that a TG-FFA cycle could exist in this tissue. The assumptions necessary for the measurement of the rate of cycling are similar to those discussed in chapter 3. The liver is able to catalyse a greater variety of metabolic interconversions than either adipose tissue or muscle, and hence the measurement of the rate of FFA reesterification would be more difficult in liver than in either of these latter tissues.

A high proportion of the fatty acids released by adipose tissue can be taken up by the liver and esterified to form triglyceride (see Robinson, 1970). The liver releases these triglycerides into the bloodstream in the form of very-low-density lipoprotein (VLDL), and they are subsequently removed as FFA by those tissues which possess an

active lipoprotein lipase (see chapter 6). Adipose tissue has lipoprotein lipase activity and hence a TG-FFA cycle operates between liver and adipose tissue. It has been suggested that the purpose of this inter-tissue substrate cycle is to increase the sensitivity of control of the concentration of plasma triglycerides. Thus the concentration of VLDL in the blood can be maintained at a relatively constant level in order to provide a continuous supply of fuel for tissues such as muscle or mammary gland. The role of this inter-tissue substrate cycle is not unlike that of the intra-tissue cycles considered in this thesis in that it provides sensitivity to the control of the concentration of a fuel despite large changes in the rate of its utilisation.

APPENDIX I

Derivation of relationship between sensitivity of forward reaction of substrate cycle and the sensitivity of a substrate cycle



At constant concentration of A and B,

$$J = F - C$$

If a change in regulator causes a change F in the forward reaction, and no change in the cycling flux, then at constant concentration of A and B,

$$J + \Delta J = (F + \Delta F) - C$$

$$\Rightarrow \quad \Delta J = \Delta F$$

$$\therefore \quad \frac{\Delta J}{J} = \frac{\Delta F}{J} = \frac{\Delta F}{F} \times \frac{F}{J} \quad .$$

Let $$\frac{\Delta J}{J} = J_{rel} \text{ and } \frac{\Delta F}{F} = F_{rel}$$

$$\therefore \quad J_{rel} = F_{rel} \times \frac{F}{J}$$

$$\Rightarrow \quad \frac{J_{rel}}{F_{rel}} = \frac{F}{J}$$

But $$J = F - C$$

$$\Rightarrow F = J + C$$

Hence $$\frac{J_{rel}}{F_{rel}} = \frac{J + C}{J} = 1 + \frac{C}{J}$$

If the forward reaction of the cycle has a sensitivity value of n to the action of a regulator, then the relative increase in the forward reaction, F_{rel}, is n times the relative increase in the concentration of regulator.

$$F_{rel} = n\ [R]_{rel}$$

where R_{rel} is the relative change in the concentration of regulator.

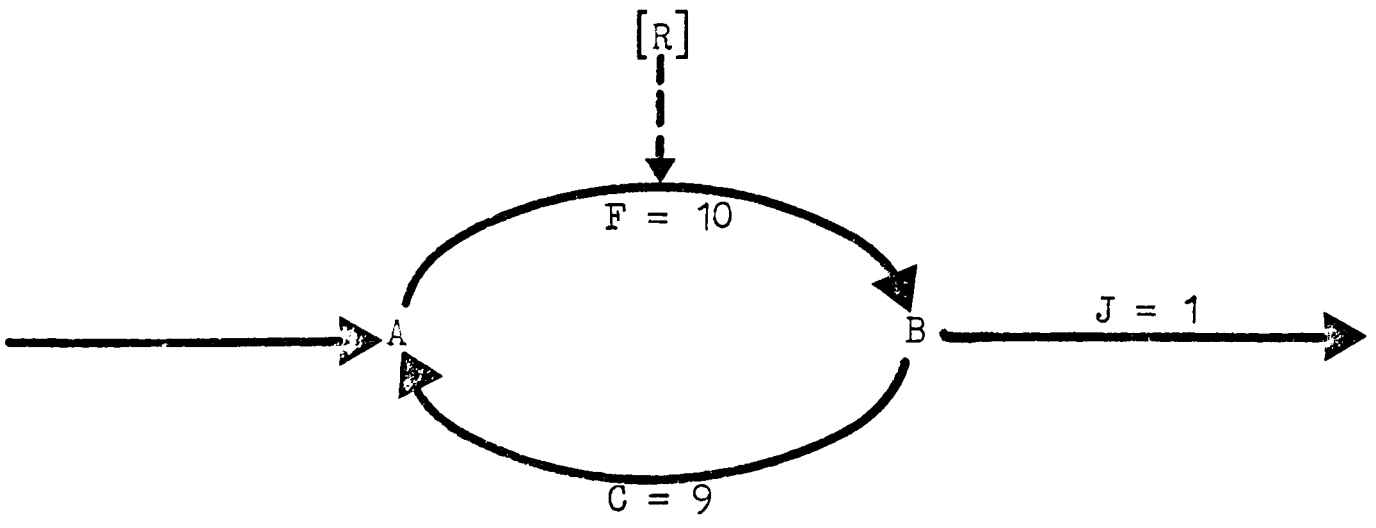
$$\frac{J_{rel}}{F_{rel}} = \frac{J_{rel}}{n\ [R]_{rel}} = 1 + \frac{C}{J}$$

$$J_{rel} = n + nC = n(1 + C)$$

This equation shows that a substrate cycle will give a (1 + C/J)-fold increase in the sensitivity of control of the existing control mechanism (of sensitivity n) used by the enzyme catalysing the forwards reaction of the substrate cycle. The sensitivity of the forward reaction to changes in the concentration of regulator might be between 0 and 1 for an enzyme with a hyperbolic response to changes in regulator concentration, and between 0 and 4 for a four subunit enzyme which exhibits positive cooperativity, ie a sigmoidal response to changes in regulator concentration (see chapter 1, section 1.3.3.). Table I.1 shows some numerical examples for the different values of n.

Table I.1 Numerical examples showing the effect of the increase in sensitivity of the forward reaction of the substrate cycle on the sensitivity of the control of pathway flux.

Sensitivity of forward reaction of cycle n	New Forward Flux F	New Pathway Flux J	New Cycling Flux C	SENSITIVITY	
				$\frac{\%\text{ change in J}}{\%\text{ change in R}}$	$n + \frac{nC}{J}$
0	10	1	9	$\frac{0}{10} = 0$	$0 + \frac{0x9}{1} = 0$
1	11	2	9	$\frac{100}{10} = 10$	$1 + \frac{1x9}{1} = 10$
2	12	3	9	$\frac{200}{10} = 20$	$2 + \frac{2x9}{1} = 20$
3	13	4	9	$\frac{300}{10} = 30$	$3 + \frac{3x9}{1} = 30$
4	14	5	9	$\frac{400}{10} = 40$	$4 + \frac{4x9}{1} = 40$



The substrate cycle is as illustrated above, R is a regulator of the forward reaction of the substrate cycle. The table shows how the new values for F, J and C after a 10% change in the concentration of R depend on the sensitivity of the forward reaction to R.

APPENDIX II

On the mechanism of the gain of tritium from tritiated water during synthesis of glycerol phosphate

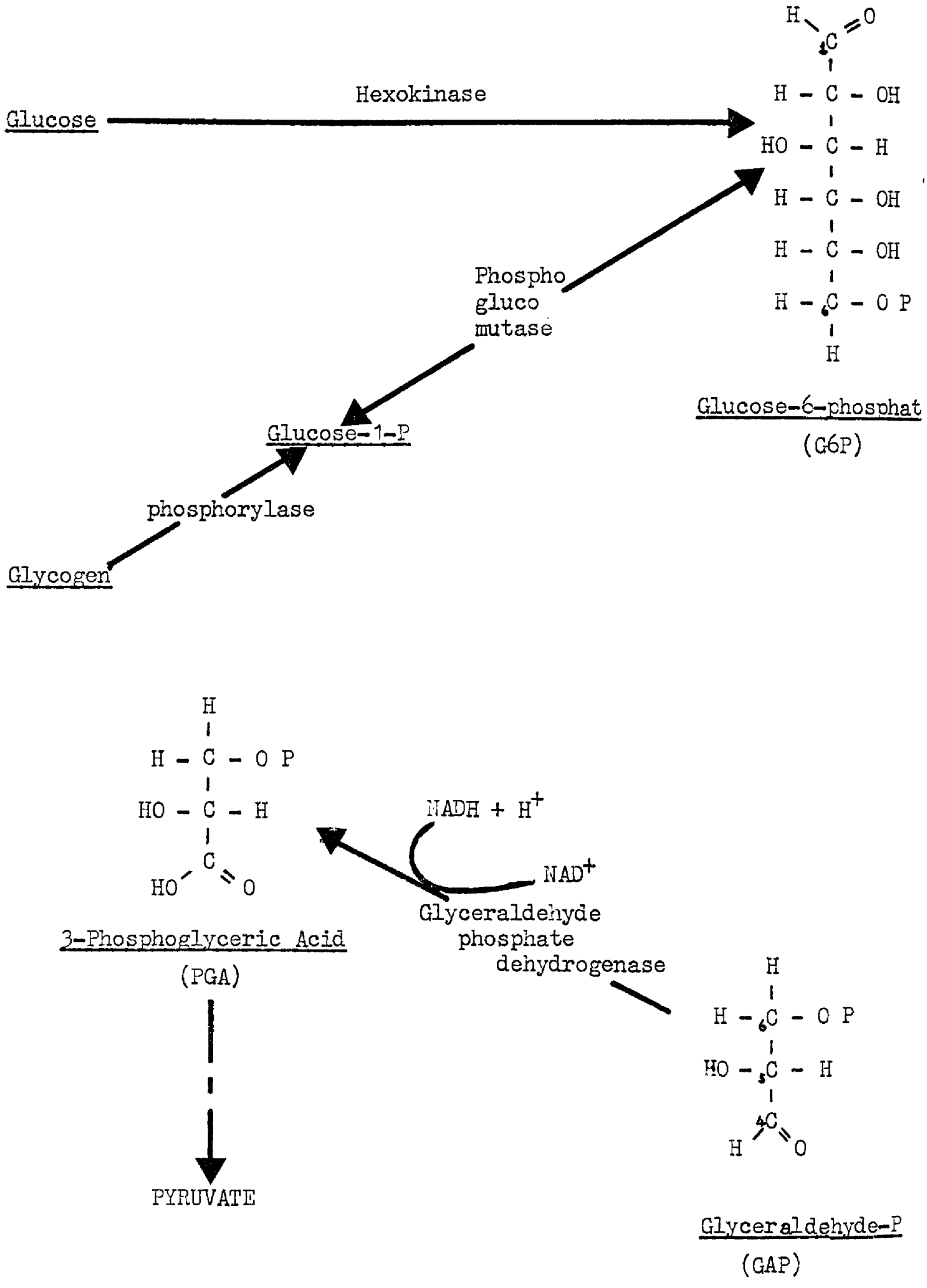
II.1 Introduction

Jungas (1968) found that when adipose tissue was incubated with tritiated water, 3.3 tritium atoms were incorporated per triglyceride-glycerol moiety synthesised. In adipose tissue, glycerol phosphate is the major precursor of the triglyceride-glycerol moiety (see section 3.3). Glycerol phosphate is derived from the glycolytic pathway by the reduction of dihydroxyacetone phosphate (DHAP). This appendix considers the mechanism of the reactions in which glycerol phosphate could gain a carbon-bound tritium atom from tritiated water.

The structure of some of the intermediates in the glycolytic pathway is given in figure II.1. The numbering of the carbon atoms in the triose phosphates is as for the parent carbon atoms in glucose. The hexose sugars are given in their straight-chain form for the sake of clarity; the internal addition reaction which produces the ring-structured sugar from the straight-chain form of the sugar does not involve carbon-hydrogen bond rupture and hence is not important to this discussion (see Edwards and Hassal, 1971). Four reactions can be implicated in the gain of tritium by glycerol phosphate. These are the phosphoglucose isomerase, aldolase, triose phosphate isomerase and glycerol phosphate dehydrogenase reactions, and the mechanisms of each of these will be considered in turn.

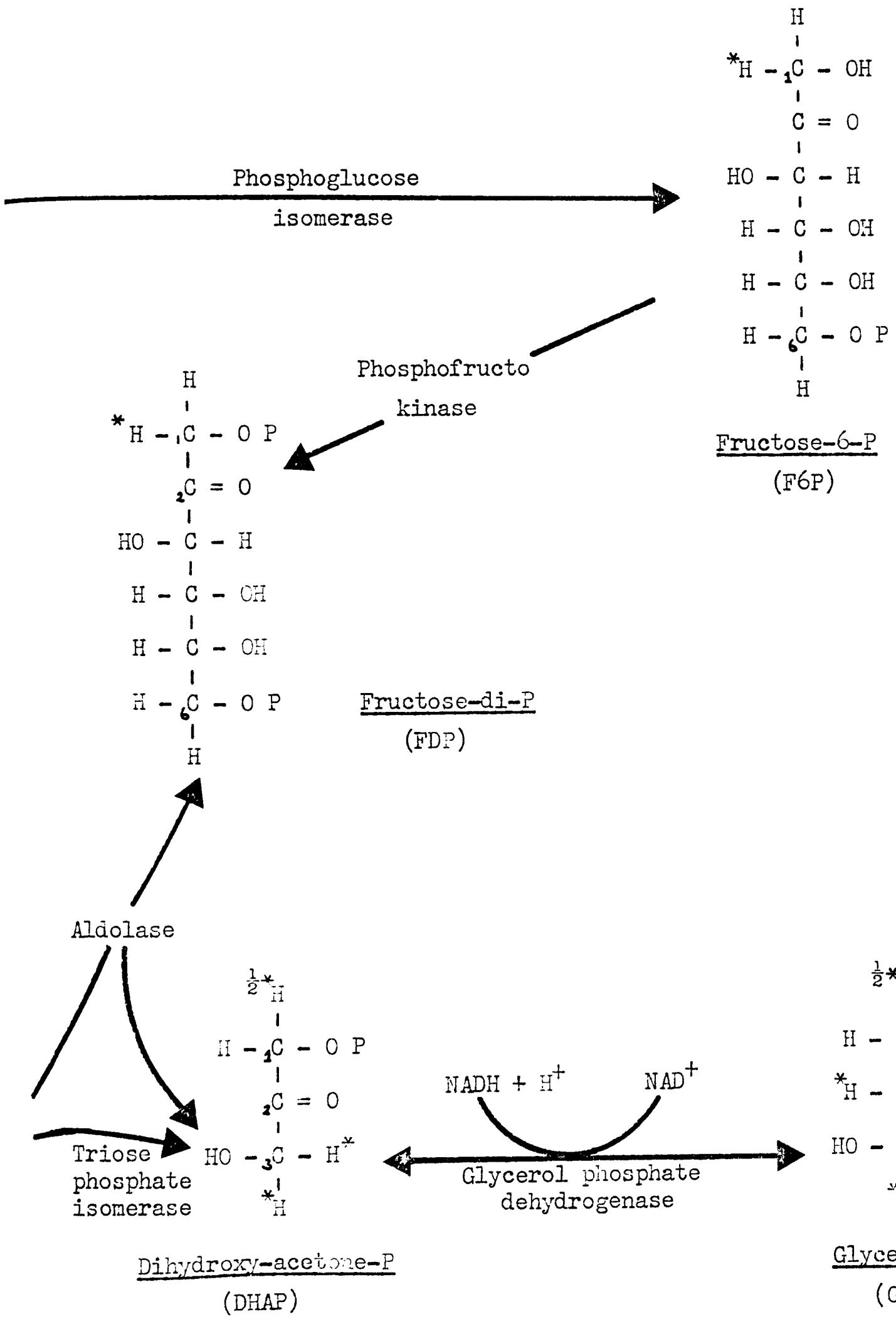
Fig. 3.3 Structure of some of the glycolytic intermediates in glycerol

Numbering of carbon atoms follows the number of the parent

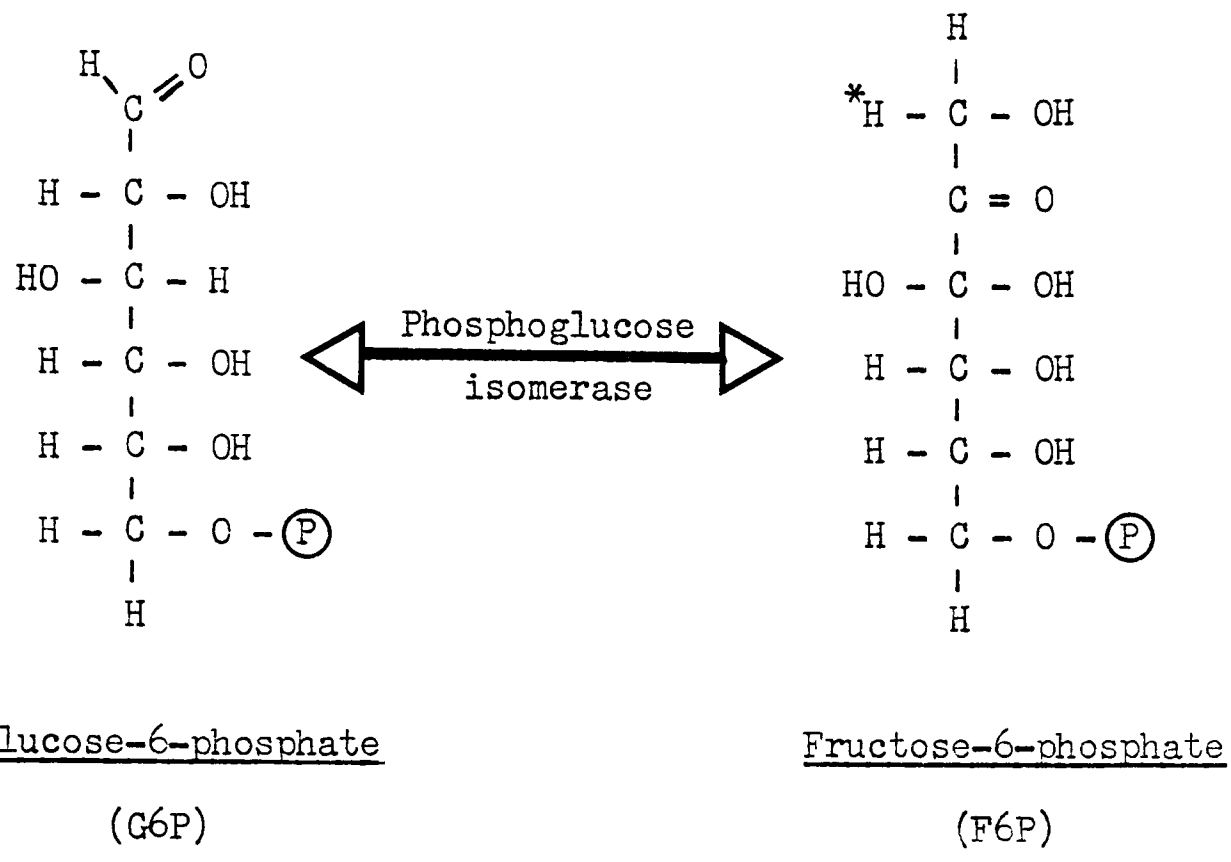


phosphate production.

carbon atom in glucose.



II.2 The Phosphoglucose isomerase reaction



Glucose-6-phosphate (G6P)

Fructose-6-phosphate (F6P)

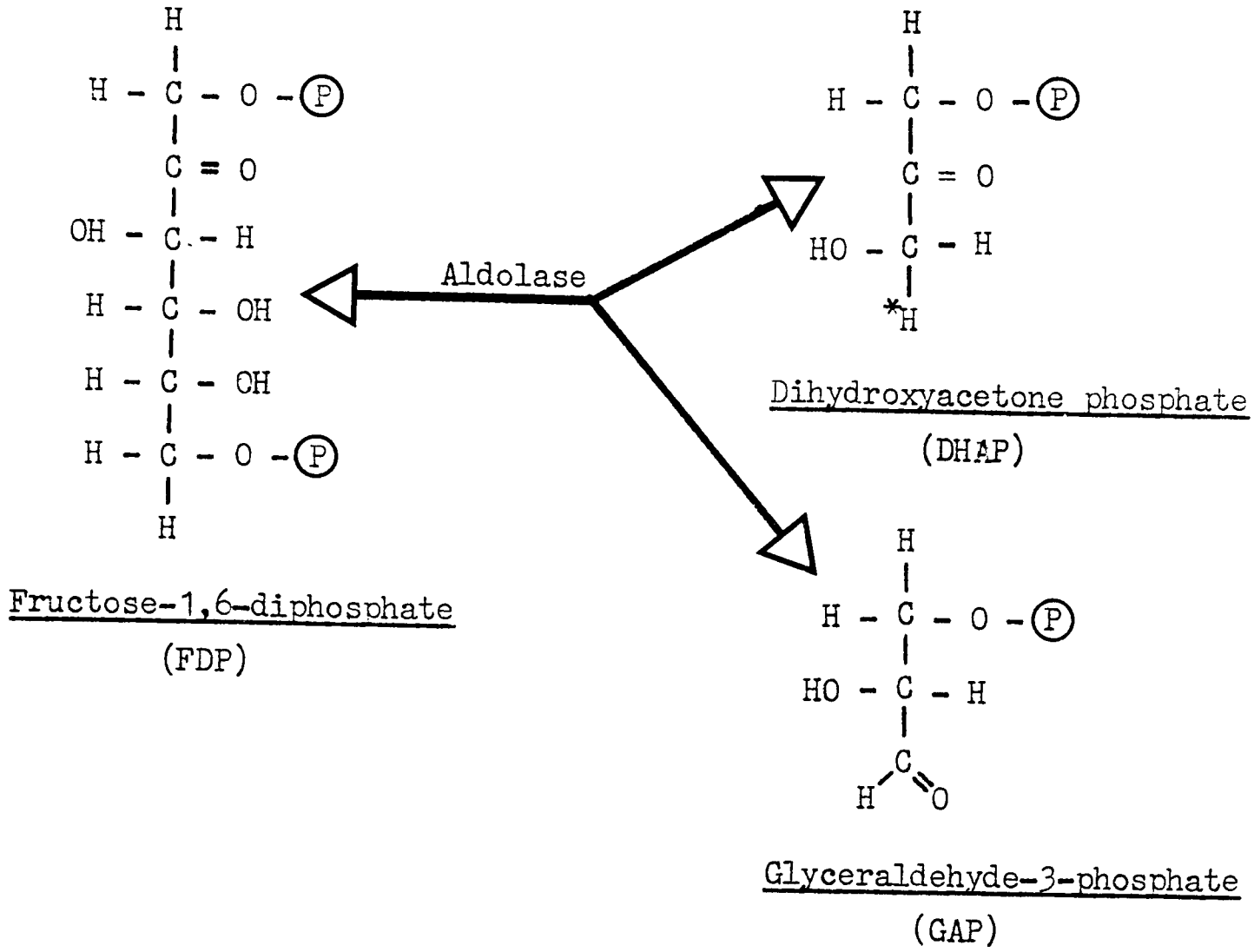
When glucose-6-phosphate (G6P) is converted to fructose-6-phosphate (F6P), the carbon-1 of G6P gains a hydrogen atom and the carbon-2 loses one. In reactions of this type, two mechanistic possibilities present themselves. Firstly the hydrogen from C-2 of G6P may shift to the C-1 as a hydride ion; this transformation would be intramolecular and would not involve any exchange between the migrating hydrogen and hydrogen in the aqueous phase. Secondly, the hydrogen may migrate as a proton, and this mechanism would allow exchange with protons (and hence tritium atoms) in the solvent (see Topper, 1957). In some enzymic isomerisations, both pathways are observed simultaneously. This appears to be the case with phosphoglucose isomerase, since both intramolecular hydrogen transfer and the uptake of tritium from tritiated water have been demonstrated (Topper, 1957; Rose and O'Connell, 1961; Katz and Rognstad, 1969). These two mechanisms appear to occur in roughly equal amounts, but the reaction is not sufficiently characterised under physiological

conditions to be able to predict the exact number of tritium atoms incorporated per F6P molecule (see Noltman, 1972; Katz and Rognstad, 1975). The number of tritium atoms gained per F6P will vary according to how close the phosphoglucose isomerase reaction is to equilibrium, since the more times the molecule passes through the G6P-F6P conversion the greater will be the opportunity for equilibration with the tritium in the medium (Katz and Rognstad, 1969). The phosphoglucose isomerase reaction in adipose tissue is thought to be close to equilibrium (Saggerson amd Greenbaum, 1970) and hence one of the hydrogens on C-1 of F6P will be more or less equilibrated with tritium in the aqueous phase.

When FDP is split by aldolase to give two triose phosphates, only one of the triose phosphates (the DHAP) will have the tritium from the C-1 of F6P. If glyceraldehyde phosphate is converted to DHAP in the triose phosphate isomerase reaction (see section II.4), then the DHAP so formed will not have the tritium that was gained in the phosphoglucose isomerase reaction. Hence on average DHAP will have gained half a tritium atom in the phosphoglucose isomerase reaction.

II.3 The Aldolase reaction

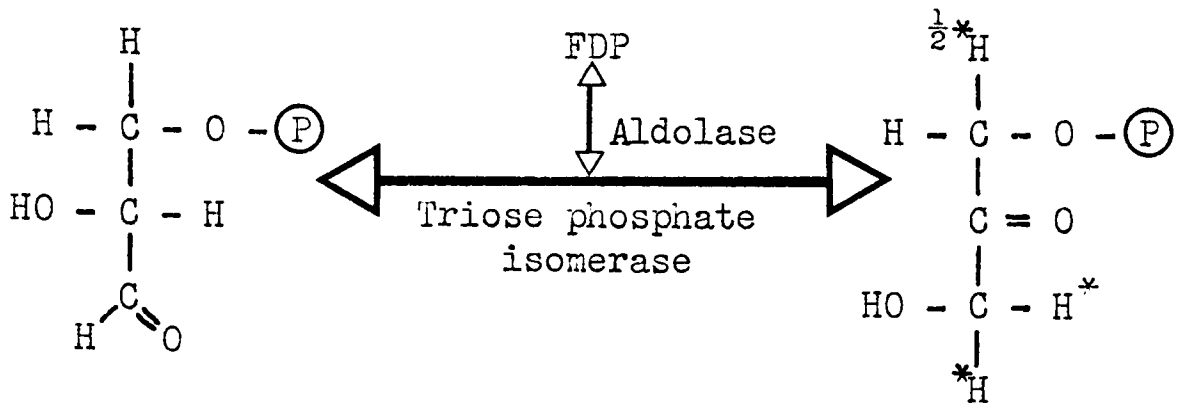
Aldolase cleaves the bond between carbon atoms three and four of fructose diphosphate (FDP). During this reaction a new C-H bond is formed on carbon-3 of dihydroxyacetone phosphate, and a hydroxyl group on C-4 is oxidised to form the aldehyde group of glyceraldehyde phosphate (GAP). Thus again there is a possibility of either an intramolecular shift of hydrogen (from the hydroxyl-group of C-4 to the C-3) or an exchange of protons with the medium. In fact, the hydrogen gained by the carbinol-carbon of DHAP is from the solvent (see Horecker et al., 1972). Aldolase is able to distinguish



between the two hydrogens on the carbinol-carbon of DHAP such that when the aldolase reaction is reversed (ie formation of FDP) it is the hydrogen that was gained from the medium (when FDP was split to give triose phosphates) which is lost. Thus incubation of FDP with aldolase and tritiated water leads to tritium incorporation into DHAP but not into FDP (Rose and Reider, 1955).

II.4 The Triose phosphate isomerase reaction

In the triose phosphate isomerase reaction, there is a simultaneous loss of hydrogen from one carbon atom and gain of hydrogen on a neighbouring carbon atom. Therefore, as was the case with the phosphoglucose isomerase and aldolase reactions, the triose phosphate isomerase reaction could proceed via two possible mechanisms. The reaction could involve either an intramolecular hydride shift of hydrogen or an extramolecular exchange of hydrogen. Experiments



Glyceraldehyde-3-phosphate (GAP)

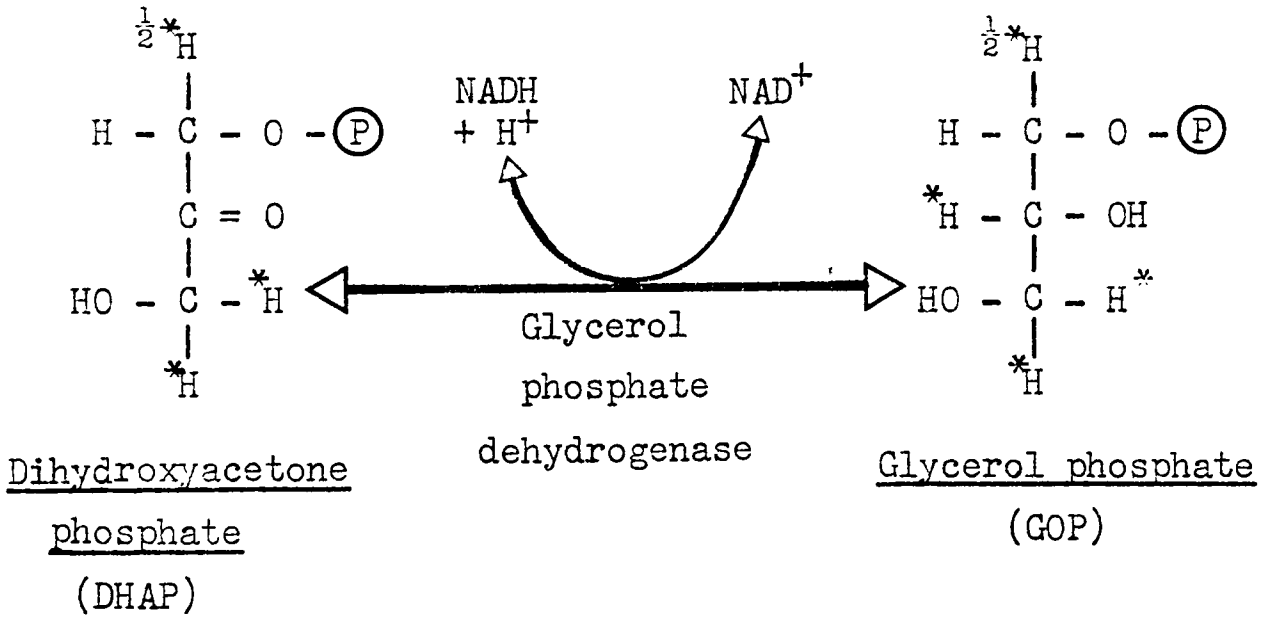
Dihydroxyacetone phosphate (DHAP)

have shown that the isomerisation between DHAP and GAP is by the second alternative, and proceeds via an exchange of protons with water (Rieder and Rose, 1959). There are two hydrogen atoms on carbinol-carbon of DHAP and the question arises as to whether these two are lost with equal frequency or whether one of the hydrogens is lost in preference to the other. In fact there is complete stereospecificity with respect to these hydrogens, meaning that it is always the same one that is lost in the triose phosphate isomerase reaction; this hydrogen is the one that was on C-3 of FDP (Katz and Rognstad, 1966). Thus, when FDP is split in the aldolase reaction, the DHAP produced gains a proton (or tritium if it is present) from the solvent. If DHAP then passes through the triose phosphate isomerase reaction, the other proton on the carbinol-carbon atom of DHAP will be exchanged with the solvent. Hence, since the triose phosphate isomerase reaction is at or near equilibrium in adipose tissue (Saggerson and Greenbaum, 1970), both of the hydrogens on the carbinol-carbon of DHAP will have been exchanged with the solvent and therefore both of them can be tritiated if the reactions are carried out in tritiated water.

It has been shown above how the two hydrogen atoms on the carbinol-carbon of DHAP can be exchanged with water in the triose phosphate

isomerase and aldolase reactions. However, when FDP is split it yields DHAP and GAP, and this reaction does not lead to exchange of the hydrogen on the carbinal-carbon of GAP. Therefore, when GAP passes through the triose phosphate isomerase reaction, only one of the hydrogens on the carbinol-carbon of DHAP will have been gained from the solvent. The other hydrogen (that is, the one that was originally on C-4 of FDP) can be exchanged if the DHAP produced from GAP is passed through the aldolase reaction to make FDP. For this to occur, the aldolase reaction must be at or near equilibrium, and this is thought to be the case in adipose tissue (Saggerson and Greenbaum, 1970).

II.5 The glycerol phosphate dehydrogenase reaction



Dihydroxyacetone phosphate (DHAP)

Glycerol phosphate (GOP)

An analysis of the distribution of the tritium atoms incorporated into the glycerol moiety of triglyceride revealed that the alpha-carbon atoms between them gained 2.40 tritium atoms and the beta-carbon gained 0.94 (Jungas, 1968). The last stage in the formation of the glycerol phosphate is the reduction of DHAP. Since there is no hydrogen bound to the beta-carbon atom of DHAP (because of the keto-group), the tritium bound to the beta-carbon of glycerol phosphate must be gained in the glycerol phosphate dehydrogenase

reaction. However, the mechanism of the glycerol phosphate dehydrogenase reaction has not been studied in detail, and it is not possible to way whether this tritium atom is gained directly from water or if it is passed on from NADH.

II.6 Summary

In summary, consider the conversion of one molecule of glucose to two molecules of glycerol phosphate (GOP). Of the ten carbon-bound hydrogen atoms present on the two GOP molecules, only seven of them can exchange with water. Six of them (ie three per GOP molecule) will be gained if the triose phosphate isomerase and aldolase reactions are at equilibrium. The seventh tritium atom will be gained by only one of the two GOP molecules (the old carbons 1, 2 and 3 of glucose) in the phosphoglucose isomerase reaction. Hence an average of 3.5 tritium atoms per glycerol phosphate can be expected. The experimental value found by Jungas (1968) of 3.3 tritium atoms per glycerol phosphate fits very well with this predicted value.

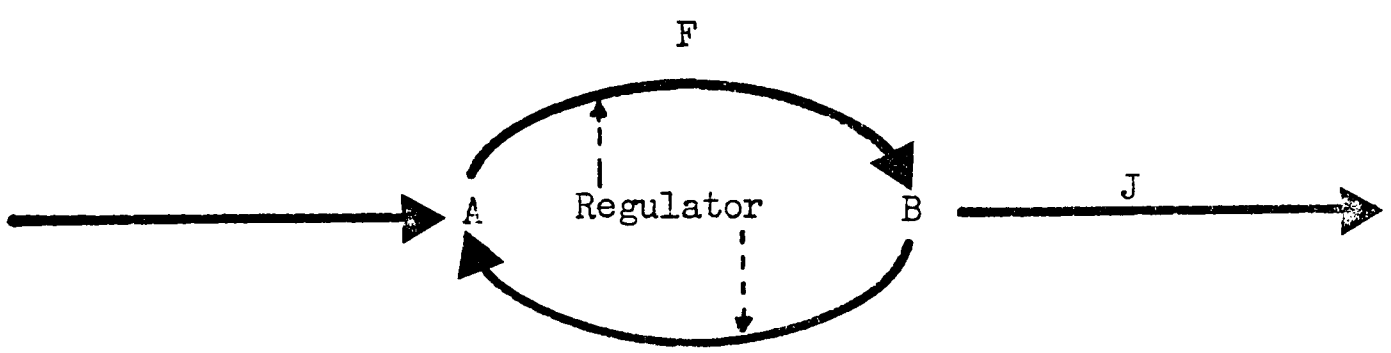
It is possible that at high rates of glycolysis or during times of high demand for glycerol phosphate the phosphoglucose isomerase, aldolase and triose phosphate isomerase reactions might depart from equilibrium. If the departure from equilibrium is pronounced, then this would lead to a smaller number of tritium atoms incorporated per glycerol phosphate molecule. However, this number would not be expected to drop below about 2½ tritium atoms per glycerol phosphate. This is because only one extra tritium atom on each of GAP and DHAP is gained if the triose phosphate isomerase and aldolase reactions are at equilibrium, and only about a half a tritium is gained per F6P molecule (equal to a quarter of a tritium per glycerol phosphate) if the phosphoglucose isomerase

reaction is at equilibrium. Hence the maximum error possible in estimating the rate of triglyceride-glycerol synthesis by using a figure of 3.3 tritium atoms per glycerol phosphate is to underestimate the rate of triglyceride-glycerol synthesis by about 30%. The extent of departure of the above reactions from equilibrium can only be determined by experiment (see section 3.5.2.2).

APPENDIX III

Derivation of the relationship between the sensitivity of the forwards and backwards reactions of a substrate cycle and the sensitivity of the response of the pathway flux to changes in the concentration of regulator

In appendix I an equation describing the sensitivity of a substrate cycle due to regulation of the activity of the forwards reaction of the cycle was derived. In this appendix, an equation to describe the sensitivity of a substrate cycle if both the forwards and reverse reactions of the cycle are regulated is derived.



At constant concentrations of A and B,

$$J = F - C$$

If a change in the concentration of regulator causes changes ΔF and ΔC in the rates of the forward reaction and cycling respectively, then at constant concentrations of A and B,

$$J + \Delta J = (F + \Delta F) - (C + \Delta C)$$

$$= F - C + \Delta F - \Delta C$$

$$\Rightarrow \quad \Delta J = \Delta F - \Delta C$$

Dividing through by J,

$$\frac{\Delta J}{J} = \frac{\Delta F - \Delta C}{J} = \frac{\Delta F - \Delta C}{FC} \times \frac{FC}{J}$$

$$= \frac{FC}{J}\left(\frac{\Delta F}{FC} - \frac{\Delta C}{FC}\right)$$

By definition, $\frac{\Delta F}{F} = F_{rel}$, $\frac{\Delta C}{C} = C_{rel}$ and $\frac{\Delta J}{J} = J_{rel}$

$$\therefore \quad J_{rel} = \frac{FC}{J}\left(\frac{F_{rel}}{C} - \frac{C_{rel}}{F}\right)$$

$$= \frac{F.F_{rel}}{J} - \frac{C.C_{rel}}{J}$$

$$= \frac{F.F_{rel} - C.C_{rel}}{J}$$

Dividing J_{rel} by the relative change in the concentration of regulator, $[R]_{rel}$, gives the sensitivity of the response of the pathway flux to a change in the concentration of regulator, i.e. $\frac{J_{rel}}{[R]_{rel}}$

$$\frac{J_{rel}}{[R]_{rel}} = [R]_{rel}^{-1}\left(\frac{F.F_{rel} - C.C_{rel}}{J}\right)$$

$$= \frac{1}{J}\left(\frac{F.F_{rel} - C.C_{rel}}{[R]_{rel}}\right)$$

But $J = F - C \quad \Rightarrow \quad F = J + C$

$$\frac{J_{rel}}{[R]_{rel}} = \frac{1}{J}\left(\frac{(J + C)\,F_{rel} - C.C_{rel}}{[R]_{rel}}\right)$$

$$= \frac{1}{J}\left(\frac{J.F_{rel} + C.F_{rel} - C.C_{rel}}{[R]_{rel}}\right)$$

$$= \frac{1}{J}\left(\frac{J.F_{rel} + C(F_{rel} - C_{rel})}{[R]_{rel}}\right)$$

$$\Rightarrow \quad \frac{J_{rel}}{[R]_{rel}} = \frac{F_{rel}}{[R]_{rel}} + \frac{C}{J}\left(\frac{F_{rel} - C_{rel}}{[R]_{rel}}\right)$$

If the sensitivities of the forwards and backwards reactions of the cycle are denoted by n_f and n_b respectively, then

$$\frac{F_{rel}}{[R]_{rel}} = n_f \quad \text{and} \quad \frac{C_{rel}}{[R]_{rel}} = n_b$$

$$\therefore \quad \frac{J_{rel}}{[R]_{rel}} = n_f + \frac{C}{J}\left(n_f - n_b\right)$$

$$= n_f \left(1 + \frac{C}{J}\left(\frac{n_f - n_b}{n_f}\right)\right)$$

This equation shows that if the sensitivity of an enzyme to a change in the concentration of regulator is denoted by n_f, then operation of a substrate cycle will improve the sensitivity of control of flux through a pathway by a factor of $\left(1 + \frac{C}{}\left(\frac{n_f - n_b}{n_f}\right)\right)$

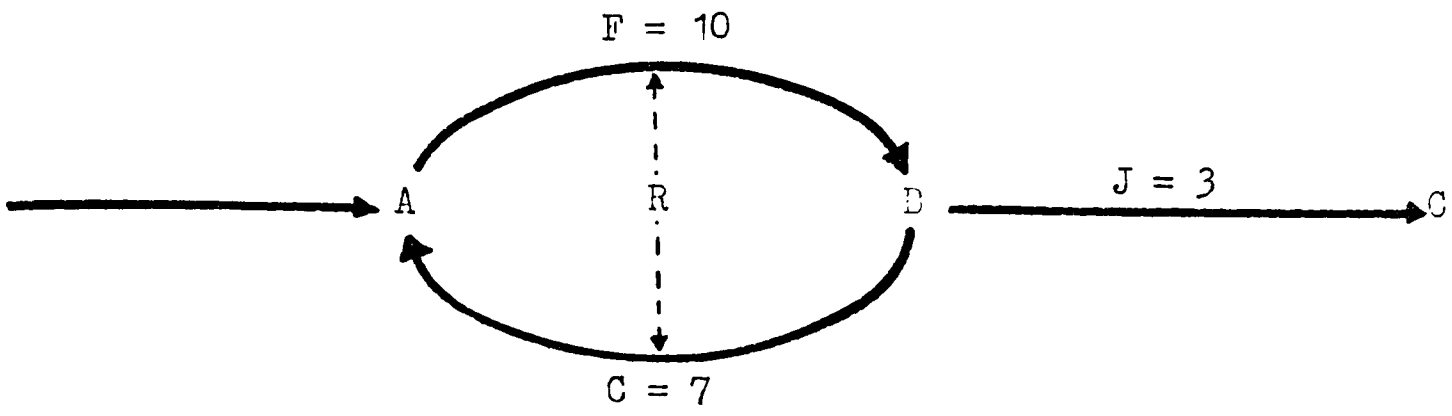
Note that if the back reaction does not change, i.e. $n_b = 0$, then the equation simplifies to that derived in appendix I.

If the flux through a pathway is known, together with the rate of cycling and the sensitivities of the forward and backward reactions to the regulator, then the response of the pathway flux to changes in the concentration of regulator can be determined. Note that the back reaction of the cycle may be activated or inhibited by the regulator molecule; if it is inhibited, then C and n_b are positive. In the former case, changes in the back reaction lead to increased sensitivity of control of pathway flux relative to the latter case, which decreases the sensitivity of control. Numerical examples illustrating the changes in pathway flux with different values of n_f and n_b are shown in table III.1.

The equation above shows how, at a given rate of cycling, the increase in sensitivity that the substrate cycle will give to the control of pathway flux can be predicted if the individual sensitivities of the forward and backward reactions are known. If these sensitivities are not known, then the response of the pathway flux to a change in the concentration of regulator cannot be predicted on the basis of a measurement of the rate of substrate cycling. However, as shown below,

Table III.1 Numerical examples showing the effect of different sensitivities of the forwards and backwards reactions of a substrate cycle on the sensitivity of control of pathway flux

Sensitivity of		New flux after 10% change in regulator			Sensitivity of pathway flux to change in regulator	
forward reaction n_f	backward reaction n_b	Forward flux	Cycling flux	Pathway flux	$\frac{\%\text{ change in J}}{\%\text{ change in R}}$	$n_f \left[1 + \frac{C}{J}\left(\frac{n_f + n_b}{n_f}\right)\right]$
1	0	11	7	4	$\frac{33}{10} = 3.3$	$1\left[1 + \frac{7}{3}\left(\frac{1-0}{1}\right)\right] = 3.3$
1	+1	11	7.7	3.3	$\frac{10}{10} = 1$	$1\left[1 + \frac{7}{3}\left(\frac{1-1}{1}\right)\right] = 1$
1	-1	11	6.3	4.7	$\frac{56.7}{10} = 5.67$	$1\left[1 + \frac{7}{3}\left(\frac{1+1}{1}\right)\right] = 5.67$
3	+1	13	7.7	5.3	$\frac{76.7}{10} = 7.67$	$3\left[1 + \frac{7}{3}\left(\frac{3-1}{3}\right)\right] = 7.67$
3	-0.5	13	6.65	6.35	$\frac{112}{10} = 11.2$	$3\left[1 + \frac{7}{3}\left(\frac{3+0.5}{3}\right)\right] = 11.2$



The substrate cycle is as illustrated above. R is a regulator of the forward and backward reactions of the cycle. Table III.[1] shows the new values for F, C and J after a 10% change in the concentration of regulator. The sensitivities assumed for the forwards and backwards reactions are given in the left-hand column; a negative sensitivity means that R inhibits the enzyme.

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it is possible to determine whether substrate cycling has increased the sensitivity of the response of the pathway flux in a tissue which is treated with two different concentrations of effector.

Suppose that a tissue is incubated with a given concentration of regulator molecule and that the rate of substrate cycling and the pathway flux are measured under these conditions. If another piece of tissue is incubated with a different concentration of regulator molecule, and the rates of substrate cycling and the pathway flux are measured under these new conditions, then it is possible to determine the fractional increase in the rate of the forwards and backwards reactions of the cycle, i.e. the sensitivities of the reactions.

Let the ratio of these increases be given by Q, i.e.

$$Q = \frac{\text{fractional increase in forwards reaction}}{\text{fractional increase in backwards reaction}} = \frac{n_f}{n_b}$$

Hence,
$$n_b = \frac{n_f}{Q}$$

Therefore,
$$\left(\frac{n_f - n_b}{n_f}\right) = \frac{n_f - \left(n_f/Q\right)}{n_f}$$

$$= \frac{Qn_f - n_f}{Qn_f}$$

$$= \frac{Q - 1}{Q}$$

The equation derived for the sensitivity of the response of the pathway flux to a change in the concentration of regulator, i.e.

$$\text{Sensitivity} = n_f \left(1 + \frac{C}{J}\left(\frac{n_f - n_b}{n_f}\right)\right)$$

requires that the sensitivity of the response of the forwards and backwards reactions be known. Taking, for example, the case of the activation of lipolysis in fat cells in response to noradrenaline, to estimate the value of n_f would require very detailed knowledge of the kinetic properties of the triglyceride lipase and protein kinase enzymes, together with the concentrations of cAMP in the cell under different hormonal conditions. Similarly, to estimate n_b, a detailed knowledge of the properties of the esterification system under the conditions found within the cell would be needed. The problem of needing to know the concentrations of regulator molecules (be they secondary messengers, substrates or hormones) can be overcome by changing the concentration of the hormone and observing the changes in the rate of forwards and backwards reactions. Thus for example one concentration of hormone could be zero and the second concentration need not be known. All that is required is to observe the changes in

the rates of cycling and pathway flux. Knowing these changes and hence the ratio Q,

$$\text{Sensitivity} = n_f \left(1 + \frac{C}{J}\left(\frac{n_f - n_b}{n_f}\right)\right) = n_f \left(1 + \frac{C}{J}\left(\frac{Q - 1}{Q}\right)\right)$$

The increase in the sensitivity of the response of the pathway flux that was actually due to the operation of a substrate cycle is therefore given by,

$$1 + \frac{C}{J}\left(\frac{Q - 1}{Q}\right)$$

Each of the variables in this equation can be determined relatively easily by experiment and hence this equation can be used to study the sensitising properties of substrate cycles.

APPENDIX IV

Summary of reactions involved in the biosynthesis of fatty acids from glucose showing how a net synthesis of ATP is achieved

In a careful accounting of the requirements for acetyl CoA and reducing power in the form of NADPH, Flatt (1970) showed that during fatty acid synthesis from glucose a net phosphorylation of ADP should occur inside the adipocyte. The exact amount of ATP produced per fatty acid molecule synthesised would depend on the relative contributions of the malate cycle (Appendix IV Fig 1) and the pentose phosphate pathway to the supply of NADPH. An example illustrating how ATP is produced by the reactions of the fatty acid synthetic pathway is given in fig IV.2; this example is summarised from McGilvery (1979). In this example, 57% of the NADPH required for fatty acid synthesis is provided by the malate cycle and the ramining reducing power comes from the pentose phosphate pathway. It should be pointed out that the actual contribution of the malate cycle to the supply of NADPH is more like 40%, with the consequence that greater than 5ATP molecules per palmitate synthesised is actually produced in the cell (Flatt, 1970). However, this example is used since it illustrates the pathways involved and also balances satisfactorily without being too dissimilar from the stoichiometry of fatty acid synthesis which is considered to occur in the adipocyte (Flatt, 1970).

In stage 1 (see fig IV.2) of the synthesis of fatty acids from glucose, glucose is phosphorylated to give G6P. Of the $4\frac{1}{2}$ molecules of glucose required to make one molecule of palmitic acid, three of them pass through the pentose phosphate pathway (stage 2a in fig IV.2) and generate 6 molecules of NADPH. The other $1\frac{1}{2}$ G6P molecules, together with 2 molecules of fructose-6-phosphate produced by the three G6P molecules passing through the pentose phosphate pathway,

pass through the glycolytic pathway to give 7 molecules of GAP (stage 2c in fig IV.2). Together with one GAP molecule produced in stage 2a, the total of 8 GAP molecules complete the glycolytic pathway to form 8 molecules of pyruvate (stage 3 in fig IV.2) and 8 molecules of NADH.

After entering the mitochondrion, the pyruvate is oxidised by pyruvate dehydrogenase to Acetyl CoA (stage 4). Transfer of the AcCoA out of the mitochondrion is achieved by forming citrate (see fig IV.1). Citrate in the cytosol is converted to AcCoA and oxaloacetate, the latter of which is reduced to malate by the NADH produced in stage 3, and then oxidised to pyruvate thus producing NADPH (see fig IV.1; stage 5 of fig IV.2). Finally, the AcCoA in the cytosol is condensed to form palmitate, using the NADPH produced in reactions 2a and 5. Overall in this example, there is a net production of 5 ATP molecules per palmitic acid synthesised, and it is this ATP production that Flatt (1970) suggested could limit the rate of fatty acid synthesis.

Fig IV.2 The reactions involved in the synthesis of fatty acids from glucose (McGilvery, 1979)

① $4\frac{1}{2}$ glucose + $4\frac{1}{2}$ ATP ⟶ $4\frac{1}{2}$G6P + $4\frac{1}{2}$ ADP

②a 3G6P + 6NADP ⟶ GAP + 2F6P + $3CO_2$ + 6NADPH

ⓑ $1\frac{1}{2}$ G6P ⟶ $1\frac{1}{2}$ F6P

ⓒ $3\frac{1}{2}$ F6P + $3\frac{1}{2}$ ATP ⟶ 7GAP + $3\frac{1}{2}$ ADP

③ 8GAP + 8NAD + 16ADP + $8P_i$ ⟶ 8 pyruvate + 8NADH + 16ATP

④ 8 pyruvate + $4O_2$ + 24(ADP + P_i) + 8CoA ⟶ $8AcCoA_{(mito)}$ + $8CO_2$ + 24ATP

⑤ $8AcCoA_{(mito)}$ + 8NADH + 8NADP + 20ATP ⟶ $8AcCoA_{(cytosol)}$ + 8NAD + 8NADPH + 20(ADP+P_i)

⑥ $8AcCoA_{(cytosol)}$ + 7ATP + 14NADPH ⟶ palmitate + 7(ADP+P_i) + 14NADP + 8CoA

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⑦ $4\frac{1}{2}$ glucose + $4O_2$ + 5(ADP+P_i) ⟶ palmitate + $11CO_2$ + 5ATP

Key for Fig IV.1

1 = Glycolytic pathway

2 = Pyruvate dehydrogenase

3 = Citrate synthase

4 = ATP citrate lyase

5 = NAD^+-linked malate dehydrogenase

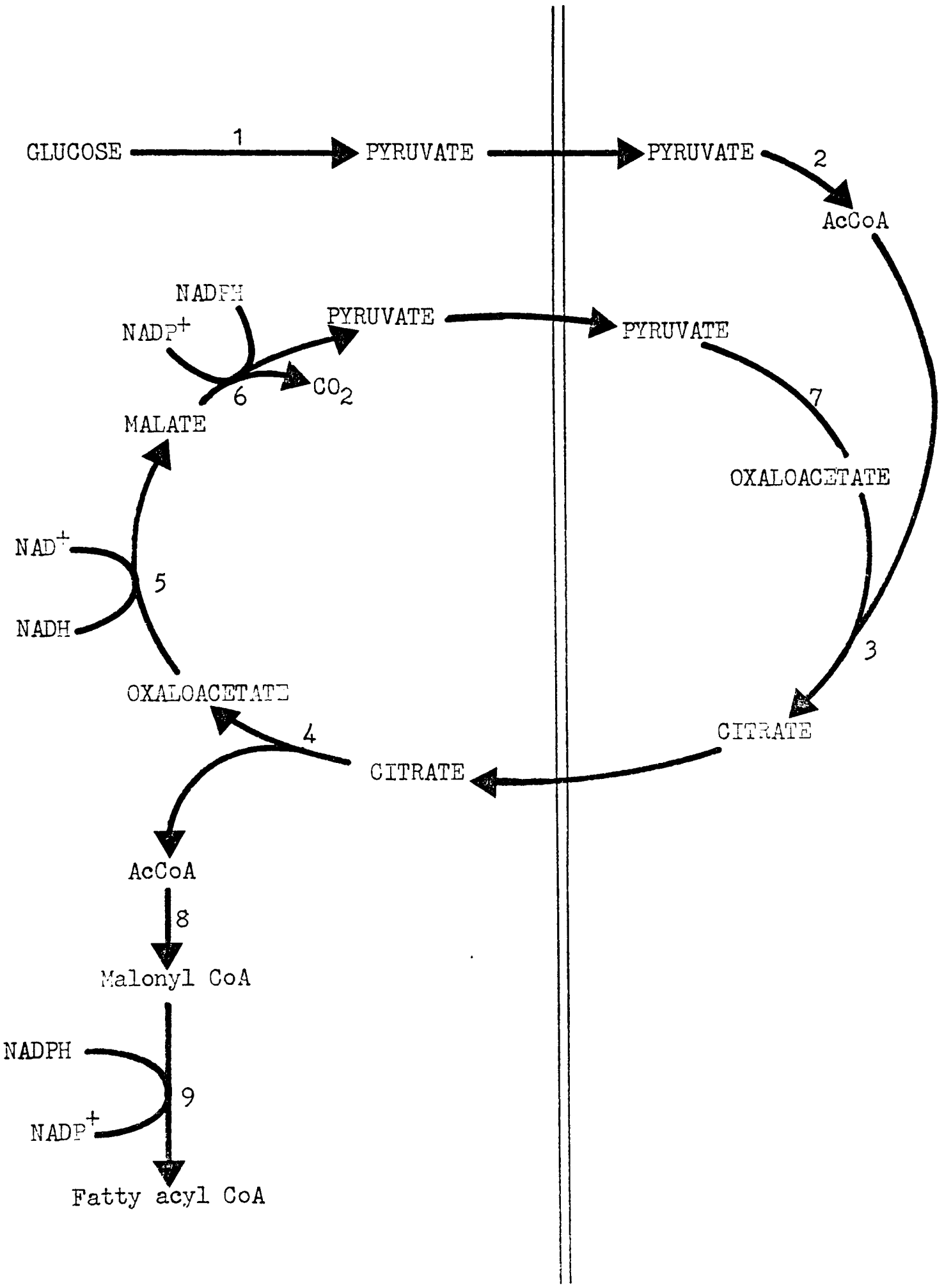
6 = $NADP^+$-linked malate dehydrogenase ("malic enzyme")

7 = Pyruvate carboxylase

8 = Acetyl CoA carboxylase

9 = Fatty acyl CoA synthetase

Fig IV.1 The malate cycle in adipose tissue showing how both carbon precursors and reducing power is transfered from the mitochondrion to the cytosol (from Newsholme & Start, 1973)



APPENDIX V

Appraisal of the use of tritiated water in vivo

To the author's knowledge, tritiated water has not been used as a means of estimating quantitatively the rate of triglyceride synthesis in the intact conscious animal. A comparison between rates of tritium and ^{14}C-glucose incorporation into lipid components in adipose tissue incubated *in vitro* has been made by Jungas (1968, see chapter 3), and tritium incorporation has been used qualitatively *in vitro* to follow triglyceride synthesis (see Sooranna & Saggerson, 1975). The problem with using tritium incorporation into the triglyceride-glycerol moiety as a quanitiative indicator of triglyceride synthesis is that the number of tritium atoms that are incorporated into the glycerol phosphate molecule during its synthesis must be known. As discussed in chapter 3, it is possible that the number of tritium atoms per glycerol phosphate molecule could vary according to the demand for glycerol phosphate. However, consideration of the reaction mechanisms of the enzymes involved in the synthesis of glycerol phosphate from glucose shows that there is a maximum and a minimum number of tritium atoms that can be incorporated into glycerol phosphate during its synthesis. The glycerol phosphate molecule can have between about 2.3 and 3.3 carbon-bound tritium atoms. The experiments discussed in chapter 3 show that the actual number of tritium atoms per glycerol phosphate is close to the maximum possible number that is predicted from the consideration of the enzyme reaction mechanisms. Also, this number was found to vary very little over the range of demands for glycerol phosphate that is seen in adipose tissue incubated *in vitro* under a variety of hormonal treatments.

In the experiments reported in chapter six , the calculations for the rate of triglyceride synthesis have all assumed 3.3 tritium

atoms to be present in each glycerol phosphate molecule incorporated into triglyceride. Hence, the results reported in chapter six will represent a minimum estimate of the rate of triglyceride synthesis. Since the rate of TG-FFA cycling is estimated by finding the rate of triglyceride synthesis and then subtracting the rate of fatty acid synthesis, the rate of TG-FFA cycling that is derived should also be a minimum estimate.

In many of the *in vivo* treatments reported in chapter six, the rate of triglyceride synthesis by far exceeds the rate of *de novo* fatty acid synthesis. If the fatty acids that are required to support this rate of triglyceride synthesis do not come from *de novo* synthesis, and they do not come from outside the cell (use of Triton WR-1339 stops the input from blood lipoproteins), then there seems little doubt that there must be TG-FFA cycling occuring *in vivo*.

An alternative explanation is that there is a flaw in the method of measuring triglyceride synthesis by tritium incorporation. One possible objection is that non-enzymatic incorporation of tritium into the glycerol moiety of lipis may be occuring. However, an experiment designed to measure the rate of non-enzymatic tritium incorporation showed that this rate was very low and could be ignored (see chapter 2). Other errors are possible if the lipid is not completely separated from the large amounts of tritiated water that are present, or if the scintillation-counting of the glycerol released from the hydrolysed lipid is not accurate. Both of these have been carefully guarded against (see chapter 2). It therefore appears that the TG-FFA cycle does exist *in vivo*.

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ABBREVIATIONS USED IN THIS THESIS

Ac CoA	Acetyl coenzyme A
ACTH	Adenocorticotrophic hormone
ADA	Adenosine deaminase
ADP	Adenosine-5'-diphosphate
AMP	Adenosine-5'-monophosphate
ATP	Adenosine-5'-triphosphate
cAMP	Adenosine-3',5'-cyclic monophosphate
CoA	Coenzyme A
DG	Diacylglycerol
DHAP	Dihydroxyacetone phosphate
EC_{50}	Concentration for 50% response
FDP	Fructose-1,6-diphosphate
FFA	Free fatty acid
F6P	Fructose-6-phosphate
GAP	Glyceraldehyde phosphate
GDP	Guanosine-5'-diphosphate
GOP	Glycerol phosphate
GOPAT	Glycerol phosphate acyl transferase
GTP	Guanosine-5'-triphosphate
G6P	Glucose-6-phosphate
Ins	Insulin
K_i	Inhibitor constant
K_m	Michaelis constant
MG	Monoacylglycerol
NA	Noradrenaline
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
NADP	Nicotinamide adenine dinecleotide phosphate

NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
PEP	Phosphoenolpyruvate
PG-E_1	Prostaglandin E_1
P_i	Inorganic phosphate
PP_i	Inorganic pyrophosphate
PTU	Propyl thiouracil
TG	Triacyl glycerol
TG-DG cycle	Triacyl glycerol - diacyl glycerol substrate cycle
TG-Glycerol moiety	Glycerol moiety of triacyl glycerol
TG-FFA cycle	Triacyl glycerol - free fatty acid sybstrate cycle
TSH	Thyroid stimulating hormone
T-3	Triiodothyronine
T-4	Thyroxine (= tetraiodothyronine)
V_{max}	Maximal velocity of enzyme reaction

ENZYMES DISCUSSED IN THE TEXT

Enzyme	E.C. No.	Systematic name
Acetyl CoA carboxylase	6.4.1.2	Acetyl CoA: carbon dioxide ligase (ADP-forming)
Adenosine deaminase	3.5.4.4	Adenosine aminohydrolase
Adenosine kinase	2.7.1.20	ATP:adenosine 5'-phosphotransferase
Adenylate cyclase	4.6.1.1	ATP pyrophosphate-lyase (cyclizing)
Aldolase	4.1.2.13	D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase
ATP citrate lyase	4.1.3.8	ATP:citrate oxaloacetate-lyase (pro-3S-CH_2COO^- → acetyl CoA: ATP dephosphorylating)
Citrate synthase	4.1.3.7	Citrate oxaloacetate-lyase (pro-3S-CH_2COO^- → acetyl CoA)
Diacyl glycerol acyl transferase	2.3.1.20	Acyl-CoA:1,2-diacyl glycerol O-acyltransferase
Diglyceride lipase	3.1.1.34	Diacylglycerol acylhydrolase
Fatty acyl CoA synthase	6.2.1.3	Acid:CoA ligase (AMP-forming)
Fructose-6-phosphatase	3.1.3.11	D-fructose-1,6-diphosphate 1-phosphohydrolase
Glucose-6-phosphatase	3.1.3.9	D-glucose-6-phosphate phosphohydrolase
Glyceraldehyde phosphate dehydrogenase	1.2.1.12	D-glyceraldehyde-3-phosphate: NAD^+ oxidoreductase (phosphorylating)
Glycogen synthase	2.4.1.11	UDP-glucose:glycogen 4-α-glucosyltransferase
Glycerol kinase	2.7.1.30	ATP:glycerol 3-phosphotransferase
Glycerol phosphate acyl transferase	2.3.1.15	Acyl-CoA:sn-glycerol-3-phosphate O-acyltransferase
Glycerol phosphate dehydrogenase	1.1.1.8	sn-glycerol-3-phosphate:NAD^+ 2-oxidoreductase (phosphorylating)
Hexokinase	2.7.1.1	ATP:D-hexose 6-phosphotransferase
Lactate dehydrogenase	1.1.1.27	L-lactate:NAD oxidoreductase

Monoacyl glycerol phosphate acyltransferase	2.3.1.51	Acyl-CoA:1-acyl-sn-glycerol-3-phosphate O-acyltransferase
NAD^+-linked malate dehydrogenase	1.1.1.37	L-malate:NAD^+oxidoreductase
$NADP^+$-linked malate dehydrogenase (malic enzyme)	1.1.1.40	L-malate:$NADP^+$ oxidoreductase (decarboxylating)
Monoglyceride lipase	3.1.1.23	Glycerol-monoester acylhydrolase
5' nucleotidase	3.1.3.5	5'-ribonucleotide phosphohydrolase
Phosphatidate phosphohydrolase	3.1.3.4	L-α-phosphatidate phosphohydrolase
Phosphodiesterase	3.1.4.17	3':5'-cyclic AMP 5'-nucleotidohydrolase
Phosphoenol pyruvate carboxykinase	4.1.1.49	ATP:oxaloacetate carboxy-lyase (transphosphorylating)
Phosphofructokinase	2.7.1.11	ATP:D-fructose-6-phosphate ^{1}-phosphotransferase
Phosphoglucomutase	2.7.5.1	α-D-glucose-^{1},6-biphosphate:α-D-glucose-^{1}-phosphate phosphotransferase
Phosphoglucose isomerase	5.3.1.9	D-glucose-6-phosphate ketolisomerase
Phosphorylase	2.4.1.1	1,4-α-D-glucan:orthophosphate glucosyltransferase
Protein kinase	2.7.1.37	ATP:protein phosphotransferase
Protein phosphatase	3.1.3.16	Phosphoprotein phosphohydrolase
Pyruvate carboxylase	6.4.1.1	Pyruvate:carbon dioxide ligase (ADP-forming)
Pyruvate dehydrogenase	1.2.4.1	Pyruvate:lipoamide oxidoreductase (decarboxylating and receptor acetylating)
Pyruvate kinase	2.7.1.40	ATP:pyruvate phosphotransferase
Triglyceride lipase	3.1.1.3	Triacylglycerol acyl-hydrolase
Triose phosphate isomerase	5.3.1.1	D-glyceraldehyde-3-phosphate ketol isomerase