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Effect of Glucose Load and of Insulin on the Metabolism of Glucose and of Palmitate in Sheep

BY C. E. WEST* AND R. F. PASSEY
School of Rural Science, University of New England, Armidale, New South Wales, Australia

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1. Simultaneous measurements of the entry rates of palmitate and glucose have been made in Merino sheep (wethers), starved for 24hr., by using constant infusions of [9,10-$^3H_2$]palmitate and [U-^{14}C]glucose. 2. The infusion of glucose into the peripheral circulation of the sheep lowered the endogenous entry of both glucose and palmitate. Since palmitate is roughly metabolically representative of the free fatty acid fraction, there was no marked change in the calories available to the sheep. 3. The infusion of insulin into either the peripheral or portal circulation increased the uptake of glucose and decreased the uptake of palmitate by the tissues of the sheep. 4. The infusion of insulin into the peripheral circulation produced a depression in glucose entry after about 80min., whereas the infusion of insulin into the portal circulation produced an almost immediate depression in glucose entry. 5. The hypoglycaemia produced gave rise to an increase in free fatty acid production followed by an increase in glucose production. 6. No direct effect of insulin on the metabolism of free fatty acids has been demonstrated by the techniques used. The effect of insulin on the metabolism of free fatty acids is apparently mediated through its effect on glucose metabolism.

The administration of glucose and insulin to animals causes a marked decline in the concentration of plasma FFA.† This has been shown in humans (Dole, 1956; Gordon & Cherkes, 1956), dogs (Shafrir, Sussman & Steinberg, 1959) and sheep (Annison, 1960). This decline in plasma FFA concentration may reflect increased utilization or decreased production of FFA (if FFA 'space' is unchanged) (for definition of entry, exit and space, see the last paragraph of the Materials and Methods section). The entry rate of FFA is markedly correlated with FFA concentration. This has been shown in dogs (Armstrong *et al.* 1961*a*) and sheep (West & Annison, 1964; C. E. West, unpublished work). The release of FFA by adipose tissue *in vitro* is depressed by raised glucose concentrations or the presence of glucose plus insulin, which appear to act by accelerating FFA esterification (see Steinberg, 1963). In work reported in the present paper the effect of raised glucose concentrations on FFA release *in vivo* was studied by measuring glucose and palmitate entry rates. The effect of insulin on glucose and FFA entry and exit rates was also studied. The simultaneous infusion of [U-^{14}C]-glucose and [9,10-$^3H_2$]palmitate allowed simultaneous measurement of both glucose and palmitate entry and exit. In the present paper it has been assumed that palmitate is metabolically representative of the FFA fraction. Studies in this Laboratory have shown that at a given individual fatty acid concentration, in sheep, the entry rate of palmitate is usually about 20% higher than that of oleate or stearate (C. E. West, unpublished work). However, the errors introduced by assuming that palmitate is metabolically representative of the FFA fraction do not invalidate the conclusions.

* Present address: Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford.

† Abbreviation: FFA, free fatty acids.

MATERIALS AND METHODS

Animals. Merino sheep (2–3-year-old wethers) were housed singly in pens indoors and fed with lucerne chaff (800g./day). The animals were trained to eat their ration in 2hr. and to stand quietly in stocks, sheep failing to respond to training (about 50%) being rejected. Sheep were starved for 24hr. before starting an experiment. Five sheep were used in these experiments.

Measurement of rate of entry of palmitate and of glucose. The general procedures described by Annison & White (1961) for the measurement of glucose entry rates and by West & Annison (1964) for the measurement of palmitate entry rates were used, but palmitate entry rates were determined by using [9,10-$^3H_2$]palmitate in place of [^{14}C]-palmitate. Labelled palmitate in albumin (0·2%) was

infused at 1–3 μc (0·02 μmole) of [9,10-$^3H_2$]palmitate/min. without using a primary dose. Labelled glucose was infused at 0·1–0·2 μc (0·04 mg.) of [U-^{14}C]glucose/min. after a priming injection of 90–120 times the amount infused in 1 min.

Aqueous solutions of sodium [9,10-$^3H_2$]palmitate in albumin were prepared by the method of Laurell (1957) and diluted to the required radioactivity/ml. Albumin (bovine serum albumin fraction V powder) obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.) was used. Palmitic acid (A grade) was obtained from the California Corp. for Biochemical Research (Los Angeles, Calif., U.S.A.). All of the labelled compounds used were obtained from The Radiochemical Centre, Amersham, Bucks.

Insulin (bovine) obtained from the Commonwealth Serum Laboratories (Parkville, N.2, Victoria, Australia) was included in the infusion solution or dissolved in 0·9% NaCl for infusion into the portal circulation. Infusions were made into and samples were taken from catheters inserted into separate jugular veins. Catheters were inserted on the day before the experiment. In experiments where infusions of insulin were made into the portal circulation, the necessary catheterization was also carried out on the day before the experiment. The operation was carried out under thiopentone anaesthesia. A left-side ventral approach was made to a ruminal vein by blunt dissection. The vessel was partially ligated and a small hole made with sharp scissors. A small catheter was fed into the vein towards the portal vein for about 15 cm. and the suture thread was tightened around the catheter. If sufficient care was taken to keep the hole in the vein as small as possible, it was not necessary to occlude the blood flow on the distal side. The catheter was led out of the body through a stab wound and the incision sutured. Such catheters usually remained patent for about a week.

Blood samples (20 ml.), taken into tubes containing 10000 i.u. of heparin, were immediately centrifuged at 8000*g* for 15 min. and the lipids extracted immediately from the plasma with the appropriate solvent mixture. Samples of plasma for the estimation of specific radioactivity and concentration of glucose were frozen as soon as possible at −20°.

Extraction of lipids and isolation of free fatty acids. Plasma was extracted with 20 vol. of chloroform–methanol (2:1, v/v) according to the method of Folch, Lees & Sloane-Stanley (1957). The organic phase was washed with aq. 0·3M-phosphate buffer, pH 6·0, to ensure quantitative recovery of FFA. The FFA fraction was isolated by the method of McCarthy & Duthie (1962), which separates lipid mixtures into neutral lipids, FFA and phospholipids.

Analysis of fatty acids. The proportion of palmitate in the FFA fraction was determined by gas–liquid chromatography as described by West & Annison (1964). The proportion of radioactivity in the fatty acids other than palmitate was determined in representative samples by the measurement of radioactivity in other fatty acids isolated by reverse-phase partition chromatography as described by West & Annison (1964).

Specific radioactivity of plasma free fatty acids. Isolated fatty acids were counted in a liquid-scintillation counter (system 725; Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.) with 10 ml. of scintillation solution [xylene containing 0·4% of 2,5-diphenyloxazole and 0·01% of 1,4-bis-(5-phenyloxazol-2-yl)benzene]. Correction for quenching was carried out by using the channels-ratio technique described by Baillie (1960). The efficiency of counting was approx. 30%. The concentration of FFA counted was determined by titration of duplicate samples in ethanol (5 ml.) with 0·02N-KOH from a Conway micro-burette under CO_2-free air with 0·002% Nile blue in 95% (v/v) ethanol (1 ml.).

Plasma free fatty acid concentration. Plasma FFA was measured by the method of Dole (1956) with modifications suggested by D. B. Lindsay (Annison, 1960).

Specific radioactivity of plasma glucose. The specific radioactivity of plasma glucose was measured by preparing the osazone derivative, which was filtered on to a planchet and counted with a Philips end-window Geiger–Müller counter as described by Annison & White (1961). Correction for self-absorption was by the method of Hendler (1959). The efficiency of counting was approx. 4%.

Plasma glucose concentration. The glucose concentration in plasma was measured by a modification of the glucose-oxidase technique of Huggett & Nixon (1957) as described by Annison & White (1961).

Calculation of entry and exit rates of palmitate and glucose. The entry of a metabolite is the inflow of that metabolite into body pool and the exit is the outflow from body pool. The metabolite space is the percentage of the animal's volume occupied by the metabolite if the concentration of the metabolite is assumed to be equal to that in the accessible pool (i.e. the plasma). Under steady-state conditions, the entry rates, which in this case equals exit rates, of glucose and palmitate were calculated by the method of Steele, Wall, de Bodo & Altzsuler (1956). When the non-steady state was studied, as during the infusion of insulin, it was assumed that the glucose space remained constant. This may not be the case (see the Discussion section), but it allows calculation of the inflow and outflow of glucose from the pool that occupied the glucose space before the infusion of insulin. On the other hand, since the FFA fraction is bound to albumin, it would be confined to plasma. The plasma volume may be assumed to remain constant and to occupy 4% of the body weight of sheep (Schambye, 1952). Such assumptions have allowed the use of the treatments of the non-steady state as described by Wall, Steele, de Bodo & Altszuler (1957) and Steele (1959) to calculate entry and exit rates.

RESULTS

Effect of glucose load on glucose and palmitate entry. In experiments where both labelled glucose and labelled palmitate were infused over a 4 hr. period, a glucose load of 63·2 mg./min. was applied during the third and fourth hours. The entry rates of glucose and palmitate were then measured by taking samples in the second and fourth hours, i.e. without and with glucose load. The results of two such experiments (Expts. 1 and 2) are shown in Table 1. As would be expected the glucose entry rate increased in response to the imposition of the glucose load. The extent of the increase was not as great as that of the load, indicating that the endogenous entry of glucose was suppressed. The entry rate of palmitate was also suppressed. If the rest of the FFA fraction were metabolized at the

Table 1. *Effect of glucose load on the entry rates of glucose and of palmitate*

Infusion rates were 3·33 μc of [9,10-$^3H_2$]palmitate/min. and 0·167 μc of [U-^{14}C]glucose/min. with a priming dose of 15 μc of [U-^{14}C]glucose. During the third and fourth hours of the infusion glucose was infused at the rate of 63·2 mg./min. Entry rates were determined in the second and fourth hours.

	Expt. 1		Expt. 2	
Sheep (wether) no.	C60		C46	
Weight (kg.)	39·5		39·2	
Rate of infusion of glucose (mg./min.)	0	63·2	0	63·2
Glucose concn. (mg./100 ml.)	55·4	72·5	88·0	139·1
Glucose entry rate* (mg./min.)	45·3	79·2	43·8	68·2
Depression of glucose entry (mg./min.)		29·3		38·8
Palmitate concn. (m-equiv./l.)	0·082	0·031	0·132	0·026
Palmitate entry rate (mg./min.)	14·1	4·2	17·3	6·9
Depression of palmitate entry (mg./min.)		9·9		10·4

* Values for glucose entry are not corrected for glucose load.

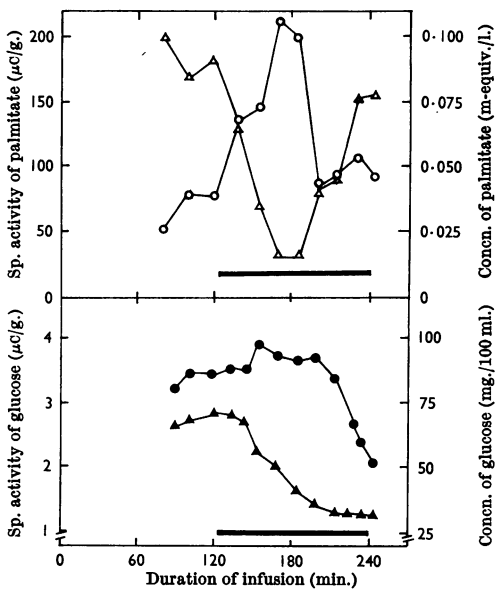

Fig. 1. Effect of a constant jugular infusion of insulin on the specific radioactivities and concentrations of glucose and palmitate during the continuous infusion of [U-^{14}C]glucose and [9,10-$^3H_2$]palmitate (Expt. 3). ○, Specific radioactivity of palmitate (μc/g.); △, concn. of palmitate (m-equiv./l.); ●, specific radioactivity of glucose (μc/g.); ▲, concn. of glucose (mg./100 ml.). The infusion rates were 0·126 μc of [U-^{14}C]glucose and 0·944 μc of [9,10-$^3H_2$]palmitate/min. with a priming dose of 15·0 μc of [U-^{14}C]glucose. The infusion rate of insulin from 125 to 240 min. (horizontal bar) was 0·1 unit/min. The weight of the wether (C46) used was 38·8 kg.

same rate as palmitate the increase in calories available from the glucose entry would roughly compensate for the decrease in the calories available from the FFA entry, producing an isocaloric shift from fatty acids to glucose.

Effect of insulin on the metabolism of glucose and of palmitate. The effects of insulin on glucose and palmitate entry were studied by using the two isotope techniques described above. From the 125th minute of the 240 min. infusion, insulin was infused into the jugular vein at the rate of 0·1 unit/min. The specific radioactivities and concentrations of glucose and of palmitate during such an experiment (Expt. 3) are shown in Fig. 1. In this experiment, before the infusion of insulin was commenced, the pool size of glucose was 4·34 g., the turnover time 118 min. and the glucose space 15·5%. Comparable values for palmitate were 45·3 mg. and 3·6 min. and the palmitate space was assumed to be 4%. The rapid depression in palmitate concentration was due to the depression of FFA release as indicated by the increase in the specific radioactivity of the palmitate. This effect occurred before there was any appreciable change in the glucose specific radioactivity or concentration. Even though there was a depression in blood glucose concentration, the specific radioactivity of plasma glucose remained constant for about 80 min., indicating that the entry of glucose was not altered. When the glucose concentration fell below about 40 mg./100 ml. (i.e. after about 65 min.), FFA was released into the circulation as indicated by the fall in the specific activity of the circulating palmitate that occurs together with an increase in the concentration of palmitate. After 80 min. the increased hypoglycaemia produced an increased rate of release of glucose into the circulation as indicated by the fall in the specific radioactivity of the glucose. The palmitate and glucose entry and exit rates are shown in Fig. 2. It is also obvious from this presentation of the results that, for the first 60–80 min. after the commencement of insulin infusion, the

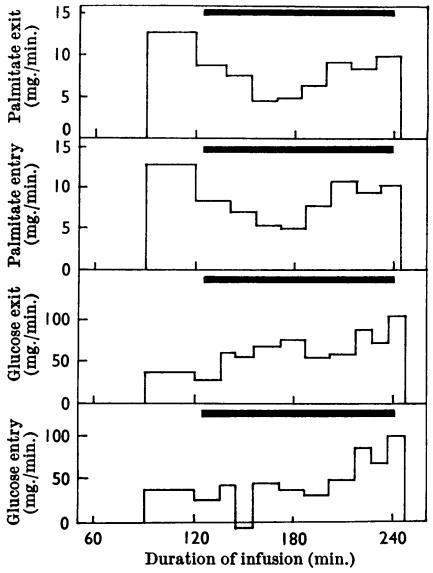

Fig. 2. Effect of a constant jugular infusion of insulin on the entry and exit rates of palmitate and glucose. The results in this Figure and Fig. 1 were obtained from Expt. 3.

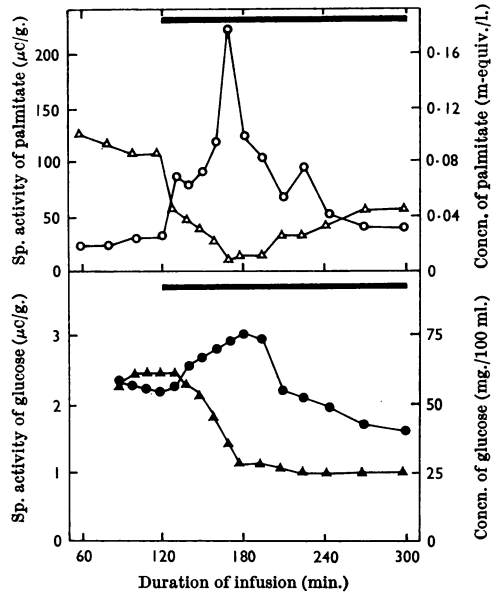

Fig. 3. Effect of a constant portal infusion of insulin on the specific radioactivities and concentrations of glucose and palmitate during the continuous infusion of [U-^{14}C]glucose and [9,10-$^{3}H_2$]palmitate (Expt. 4). ○, Specific radioactivity of palmitate (μc/g.); △, concn. of palmitate (m-equiv./l.); ●, specific radioactivity of glucose (μc/g.); ▲, concn. of glucose (mg./100 ml.). The infusion rates were 0·167 μc of [U-^{14}C]glucose and 1·18 μc of [9,10-$^{3}H_2$]palmitate/min. with a priming dose of 15·0 μc of [U-^{14}C]glucose. The infusion rate of insulin from 120 to 240 min. (horizontal bar) was 0·1 unit/min. The weight of the wether (C8) used was 34·4 kg.

entry of glucose was relatively unaffected whereas the exit rate was increased. The negative entry rate between 144 and 156 min. appears to be an aberrant result. As insulin is secreted into the portal circulation it was decided to test whether insulin infused into the portal circulation had the same effect on glucose and palmitate entry and exit as insulin infused into the peripheral circulation. The results of such an experiment (Expt. 4), expressed as before, are shown in Figs. 3 and 4. The main difference was the almost immediate depression in glucose entry after the commencement of insulin infusion into the portal circulation.

In two other experiments (Expts. 5 and 6), insulin was infused into the portal circulation at lower rates in an attempt to separate the effects of insulin on fatty acid metabolism from those on glucose metabolism. In Expt. 5, three rates of infusion were used, in the third hour 0·004 unit/min., in the fourth hour 0·02 unit/min. and in the fifth hour 0·1 unit/min., which was the rate used previously. In this experiment the metabolism of glucose and palmitate was influenced by insulin but the time-course of events was difficult to interpret since the effect of infusing insulin into the sheep at one rate for an hour was superimposed on the effect produced in previous periods. In Expt. 6 insulin was infused into the portal circulation at the rate of 0·004 unit/min. in the third, fourth and fifth hours. However, the concentration of insulin was too low to show any

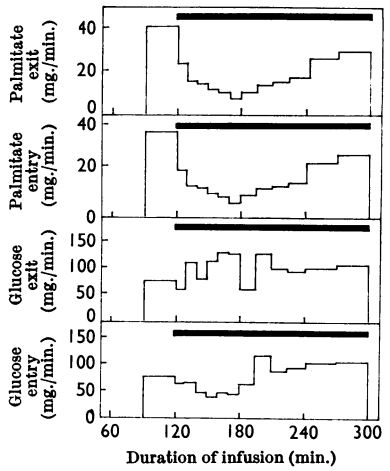

Fig. 4. Effect of a constant portal infusion of insulin on the entry and exit rates of palmitate and glucose. The results in this Figure and Fig. 3 were obtained from Expt. 4.

appreciable effect on glucose and palmitate metabolism.

DISCUSSION

Effect of glucose load on glucose entry. The application of a glucose load decreased the release of endogenous glucose into the circulation (Table 1). In the literature, there is much controversy as to whether hepatic glucose output is depressed by raised concentrations of plasma glucose. Studies based on the application of a glucose load during the decline in specific radioactivity of blood glucose in dogs after the injection of [^{14}C]glucose (Searle & Chaikoff, 1952; Reichard, Freidmann, Maass & Weinhouse, 1958) suggested that hepatic output of glucose was abolished in respose to high blood glucose concentrations. On the other hand Steele and his associates have shown that the constant specific activity observed after the injection of [^{14}C]glucose and the imposition of a glucose load was not necessarily due to inhibition of endogenous glucose entry (see Steele, 1959). Steele & Marks (1958) produced evidence that hepatic output of glucose in dogs is not inhibited by the sudden application of a glucose load. Landau, Leonards & Barry (1961) studied the output of glucose from the livers of dogs at increasing glucose loads by the balance method, applying the Fick principle. They found that hepatic glucose production in protein-fed dogs was inhibited only at hyperglycaemic concentrations, although in carbohydrate-fed dogs, where there was little rise in blood glucose concentrations, the livers actually took up glucose. Much of the controversy about whether glucose loads inhibit the hepatic output of glucose in dogs probably results from the use of different diets.

The inhibition of glucose output by a glucose load in the present experiments in sheep has confirmed the results of experiments carried out by Annison & White (1961). Ruminants, which depend to a large extent on endogenous glucose, may have a more effective mechanism than non-ruminants for the control of glucose production. The metabolic economy of ruminants appears to be delicately balanced with respect to glucose availability, particularly in the pregnant or lactating animal, and any mechanism that conserves glucose or glucose precursors is of obvious value. Although the requirement for glucose may be less in ruminants than in non-ruminants, it is still essential for the metabolism of the brain and mammary gland and as a foetal requirement.

Effect of glucose load on palmitate entry. A decrease in FFA concentration may be produced either by a decrease in the rate of FFA entry or an increase in the rate of FFA exit from the body pool. The results expressed in Table 1 show that the infusion of glucose into the peripheral circulation depressed the concentration and rate of entry of FFA. The work discussed below indicates that this was due to the effect of glucose on the adipose tissues of the sheep. Gordon (1957) has shown that in starved human subjects arterial and saphenous-vein samples reveal large negative arteriovenous differences, which indicate a net release of fatty acid from adipose tissue and which are abolished by the administration of glucose or insulin (Gordon, 1957; Estes, Bogdonoff, Freidberg, Harlan & Trout, 1959). Experiments *in vitro*, mainly with the epididymal fat pads of rats, have shown that glucose strongly stimulates the incorporation of [^{14}C]glucose into glyceride glycerol and fatty acids (Lynn, MacLeod & Brown, 1960; Cahill, Leboeuf & Flinn, 1960; Jeanrenaud & Renold, 1959; Winegrad, Shaw, Lukens, Stadie & Renold, 1959). It has been shown in other tissues that the control of fatty acid synthesis is through the acetyl-CoA carboxylase reaction, which is stimulated by citrate and to a smaller extent by other compounds of the tricarboxylic acid cycle and is inhibited by the presence of long-chain fatty acids (Waite & Wakil, 1963; Bortz, Abraham & Chaikoff, 1963). In other experiments *in vitro* glucose and glucose plus insulin have been shown to stimulate the conversion of fatty acids into triglycerides (Raben & Hollenberg, 1960; Bally, Cahill, Leboeuf & Renold, 1960; Steinberg, Vaughan & Margolis, 1960) without having any significant effect on the rate of lipolysis (Leboeuf, Flinn & Cahill, 1959; Vaughan, 1962). Glucose and insulin seem to suppress FFA release by accelerating esterification without altering the rate of lipolysis. The net result is a decrease in the release of FFA into the plasma, producing a caloric shift from fatty acids to glucose.

Effect of insulin on glucose entry and exit. When insulin was infused into the peripheral circulation, the marked decrease in glucose concentration at a constant specific activity indicated an outflow of glucose from the plasma (Figs. 1 and 2). The glucose that passes out of plasma has been shown by many workers to remain in equilibrium with plasma glucose so that there is an increase in the volume occupied by glucose, or the glucose 'space'. Hetenyi, Rappaport & Wrenshall (1963), using normal and hepatectomized dogs, found that the liver plays a central role in this effect of insulin in causing an apparent increase in glucose space. However, glucose uptake and space in other tissues are also increased by insulin. Park, Reinwein, Henderson, Candenas & Morgan (1959) found that insulin increased the calculated concentration of intracellular free glucose and thus the size of the calculated glucose space in the perfused rat heart. It has also been shown that insulin increases the permeability of adipose tissue to glucose (Jeanrenaud & Renold, 1959), thus increasing the glucose space.

Effect of insulin on palmitate entry and exit. The rapid depression of FFA release in response to insulin preceded any noticeable effect on glucose metabolism (Fig. 2). This could be interpreted as a direct effect of insulin on fatty acid metabolism in adipose tissue, i.e. an effect that was not mediated through glucose. On the other hand, since the size and turnover time of the glucose pool are many times greater than those of the palmitate pool (see the Results section), it is clear that a comparable change in the uptake of glucose and palmitate would affect the concentration and specific radioactivity of glucose more slowly than it would those of palmitate, and is in line with the view that the primary effect of insulin on fat mobilization by adipose tissue is the effect of insulin on glucose uptake. This is supported by studies with rat epididymal fat pads by Engel & White (1960), where in the absence of glucose insulin was shown not to suppress FFA release. The results showed that the exit rate of palmitate from plasma closely follows the entry rate, and the infusion of insulin has no effect on the exit of palmitate, which is dependent on the palmitate concentration. This is in line with the results obtained in dogs by Bierman, Schwartz & Dole (1957). They found that the rate of disappearance from blood of a single injection of [^{14}C]palmitate was the same before and after intravenous injection of insulin (0·1 unit/kg. body wt.), although the expected significant fall in FFA concentration occurred. When a steady concentration of labelled FFA was maintained by a constant infusion, the administration of glucose produced a significant increase in specific radioactivity. On the other hand, Shoemaker, Ashmore, Carruthers & Schulman (1960) advanced the view that increased hepatic uptake of FFA under the influence of insulin might explain the fall in FFA concentration. They based their conclusions on catheterization experiments in which blood samples were obtained from the portal vein, the hepatic vein and an artery of the unanaesthetized dog. However, Fine & Williams (1960), using the hepatic catheterization technique, showed a decrease in hepatic uptake in proportion to the decrease in plasma FFA concentration. Armstrong *et al.* (1961*b*) have carried out experiments in dogs, similar to those described in the present paper, by using a constant infusion of [^{14}C]glucose. Their results were very similar to those obtained in the present work. Hence there is strong evidence from experiments both *in vitro* and *in vivo* that the effect of insulin on fatty acid release from adipose tissue is mediated through the effect on glucose metabolism. When the plasma glucose concentrations fell below about 40 mg./100 ml. the effect of insulin on fatty acid release was overcome and reversed, presumably by a sympathoadrenal response to hypoglycaemia as glucose production was also increased. Such an effect was shown to occur in dogs by Armstrong *et al.* (1961*b*).

Effect of portal infusions of insulin on glucose and palmitate entry and exit. When insulin is infused into the portal circulation, the liver is exposed to high concentrations of insulin. This may explain the observed inhibition of glucose output by intraportal infusions of insulin. Inhibition of hepatic glucose output by insulin is widely accepted as an established fact (see the discussion following the paper of de Bodo, Steele, Altszuler, Dunn & Bishop, 1963). Portal and peripheral infusions of insulin produce similar effects on FFA metabolism as adipose tissue is probably subjected to similar concentrations of exogenous insulin in the two cases. The rate of insulin infusion used (26–29 milliunits/kg. body wt.) is about ten times that required to produce similar effects on glucose and FFA metabolism in humans or dogs (Gordon, 1957; Estes *et al.* 1959; Armstrong *et al.* 1961*b*). That ruminants are less sensitive to insulin in many respects has been known for many years (Reid, 1951*a*,*b*, 1952; Jarrett & Potter, 1953; Lindsay, 1959).

The entry rates for palmitate reported in the present paper may be overestimated. More recent experiments (C. E. West, unpublished work) have shown that during the infusion of either ^{14}C or ^{3}H-labelled FFA there is a substantial arteriovenous difference in specific radioactivity across the head as measured by samples taken from the carotid artery and jugular vein. No significant arteriovenous difference in FFA concentration was observed. On the other hand, during the infusion of [^{14}C]glucose there was an arteriovenous difference in concentration but not in specific radioactivity of glucose. Lipolysis of triglycerides in or near the capillary bed with incomplete mixing of the FFA so produced with the plasma FFA might also underestimate the exit rate. However, although these differences may produce overestimations of the entry and exit rates of palmitate and of the glucose space, it is unlikely that the conclusions drawn from these measurements would be markedly altered.

Hence the present results with sheep support results of previous work with dogs suggesting that lowered FFA release and not increased FFA uptake accounts for the depression of plasma FFA concentration caused by insulin. The results obtained are in line with studies *in vitro* of adipose tissue which show that the effect of insulin on fatty acid release by adipose tissue is mediated through its effect on glucose metabolism.

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