Glycerol production and utilization measured using stable isotopes

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The rate of appearance of glycerol in the systemic circulation is determined from the enrichment of arterial blood glycerol when labelled glycerol is infused intravenously. This value provides a good measure of whole-body lipolysis during fasting, except that arterial infusion and venous sampling, if feasible, would probably give a higher more-accurate value. Lipolysis occurs primarily in adipose tissue, although other tissues contribute, notably muscle. Measurement is based on the difference in the enrichment of the glycerol entering and leaving the tissue. Lipolysis is underestimated by the extent to which glycerol released by lipolysis does not enter the systemic circulation, as occurs when lipolysis takes place in the non-hepatic tissue of the splanchnic bed. Glycerol released into the systemic circulation is utilized mainly by liver, although kidney and muscle are also major users of glycerol. Measurement of glycerol utilization is based on the amount of labelled glycerol taken up by the tissues. Other tissues probably utilize glycerol to a smaller extent, but in total this represents a significant amount. Most glycerol taken up by liver is converted to glucose. Glucose is probably the major source of glycerol-3-phosphate used in the esterification of fatty acids by adipose tissue.

Glycerol: Lipolysis: Stable isotopes

Whole-body production of glycerol is measured, using stable isotopes, from the enrichment in blood glycerol when ^{2}H- or ^{13}C-labelled glycerol is infused. Net production or utilization (uptake) of glycerol by an organ or tissue is measured by multiplying the difference between the concentration of blood glycerol entering and leaving the organ or tissue by blood flow through the organ or tissue. When the blood glycerol is labelled, uptake of the label across the organ or tissue provides a measure of the amount of glycerol utilized by the organ or tissue.

The enrichment of the glycerol leaving the organ or tissue is less than that of glycerol entering the organ or tissue and the difference is a measure of the extent of glycerol production by the organ or tissue. (When ^{3}H ^{14}C-labelled glycerol is infused, glycerol production is determined from the specific activity of blood glycerol. The difference between the level of radioactivity in blood entering and leaving the tissue or organ is then a measure of glycerol utilized (i.e. taken up).) The methods of measurement will now be examined and examples given, particularly in human subjects after long-term fasting.

Whole-body glycerol production

The rate of appearance (Ra) of endogenous glycerol in the systemic circulation is determined by infusing ^{2}H- or ^{13}C-labelled glycerol into a peripheral vein and sampling arterial blood (the venous–arterial mode). Ra at steady-state is calculated using the equation:

$$Ra = ((E_i / E_a) - 1) R,$$

where E_i is the enrichment of the infused glycerol, E_a is the enrichment of glycerol in the arterial blood and R is the rate of infusion of the glycerol (Hetenyi *et al.* 1983). Arterial infusion and venous sampling (the arterio–venous mode) is theoretically preferred, but is not feasible in human subjects (Katz & Wolfe, 1988). In the rat a higher Ra is obtained when using the arterio–venous mode than when using the venous–arterial mode (Peroni *et al.* 1996). Glycerol may be produced and then utilized within an organ or tissue. For example, glycerol released by lipolysis of triacylglycerol in splanchnic adipose tissue will be cleared almost completely by the liver (Larsen, 1963; Coppack *et al.* 1994). This

Abbreviation: Ra, rate of appearance.

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equivalent amount will not then appear in the systemic circulation and therefore will not be included in the Ra.

The Ra of glycerol in healthy subjects fasted long term is estimated to be about 5 μmol/kg body weight per min and this value is about twice that after an overnight fast (Bortz *et al*. 1972; Hetenyi *et al*. 1983; Klein *et al*. 1986).

Regional glycerol utilization

Utilization of glycerol during fasting is generally accepted to occur mainly in the liver, to a lesser extent in the kidney, and to minimal extents in other tissues. Thus, there is little utilization of glycerol in eviscerated animals (Larsen, 1963). In addition, glycerol kinase (*EC* 2.7.1.30) activity is low in tissues other than liver and kidney (Thorner & Paulus, 1973). While phosphorylation need not be the initial step in the utilization of glycerol, enzymic activities that would catalyse alternative initial steps appear minimal (Vaughan & Steinberg, 1965; Hagenfeldt & Wahren, 1968). Glycerol uptake has been reported to occur in many tissues *in vitro* including nerve, adipose and muscle tissues, but to relatively small extents (Winkler *et al*. 1969).

Tracer balance techniques offer a means of quantifying glycerol utilization. Table 1 records mean concentrations and enrichments in blood glycerol when [U-$^{13}C_3$]glycerol was infused for 5 h into subjects fasted for 60 h (Landau *et al*. 1996). The concentration of glycerol in hepatic-vein blood was 20 % and the ^{13}C enrichment 25 % that in the arterial blood, while the corresponding values in renal-vein blood were 50 % and 75 % that in arterial blood; in deep-vein blood, assumed to drain muscle, the glycerol concentration was the same as that in arterial blood and the enrichment was 66 % that in the arterial blood. From these data the net uptake (equated with utilization) of glycerol from the systemic circulation by the splanchnic bed, kidneys and muscle can be calculated, together with the amount of glycerol they produced that was released into the systemic circulation (Table 2).

The net balance across the splanchnic bed is calculated from the expression:

$$(\text{glycerol}_a - \text{glycerol}_{hv}) \times \text{SBF/body weight},$$

where glycerol_a and glycerol_{hv} are the concentrations of glycerol in arterial and hepatic vein blood respectively and SBF is splanchnic blood flow. Using the data in Table 1 the net glycerol uptake by the splanchnic bed is 1·4 μmol/kg per min. The unlabelled glycerol released into the circulation that was produced within the splanchnic bed is:

$$(\text{glycerol}_{hv} \times (E_a - E_{hv})/E_a) \times \text{SBF/body weight},$$

where E_a and E_{hv} are the enrichments in arterial and hepatic-vein glycerol respectively. Using the data from Table 1 the rate of release from the splanchnic bed is 0·3 μmol/kg per min. The total amount of glycerol from the systemic circulation that was taken up by the splanchnic bed is:

$$((\text{glycerol}_a \times E_a - \text{glycerol}_{hv} \times E_{hv})/\text{glycerol}_a \times E_a) \times \text{glycerol}_a \times \text{SBF/body weight, i.e. } 1{\cdot}7\ \mu\text{mol/kg per min.}$$

Presumably, therefore, the liver utilized 100(1·7/5·1) = 30 % of the glycerol entering the systemic circulation, the kidneys about 20 %, and muscle 10 %. In dogs which had been fasted long-term the results were similar (Previs *et al*. 1996). Balance studies have suggested that there is uptake of glycerol by muscle of human subjects fasted overnight, although results have been variable (Frayn *et al*. 1991). Sacchetti & van Hall (1999) infused [$^2H_5$]glycerol with a primer into subjects at rest for 2 h and then during 2 h of exercise. There was a release of glycerol as well as glycerol uptake by the leg, both of which increased with exercise. Coppack *et al*. (1999) have also reported uptake of glycerol by muscle. Hagenfeldt & Wahren (1968) observed uptake of [1-^{14}C]glycerol and oxidation to $^{14}CO_2$.

The site(s) of utilization of the 40 % of the Ra presumably remaining is unknown. Klein *et al*. (1996) and Coppack *et al*. (1999) found no uptake of glycerol across the adipose tissue. Elia *et al*. (1993) and Kurpad *et al*. (1994) found uptake and dilution of [$^2H_5$]glycerol across the adipose tissue and muscle. However, infusion was only for 1 h, so that steady-state may not have been achieved. In the study of Samra *et al*. (1999) subjects fasted overnight were infused with [$^2H_5$]glycerol for 7 h and arterial and adipose venous glycerol concentrations and enrichments during the last 6 h were determined. The measurements are in accordance with glycerol uptake by subcutaneous adipose tissue. In individuals fasted overnight the mean uptake of glycerol across brain was about 0·25 μmol/kg per min, but not significantly different from zero (Ahlborg & Wahren, 1972). Uptake by pulmonary tissues remains a possibility, but because of the large blood flow through the lungs, uptake could not be determined with any confidence (Landau *et al*. 1996). There was no demonstrable change in the concentration of glycerol across the lungs of fasted dogs (Borchgrevink & Havel, 1963).

Table 1. Glycerol concentration and ^{13}C enrichments of glycerol in blood collected from blood vessels on infusion of [U-$^{13}C_3$]glycerol in subjects fasted for 60 h* (From Landau *et al.* 1996)

Blood vessel	Glycerol concentration (μM/l)	^{13}C enrichment of glycerol (%)
Brachial artery	94	6·3
Hepatic vein	18	1·4
Renal vein	49	4·8
Deep vein	95	4·1

*The average blood flow through the splanchnic bed was 1·5 litre/min, 1·4 litre/min through the kidneys, and estimated to be 1·3 litre/min through total-body muscle. The rate of appearance of glycerol averaged 5·1 μmol/kg body weight per min. Average weight of the subjects was 82 kg.

Table 2. Net glycerol uptake, glycerol production (release into the systemic circulation), and utilization (μmol/kg body weight per min) by the splanchnic bed, kidneys and muscle*

Site	Net uptake	Production	Utilization
Splanchnic bed	1·4	0·3	1·7
Kidneys	0·8	0·2	1·0
Muscle	0·0	0·5	0·5
Total	2·2	1·0	3·2

*Values were calculated from data shown in Table 1 (for details of calculations, see p. 974).

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Regional glycerol production

Of the 5·1 μmol glycerol/kg body weight per min appearing in the systemic circulation, 1·0 μmol/kg body weight per min, i.e. 20 %, was produced by the splanchnic bed, kidneys and muscle. This finding is in accordance with lipolysis of triacylglycerol in adipose cells being the major source of glycerol in the fasted state. As a result of the uptake by the liver, more glycerol was produced in the splanchnic bed than was released. To a lesser extent more glycerol must have been produced than was released by kidney and muscle.

Essentially all glycerol production in the splanchnic bed appears to originate from omental and mesenteric adipose tissue (Jensen & Johnson, 1996). However, in post-operative patients fasted overnight there was no net release of glycerol into the portal circulation (Bjorkman *et al*. 1990). In addition, evidence for minimal production (at most) by liver was reported by Basso & Havel (1970), who infused [1-^{14}C]palmitate into fasted dogs and found that the specific activity of free fatty acid leaving the liver was the same as that entering the liver. In the perfused liver, glycerol formation by hydrolysis of glycerol-3-phosphate has been suggested to occur (Previs *et al*. 1995), but this process was not found to occur in human subjects (Diraison & Beylot, 1998). In fasted dogs less than 10 % of the free fatty acid flux has been estimated to be derived from mesenteric lipolysis (Basso & Havel, 1970; Wasserman *et al*. 1989). However, in the subjects fasted for 60 h, assuming glycerol was not taken up by the non-hepatic splanchnic tissues and dilution of the enrichment of the glycerol entering the splanchnic bed occurred from glycerol released from the non-hepatic splanchnic tissues, glycerol production by those tissues was:

$(94 \times 1{\cdot}5 \times ((6{\cdot}3 - 1{\cdot}4)/6{\cdot}3))/82 = 1{\cdot}3$ μmol/kg per min,
i.e. glycerol$_a$ × SBF $((E_a - E_{hv})/E_a)$/body weight,

i.e. about 30 % of the Ra of glycerol, even though only 0·3 μmol/kg per min was released into the systemic circulation.

Glycerol and Re-esterification

Whole-body glycerol production, measured as the Ra, has been taken as the measure of whole-body lipolysis. The amount of fatty acid released into the circulation would therefore be three times the rate of glycerol production, i.e. triacylglycerol → glycerol + three fatty acids, which is supported by measurements of free fatty acid turnover (Klein *et al*. 1986; Coppack *et al*. 1994). However, measurements of glycerol and fatty-acid turnover, and the amount of fatty acid oxidized (as determined by indirect calorimetry) during fasting have shown that the level of fatty acids released into the circulation is greater than that utilized (Klein *et al*. 1986; Bonadonna *et al*. 1990; Wolfe *et al*. 1990; Campbell *et al*. 1992; Wolfe, 1992*a*,*b*). During long-term fasting 60–70 % of the fatty acids released are estimated to be re-esterified. Re-esterification has been assumed to occur only in the liver (Hellerstein *et al*. 1993), but there is evidence that the major site is the extra-hepatic tissues (Diraison & Beylot, 1998). Balance studies indicate fatty acid uptake by the splanchnic bed may represent only about 25 % of the amount re-esterified during long-term fasting (Landau *et al*. 1996). The major site of this re-esterification is presumably adipose tissue, with some re-esterification occurring in muscle (Figs. 1 and 2).

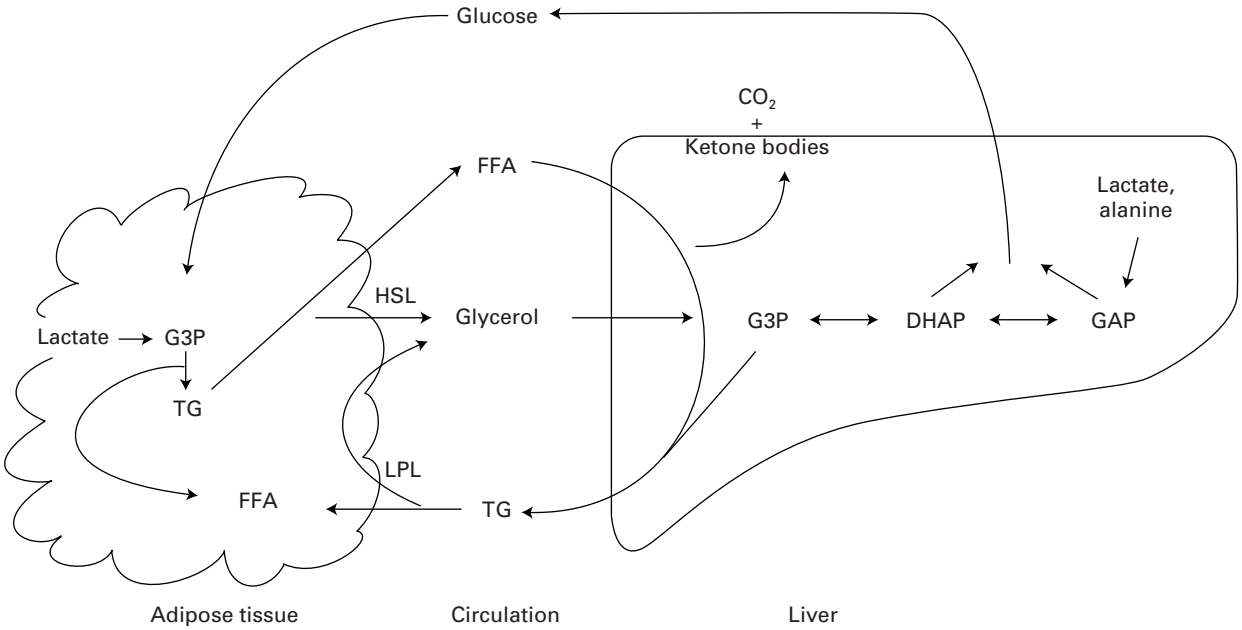

Fig. 1. Relationship between liver and adipose tissue with respect to pathways of glycerol, free fatty acid (FFA), triacylglycerol (TG), and glycerol-3-phosphate (G3P) metabolism. DHAP, dihydroxyacetone-3-phosphate; GAP, glyceraldehyde-3-phosphate; HSL, hormone-sensitive lipase; LPL, lipoprotein lipase (*EC* 3.1.1.34).

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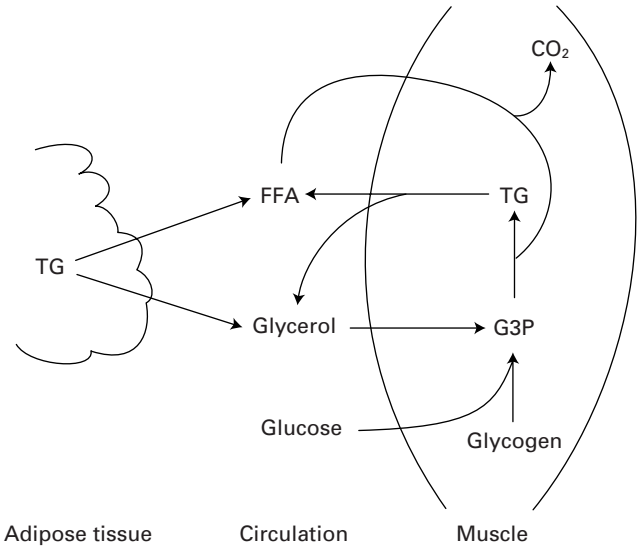

Fig. 2. Relationship between muscle and adipose tissue with respect to pathways of glycerol, free fatty acid (FFA) triacylglycerol (TG) and glycerol-3-phosphate (G3P) metabolism.

The following calculations illustrate the implications of the re-esterification. Since after 60 h of fasting the Ra of glycerol is 5 μmol/kg per min (measured by infusing labelled glycerol), 15 μmol free fatty acid/kg per min are released into the circulation and about 10 μmol/kg per min are re-esterified. About 25 %, i.e. 2·5 μmol/kg per min, is then re-esterified in the liver. Since essentially all glycerol entering the splanchnic bed is removed during a single passage, the 0·8 μmol glycerol-3-phosphate/kg per min used in re-esterification in the liver is essentially unlabelled (see glycerol synthesis). The fatty acids which are present in triacylglycerol thus formed and released into the circulation are then presumably mainly deposited as triacylglycerol in adipose tissue. Uptake of triacylglycerol across subcutaneous abdominal tissue in subjects fasted overnight has been demonstrated by arterio–venous balance (Frayn *et al*. 1993). Hydrolysis of this triacylglycerol to fatty acid and glycerol is catalysed by lipoprotein lipase (*EC* 3.1.1.34). The fatty acid released from this triacylglycerol is the same fatty acid that has been esterified with glycerol on two occasions, first before lipolysis occurred and second when the re-esterified fatty acid was redeposited in adipose tissue. The result, assuming no further processing occurs, would be a value for fatty acid Ra : glycerol Ra of < 3 (i.e. approximately 100(0·8/5) = 16 % less). However, if some free fatty acids from the splanchnic bed are released into the circulation, the ratio would be > 3. The Ra for glycerol across the subcutaneous adipose tissue has been estimated to be twice the amount of glycerol released, when determined by arterio–venous balance (Kurpad *et al*. 1994). However, glycerol released into the capillaries by the action of lipoprotein lipase will be included in the Ra of glycerol, but it will result in the underestimation of glycerol released when this is determined using the balance technique because the hydrolysis occurs after arterial sampling. The glycerol released into the capillaries would dilute the enrichment of labelled glycerol passing through the tissue, while raising the concentration of the glycerol in venous blood leaving the tissue and hence decreasing the arterio–venous difference.

Glycerol synthesis

When labelled glycerol is infused, the glycerol used for re-esterification in the liver is essentially unlabelled, because the labelled glycerol-3-phosphate formed equilibrates with dihydroxyacetone-3-phosphate and glyceraldehyde-3-phosphate (intermediates in the conversion of the other gluconeogenic substrates, e.g. unlabelled lactate and alanine, to glucose). Experimental evidence for this process was obtained when ^2H_2O was ingested by normal subjects fasted for 60 h. The enrichment of the two H bound to C-6 of blood glucose was compared with the enrichment of the two H bound to C-3 of blood glycerol (Jensen *et al*. 1999). If isotopic equilibration of glyceraldehyde-3-phosphate with glycerol-3-phosphate was complete and all the glycerol in the blood was synthesized from glycerol-3-phosphate in liver, the enrichments at C-3 of glycerol and at C-6 of glucose should be the same. The enrichment at C-3 was 17 (SE 3) % (*n* 4) that at C-6. Thus, about 17 % of the 5 μmol glycerol/kg per min entering the systemic circulation (i.e. 0·8 μmol/kg per min) was synthesized in the liver rather than released from triacylglycerol stores. This amount is in agreement with the 0·8 μmol glycerol/kg per min estimated to be released during re-esterification of the fatty acids. In a study using labelled glycerol and glucose, 17 % of the glycerol in the circulation of rats fasted for 24 h was attributed to synthesis (DeFreitas & Depocas, 1970).

Glycerol-3-phosphate required for the esterification of the fatty acids in adipose tissue cannot be derived from glycerol to any significant extent. Thus, as previously noted, Klein *et al*. (1996) could not detect uptake of glycerol by adipose tissue. Based on the low activity of glycerol kinase, glycerol utilization by adipose tissue has been estimated to account for < 1 % of the glycerol production (Kurpad *et al*. 1994). In order to esterify the 10 μmol fatty acids calculated to be cycled in a subject fasted for 60 h, 1·67 μmol glucose would need to be converted to glycerol-3-phosphate (about 20 % of the glucose production for a human subject fasted for 60 h). Approximately 80 % of the glycerol taken up by the liver (and kidney) is converted to glucose in a human subject fasted for 60 h (Landau *et al*. 1996). A glucose–glycerol cycle then appears to be active, with glycerol from adipose tissue triacylglycerol being converted to glucose in liver (and kidney) and the glucose being converted to glycerol-3-phosphate in adipose tissue to form triacylglycerol. The uptake of glucose by adipose tissue would have to occur in the presence of a high blood glucagon : insulin value. Alternatively, the glycerol-3-phosphate could be formed by glyceroneogenesis from lactate and/or alanine (Reshef *et al*. 1970; Hanson *et al*. 1971). However, blood flow through adipose tissue is not sufficient to provide these substrates in sufficient quantities for them to make a major contribution. In addition, net production of lactate has been reported in adipose tissue of human subjects fasted for 60 h (Landau *et al*. 1996), and also in adipose tissue from rats fasted for 48 h (Newby *et al*. 1990). Glycerol-3-phosphate used in esterification in muscle

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could be derived from muscle glycogen, as well as from glucose (Fig. 2).

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