

The Regulation of Triglyceride Synthesis and Fatty Acid Synthesis in Rat Epididymal Adipose Tissue

EFFECTS OF INSULIN, ADRENALINE AND SOME METABOLITES *IN VITRO*

BY E. D. SAGGERSON AND A. L. GREENBAUM

Department of Biochemistry, University College London, Gower Street, London W.C.1, U.K.

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1. Adipose tissues from rats fed a balanced diet were incubated in the presence of glucose (20 mM) with the following additions: insulin, anti-insulin serum, insulin + acetate, insulin + pyruvate, insulin + lactate, insulin + phenazine methosulphate, insulin + oleate + albumin, insulin + adrenaline + albumin, insulin + 6-*N*-2'-*O*-dibutyryl 3':5'-cyclic AMP + albumin. 2. Measurements were made of the whole tissue concentrations of adenine nucleotides, hexose phosphates, triose phosphates, glycerol 1-phosphate, 3-phosphoglycerate, 6-phosphogluconate, long-chain fatty acyl-CoA, acid-soluble CoA, citrate, isocitrate, malate and 2-oxoglutarate, and of the release into the incubation medium of lactate, pyruvate and glycerol after 1 h of incubation. 3. Fluxes of [^{14}C]glucose carbon through the major pathways of glucose metabolism were calculated from the yields of ^{14}C in various products after 2 h of incubation. Fluxes of [^{14}C]acetate, [^{14}C]pyruvate or [^{14}C]lactate carbon in the presence of glucose were also determined. 4. Measurements were also made of the whole-tissue concentrations of metabolites in tissues taken directly from Nembutal-anaesthetized rats. 5. Whole tissue mass-action ratios for phosphofructokinase, phosphoglucose isomerase and the combined (aldolase \times triose phosphate isomerase) reaction were similar *in vivo* and *in vitro*. The reactants of phosphofructokinase appeared to be far from mass-action equilibrium. *In vitro*, the reactants of hexokinase also appeared to be far from mass-action equilibrium. 6. Correlation of observed changes in glycolytic flux with changes in fructose 6-phosphate concentration suggested that phosphofructokinase may show regulatory behaviour. The enzyme appeared to be activated in the presence of oleate or adrenaline and to be inhibited in the presence of lactate or pyruvate. 7. Evidence is presented that the reactants of lactate dehydrogenase and glycerol 1-phosphate dehydrogenase may be near to mass-action equilibrium in the cytoplasm. 8. No satisfactory correlations could be drawn between the whole-tissue concentrations of long-chain fatty acyl-CoA, citrate and glycerol 1-phosphate and the observed rates of triglyceride and fatty acid synthesis. Under the conditions employed, the concentration of glycerol 1-phosphate appeared to depend mainly on the cytoplasmic $[\text{NAD}^+]/[\text{NADH}]$ ratios. 9. Calculated hexose monophosphate pathway flux rates roughly correlated with fatty acid synthesis rates and with whole tissue $[6\text{-phosphogluconate}]/[\text{glucose } 6\text{-phosphate}]$ ratios. The relative rates of production of NADPH for fatty acid synthesis by the hexose monophosphate pathway and by the 'malic enzyme' are discussed. It is suggested that all NADH produced in the cytoplasm may be used in that compartment for reductive synthesis of fatty acids, lactate or glycerol 1-phosphate.

Adipose tissue is an important site of interaction between the metabolism of carbohydrate and lipid. Rat epididymal adipose tissue in particular has been studied, since the whole tissue *in vitro* will readily carry out metabolic functions and exhibits an extreme sensitivity to hormones.

Measurements of the steady-state concentrations

of metabolic intermediates in various tissues *in vivo* and *in vitro* have yielded information concerning the regulation of metabolism in those tissues. The glycolytic pathway in particular has been studied (Hess, 1962; Lowry, Passonneau, Hasselberger & Schulz, 1964; Williamson, 1965; Rolleston & Newsholme, 1967). Newsholme &

Gevers (1967) have reviewed the techniques of locating possible regulatory enzymes from whole-tissue enzyme mass-action ratios and of correlating changes of substrate concentrations with changes in flux rates through these reactions. Methods are available for estimating rates of flux through the major pathways of glucose metabolism in adipose tissue (Flatt & Ball, 1964; Katz, Landau & Bartsch, 1966).

Denton, Yorke & Randle (1966), Denton & Halperin (1968) and Halperin & Denton (1969) have reported measurements of a limited range of metabolites in adipose tissues *in vitro* under certain steady-state conditions and have attempted to relate these to observed rates of glucose metabolism. The results of the present investigation generally extend and complement the findings of Denton and co-workers.

In this study it has been possible to extend the range of steady states examined and to increase the number of metabolites measured. Flux rates through various metabolic pathways have also been calculated in an attempt to correlate these with the metabolite measurements and to obtain information on the relationships between the observed rates of flux through the various pathways.

MATERIALS AND METHODS

Chemicals. Nucleotides, sugar phosphates, triethanolamine hydrochloride and tris base were obtained from the Boehringer Corp. (London) Ltd., London W.5, U.K. All enzymes were also obtained from Boehringer except for 2-oxoglutarate dehydrogenase which was prepared by the method of Sanadi, Littlefield & Bock (1952) as modified by Hirashima, Hayakawa & Koike (1967). The preparation was taken as far as the 16000g supernatant obtained by centrifugation of the dialysed, resuspended 2% (w/v) protamine sulphate precipitate. 2-Oxoglutaric acid, phenazine methosulphate, adrenaline and 6-*N*-2'-*O*-dibutylryl 3':5'-cyclic AMP were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. Dithiothreitol and oleic acid (grade A) were products of Calbiochem, Los Angeles, Calif, U.S.A. Sodium pyruvate was from E. Merck A.-G., Darmstadt, Germany. Insulin was obtained from Boots Pure Drug Co. Ltd., Nottingham, U.K., and anti-insulin serum from the Wellcome Research Laboratories, Beckenham, Kent, U.K. Bovine plasma albumin (fraction V), from the Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K. was purified as described by Chen (1967). Hyamine hydroxide was obtained from Nuclear Enterprises Ltd., Edinburgh, U.K. 2,5-Bis-(5-*tert*.-butylbenzoxazol-2-yl)thiophen was from CIBA (A.R.L.) Ltd., Duxford, Cambs., U.K. All radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were of A.R. grade and were used without further purification.

Animals. Male albino rats of an inbred strain were used in all experiments. The animals were maintained on cube

diet 41B (Bruce & Parkes, 1949) supplied with water *ad libitum*, and weighed between 150 and 180g at the time of death.

Incubation media. All incubations of adipose tissues were performed at 37°C in Krebs-Ringer bicarbonate buffer (Krebs & Henseleit, 1932) containing 20 mM-glucose and pregassed with O₂+CO₂ (95:5) for 30 min. Other additions to this basic medium, as specified in the Tables, were made as follows. Insulin was dissolved in 3.3 mM-HCl (50 units/ml) and added to media to give a final concentration of 200 m-units/ml. Adrenaline was dissolved in 3.3 mM-HCl (1.25 mg/ml) and diluted in media to 5 µg/ml. Anti-insulin serum (1 unit/ml) was added to incubation media to give a final concentration of 5 m-units/ml. Albumin was used at a final concentration of 10 mg/ml, the incubation media being readjusted to pH 7.4 with *m*-NaOH. Sodium pyruvate, acetate or lactate were all added as *m* solutions at pH 7.4 to give a final concentration in the media of 20 mM. Phenazine methosulphate (10 mM) was added to media to give a final concentration of 0.1 mM. Ammonium oleate (bound to albumin) was added to give a final concentration of 1.5 mM. 6-*N*-2'-*O*-Dibutylryl 3':5'-cyclic AMP was used at a final concentration of 1.5 mM. In determinations of tissue [¹⁴C]sorbitol and [³H]water spaces, sorbitol was used at a concentration of 5 mM and specific radioactivity of 80 µCi/mmol. [³H]Water was used at a final specific radioactivity of 5 µCi/ml.

Incubation of fat pads for determination of metabolite contents and [¹⁴C]sorbitol and [³H]water spaces. Rats were killed by cervical dislocation and the epididymal fat pads were rapidly excised, trimmed free of blood vessels as far as possible, weighed and preincubated for 10 min at 37°C in open vessels under conditions identical with those of the final incubation; i.e. all required substrates and hormones were present, except for radioactive compounds. The actual incubations were started when the tissues were transferred from the open vessels to 50 ml Erlenmeyer flasks. In experiments to determine the tissue contents of metabolites, the combined fat pads of two rats were incubated in 10 ml of the required medium in a flask, whereas in determinations of [¹⁴C]sorbitol and [³H]water spaces, each flask contained 5 ml of incubation medium containing 5 mM-[¹⁴C]sorbitol and the two fat pads of a single rat. The flasks were sealed with rubber vaccine caps and incubated with shaking at 37°C for 1 h. Gassing with a stream of O₂+CO₂ (95:5) was carried out for the first 15 min of incubation. [³H]Water was injected into the flasks after 40 min when [³H]water-space determinations were to be made (Crofford & Renold, 1965). Control experiments showed that rates of glucose uptake and metabolism by the incubated tissues were constant for at least 2 h under the conditions employed. It was therefore assumed that the tissues were in a steady state of metabolism at the time of sampling.

Preparation of extracts of incubated fat pads for determination of metabolite contents or [¹⁴C]sorbitol and [³H]water spaces. This was carried out in all cases after 1 h of incubation. The procedure adopted depended on the measurements to be made. For the assay of acid-soluble metabolic intermediates, the methods used by Denton *et al.* (1966) and by Denton & Halperin (1968) were used with minor modifications. Fat pads were removed from the incubation flasks with forceps and rapidly freeze-

clamped (Wollenberger, Ristau & Schoffa, 1960). The tissues were not blotted before freezing. The combined frozen fat pads of four rats were collected in a stone mortar, ground to a fine powder under liquid N_2 and well mixed with 10g of previously frozen and powdered 5% (v/v) $HClO_4$, 2mM-EDTA (Lowry *et al.* 1964). The frozen powder was homogenized with 15ml of light petroleum (b.p. range 40–60°C) in a glass Potter–Elvehjem homogenizer fitted with a motor-driven Teflon pestle. The homogenate was transferred to a 50ml plastic centrifuge tube with one washing of the homogenizer with 5ml of 5% (v/v) $HClO_4$, 2mM-EDTA and 5ml of light petroleum, and then centrifuged at 6000g in an MSE angle 13 centrifuge for 10 min at 2°C. The resulting light-petroleum layer was removed by aspiration and discarded. The lower, aqueous layer was decanted from the floating protein plug, re-extracted with 15ml of light petroleum and stored on ice. Light petroleum appeared to be a more efficient lipid extractant than diethyl ether under the conditions employed. m-Triethanolamine hydrochloride (0.5ml) and 0.1M-dithiothreitol (0.5ml) were added to the acid extract which was then neutralized with KOH, centrifuged to remove $KClO_4$, and freeze-dried. The freeze-dried material was dissolved in 1ml of water/g of original tissue, stored at –20°C and used within 2 days for assay of metabolites. Analysis for acid-insoluble CoA was performed as follows. The protein precipitate of the $HClO_4$ extract obtained as described above was suspended in 3.0ml of 0.4M-KOH–10mM-dithiothreitol in a small glass homogenizer and kept at 25°C for 15 min. Frequent strokes of the homogenizer pestle ensured complete penetration of the insoluble material by the alkali. The contents of the tube were finally acidified with 0.2ml of 60% (v/v) ice-cold $HClO_4$, cooled in ice and centrifuged. The resulting precipitate was washed with 3.0ml of 5% (v/v) $HClO_4$ and recentrifuged. Saturated KH_2PO_4 (1.0ml) was added to the combined acid supernatants, which were adjusted to pH 7.0 with 10M-KOH. The $KClO_4$ was removed by centrifugation. The volume of the neutral supernatant was noted and this was used directly, without freeze-drying, for assay of acid-soluble CoA liberated by hydrolysis of long-chain fatty acyl-CoA. As noted by Denton *et al.* (1966) and by Denton & Halperin (1968), recoveries of phosphorylated sugars, adenine nucleotides, citrate, malate, acid-soluble CoA and long-chain fatty acyl-CoA from these extracts were satisfactory (>90%). However, as reported by Denton & Halperin (1968), the recovery of acetyl-CoA from freeze-dried extracts was unsatisfactory and the CoASH measured in acid-soluble extracts was taken to represent the sum of the tissue acid-soluble CoA + acetyl-CoA.

For the determination of $[^3H]$ water, $[^{14}C]$ sorbitol and glucose spaces, the fat pads were removed from the incubation flasks, blotted and freeze-clamped. The tissues were powdered with frozen 5% (v/v) $HClO_4$ and homogenized without light petroleum. The contents of the homogenizer were centrifuged and a 2–3ml portion of the aqueous extract was decanted into a 15ml centrifuge tube. These samples were then neutralized by addition of solid $KHCO_3$, and $KClO_4$ was removed by centrifugation after the samples had been kept on ice. The clear supernatant from this centrifugation was used directly for determination of $[^{14}C]$ sorbitol, $[^3H]$ water and glucose.

Generally one extract was made by the homogenization of the fat pads of one rat with 5ml of 5% (v/v) $HClO_4$.

Treatment of incubation media after incubation of pooled whole fat pads. After the removal of incubated tissues, 4ml samples of incubation media were deproteinized by the addition of sufficient ice-cold 60% (v/v) $HClO_4$ to give a final concentration of 5% (v/v). Precipitated protein was removed by centrifugation and the supernatant was neutralized by addition of 0.1 ml of m-tris base and sufficient saturated K_2CO_3 . The $KClO_4$ was removed by centrifugation.

Preparation of extracts of non-incubated fat pads. Rats were injected intraperitoneally with 9mg of Nembutal in a volume of 0.15ml. After 5 min the fat pads were exposed, excised and immediately freeze-clamped. Acid-soluble and -insoluble extracts of the combined frozen fat pads of sets of three rats were prepared as described above for incubated fat pads.

Assays of metabolites in tissue extracts and in incubation media. Assays were made by the production or disappearance of NADH or NADPH, as appropriate, in specific enzymic reactions. Tissue contents of ATP, ADP, AMP and those of lactate, pyruvate and glycerol in incubation media were assayed spectrophotometrically at 340nm in a 3.0ml reaction volume with a Unicam SP.800 recording spectrophotometer by using the methods of Lamprecht & Trautshold (1963), Adam (1963), Hohorst (1963a), Bücher, Czok, Lamprecht & Latzko (1963) and Garland & Randle (1962). NADH used in the estimation of AMP was pretreated with alkaline phosphatase as recommended by Lowry *et al.* (1964). All other assays were fluorimetric and were performed in a 2.0ml volume (1 cm light-path) by using a modified Eppendorf fluorescence accessory (Garland, Shepherd & Yates, 1965). Glucose 6-phosphate, fructose 6-phosphate and 3-phosphoglycerate, dihydroxyacetone phosphate, glyceraldehyde 3-phosphate and fructose 1,6-diphosphate, glycerol 1-phosphate, and 6-phosphogluconate were assayed by the methods of Maitra & Estabrook (1964), Bücher & Hohorst (1963), Bublitz & Kennedy (1954), and Hohorst (1963b) respectively. CoASH was determined by the method of Garland *et al.* (1965), malate by the method of Hohorst (1963c) and 2-oxoglutarate by the method of Bergmeyer & Bernt (1963). Citrate and isocitrate were determined as described by Moellering & Gruber (1966) and Ochoa (1948).

Determination of $[^{14}C]$ sorbitol, $[^3H]$ water and glucose spaces. Simultaneous determinations of $[^{14}C]$ sorbitol and $[^3H]$ water in tissue extracts or in incubation media were made with a Beckman liquid-scintillation counter by using a scintillator consisting of 4g of 2,5-bis-(5-*tert.*-butylbenzoxazol-2-yl)thiophen/l of toluene-methanol (1:1, v/v). Glucose was determined in the same extracts by the method of Slein (1963). Tissue $[^{14}C]$ sorbitol, $[^3H]$ water and glucose spaces were calculated as described by Randle & Smith (1958) and by Crofford & Renold (1965).

Determination of tissue nitrogen. Samples (20–30mg) of powdered frozen fat pads were taken from each group of pooled tissues before $HClO_4$ extraction and were digested in 2.0ml of 50% nitrogen-free H_2SO_4 . The $(NH_4)_2SO_4$ so produced was assayed as ammonia by the method of Fawcett & Scott (1960), modified by the use of potassium tartrate (Lubochinsky & Zalta, 1954).

Incubation of fat-pad segments for determination of [^{14}C]fluxes. The distal portions of the fat pads of each rat were cut into four or five weighed segments of approx. 100mg each and preincubated as described above for whole fat pads. After preincubation, the segments were randomly distributed, one to a flask, among 50ml Erlenmeyer flasks fitted with glass centre-wells. The flasks contained 5ml of the incubation medium with all required hormones and radioactive substrates added. All radioactive substrates were present at a specific radioactivity of $10\mu\text{Ci}/\text{mmol}$. The flasks were sealed and incubated, with shaking, for 2h at 37°C . Gassing with a stream of $\text{O}_2 + \text{CO}_2$ (95:5) was carried out for the first 5min of incubation. After 2h, 0.5ml of $\text{M-Hyaminate hydroxide}$ in methanol was injected through the rubber cap into the centre-well of each flask to trap $^{14}\text{CO}_2$, and 1.0ml of 2.5M-HCl was injected into each outer well. The flasks were then shaken for 1h at 37°C .

In experiments designed to measure the flux of glucose carbon through pathways in the presence of insulin+adrenaline, insulin+oleate, insulin+phenazine methosulphate, insulin+dibutylryl cyclic-AMP or anti-insulin serum, compared with control tissues incubated with insulin alone, four fat-pad segments from each rat were incubated in flasks as follows: (i) [$\text{U-}^{14}\text{C}$]glucose+insulin; (ii) [$6\text{-}^{14}\text{C}$]glucose+insulin; (iii) [$\text{U-}^{14}\text{C}$]glucose and the required additions; (iv) [$6\text{-}^{14}\text{C}$]glucose and the required additions.

In experiments designed to measure the flux of glucose carbon in the presence of acetate, lactate or pyruvate, and to measure the fluxes of acetate, lactate or pyruvate in the presence of glucose compared with control tissues incubated with glucose alone, five fat-pad segments from each rat were incubated as follows: (i) [$\text{U-}^{14}\text{C}$]glucose+insulin; (ii) [$6\text{-}^{14}\text{C}$]glucose+insulin; (iii) [$\text{U-}^{14}\text{C}$]glucose+acetate, lactate or pyruvate+insulin; (iv) [$6\text{-}^{14}\text{C}$]glucose+acetate, lactate or pyruvate+insulin; (v) glucose+[$\text{U-}^{14}\text{C}$]acetate, [$\text{U-}^{14}\text{C}$]lactate or [$\text{U-}^{14}\text{C}$]pyruvate+insulin.

Determination of ^{14}C -flux rates in incubated tissue segments. On completion of incubation the flasks were opened and 1ml of dry methanol was added to each centre-well. The Hyamine solutions in the centre-wells were then transferred to a scintillation bottle containing 15ml of scintillator. Each centre-well was washed with a further 1ml of methanol which was also transferred to the scintillation bottle.

The pieces of tissue were removed from the flasks with forceps, briefly rinsed in 0.9% NaCl and left overnight at $2\text{--}4^\circ\text{C}$ in 30ml of chloroform-methanol (1:2, v/v). The tissues were homogenized in the chloroform-methanol with a ground glass homogenizer and the chloroform phase was separated by addition of 10ml of chloroform and 18ml of water (Bligh & Dyer, 1959). A 5ml sample of this chloroform fraction was evaporated to dryness on a rotary film evaporator at $50\text{--}60^\circ\text{C}$ and the residue was dissolved in 8ml of 5% ethanolic KOH and heated at 60°C for 3h. After cooling, 3ml of water was added and the sample was extracted twice with 15ml of light petroleum (b.p. range $40\text{--}60^\circ\text{C}$). The light petroleum fractions were discarded. The remaining ethanolic KOH layer was acidified by addition of 1ml of 10M- H_2SO_4 and was extracted three times with 15ml of light petroleum. The combined light petroleum fractions (the fatty acid fraction)

were evaporated to dryness on a rotary film evaporator, the residues dissolved in 4ml of light petroleum, and a 2ml sample of this solution was added to 15ml of scintillator. The scintillator used for measurement of $^{14}\text{CO}_2$ in Hyamine and of fatty acid fractions consisted of 4g of 2,5-bis-(5-*tert.*-butylbenzoxazol-2-yl)thiophen/l of toluene.

The remaining acid aqueous fraction after light-petroleum extraction of fatty acids was designated the glyceride-glycerol fraction, and a sample was taken directly for measurement of radioactivity in 15ml of a scintillator consisting of 4g of 2,5-bis-(5-*tert.*-butylbenzoxazol-2-yl)thiophen/l of Triton X-100-toluene (1:2, v/v). The Triton X-100 was pretreated with silica gel to decrease a high phosphorescence (Patterson & Greene, 1965).

The acidified incubation media remaining in the flasks used for incubation of tissues with uniformly labelled substrates were assayed for lactate+pyruvate by one of two methods. Generally, the media were deproteinized with HClO_4 and then neutralized as described above for pooled whole fat pads. Lactate and pyruvate were assayed enzymically in these neutralized preparations. When [$\text{U-}^{14}\text{C}$]glucose or [$\text{U-}^{14}\text{C}$]glucose+acetate were the sole substrates added to incubation media, it was assumed that the specific radioactivities of lactate and pyruvate released by the tissues were the same as that of [$\text{U-}^{14}\text{C}$]glucose as shown by Denton & Randle (1967). When an assay of [^{14}C]lactate and [^{14}C]pyruvate production from [$\text{U-}^{14}\text{C}$]glucose in the presence of added lactate or pyruvate was required, [$\text{U-}^{14}\text{C}$]glucose was precipitated from the acidified media by the copper-lime method of Katz *et al.* (1966) and a sample of the remaining supernatant was counted for radioactivity by using the Triton X-100-toluene (1:2, v/v) scintillator. Control experiments showed that agreement between the two methods of lactate+pyruvate determination was good. For example, the mean value for the rate of release of lactate+pyruvate by five incubated tissues was estimated to be $5.15 \pm 1.41 \mu\text{mol}/\text{h}$ per g of tissue if the products were determined enzymically. With the copper-lime method the mean rate of lactate+pyruvate release by the same tissues was found to be $5.84 \pm 1.41 \mu\text{mol}/\text{h}$ per g of tissue.

Fluxes of glucose carbon through various metabolic pathways in tissues incubated under the various conditions considered in this study were calculated from the yields of ^{14}C in fatty acids and CO_2 derived from [$\text{U-}^{14}\text{C}$]glucose and [$6\text{-}^{14}\text{C}$]glucose and from the yields of ^{14}C in glyceride glycerol, lactate and pyruvate derived from [$\text{U-}^{14}\text{C}$]glucose as described by Flatt & Ball (1964, 1966). Fluxes of acetate carbon into $^{14}\text{CO}_2$ and fatty acids in the presence of glucose were measured as described by Flatt & Ball (1966). Fluxes of [^{14}C]pyruvate and [^{14}C]lactate carbon through various metabolic pathways in the presence of glucose were calculated from the yields of ^{14}C in fatty acids, CO_2 and glyceride glycerol derived from [$\text{U-}^{14}\text{C}$]pyruvate or [$\text{U-}^{14}\text{C}$]lactate as follows. It was assumed that pyruvate derived from exogenous non-radioactive glucose and pyruvate derived from exogenous [$\text{U-}^{14}\text{C}$]pyruvate or [$\text{U-}^{14}\text{C}$]lactate equilibrated in the same intracellular pool, and therefore that

$$\text{Fa}^{\text{U}}/\text{Fa}^{\text{P}} = \text{Z}'/\text{Z}^{\text{P}} = \text{Y}'/\text{Y}^{\text{P}}$$

where Fa^{U} is d.p.m. from [$\text{U-}^{14}\text{C}$]glucose found in fatty

acids, Z' is d.p.m. from [U-¹⁴C]glucose found in CO₂ produced in the citric acid cycle, Y' is d.p.m. from [U-¹⁴C]glucose found in CO₂ produced by decarboxylation of pyruvate, Fa^P is d.p.m. from [U-¹⁴C]pyruvate found in fatty acid, Z^P is d.p.m. from [U-¹⁴C]pyruvate found in CO₂ produced in the citric acid cycle and Y^P is d.p.m. from [U-¹⁴C]pyruvate found in CO₂ produced by decarboxylation of pyruvate.

The relationship would be equally true if [U-¹⁴C]lactate were used instead of [U-¹⁴C]pyruvate.

Since Fa^U and Fa^P may be directly determined, and Z' and Y' may be calculated by the method of Flatt & Ball (1964), Z^P and Y^P may also be calculated. All flux rates were calculated as $\mu\text{g-atoms of glucose-, acetate-, lactate- or pyruvate-derived carbon/h per g wet wt. of tissue.}$

RESULTS

Measurements of metabolites in incubation media and in incubated adipose tissues. Nine experiments in all were performed. Table 1 records data concerning the body weight and epididymal adipose tissue weight of the animals used. The tissues incubated with insulin alone are taken throughout as controls for the purpose of statistical analysis. Table 1 shows that exclusion of insulin from, or addition of anti-insulin serum to, the incubation medium lowered the efflux of lactate and pyruvate with respect to the control; adrenaline in the presence of insulin considerably increased the efflux of lactate, pyruvate and glycerol. These results agree with those of Denton & Halperin (1968). A significant decrease with respect to the control in the release of lactate, and a significant increase in the release of pyruvate is recorded for tissues incubated in the presence of phenazine methosulphate+insulin. Tissues incubated in the presence of insulin+phenazine methosulphate and in the presence of insulin+lactate showed increased rates of glycerol production.

Table 2 shows measurements of the whole-tissue concentrations of several phosphorylated sugars under the nine experimental treatments. Exclusion of insulin from, or addition of anti-insulin serum to, the incubation medium decreased the contents of all measured phosphorylated sugars with respect to the insulin-treated control. The tissue concentrations of these intermediates were lower in the presence of anti-insulin serum than in tissues merely incubated in the absence of added insulin. The whole-tissue concentration of 6-phosphogluconate (in fact this represents the sum of 6-phosphogluconate+6-phosphogluconolactone in the intact tissue) appeared to be extremely responsive to changes in the components of the incubation medium in the presence of insulin. Significant increases in the content of 6-phosphogluconate with respect to the control were observed in the presence of acetate, lactate, pyruvate or phenazine metho-

Table 1. *Effects of insulin, adrenaline and various substrates on the release of lactate, pyruvate and glycerol by epididymal adipose tissues in vitro*
Details of incubation procedures are described in the Materials and Methods section. The results are given as mean values \pm s.e.m. * $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$ (versus group 1).

Expt. no.	Additions to incubation medium	Metabolite released ($\mu\text{mol/h per mg of tissue N}$)			Mean body wt. of animals (g)	Total no. of animals	No. of groups of pooled tissues	Mean wt. of tissues from each animal (mg)
		Pyruvate	Lactate	Glycerol				
1	Insulin	0.19 \pm 0.02	1.40 \pm 0.20	0.19 \pm 0.04	177 \pm 2	28	5	1045
2	None	0.13 \pm 0.01*	0.78 \pm 0.21	0.27 \pm 0.05	177 \pm 2	28	5	1113
3	Anti-insulin serum	0.069 \pm 0.003†	0.48 \pm 0.04†	0.23 \pm 0.02	172 \pm 2	24	6	1121
4	Insulin+acetate	0.27 \pm 0.03	1.11 \pm 0.14	0.13 \pm 0.02	171 \pm 2	22	5	1018
5	Insulin+pyruvate	—	—	—	165 \pm 2	24	6	978
6	Insulin+lactate	—	—	0.39 \pm 0.02†	166 \pm 2	24	6	1090
7	Insulin+phenazine methosulphate	0.45 \pm 0.01‡	0.59 \pm 0.07†	0.34 \pm 0.01†	166 \pm 2	24	6	977
8	Insulin+oleate+albumin	0.20 \pm 0.01	1.06 \pm 0.07	0.20 \pm 0.02	165 \pm 2	24	6	961
9	Insulin+adrenaline+albumin	0.91 \pm 0.09†	2.71 \pm 0.35†	4.64 \pm 0.85†	171 \pm 2	22	5	944

Table 2. *Effects of insulin, adrenaline and various substrates on whole-tissue concentrations of phosphorylated sugars in incubated epididymal adipose tissues*

Details of incubation procedures are described in the Materials and Methods section. The results are given as mean values \pm S.E.M. * $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$ (versus group 1). The tissues used were the same as those used in Table 1. The following abbreviations are used in this and subsequent tables: 6PG, 6-phosphogluconate; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; FDP, fructose 1,6-diphosphate; DHAP, dihydroxyacetone phosphate; G1P, glycerol 1-phosphate; 3PG, 3-phosphoglycerate.

Expt. no.	Additions to incubation medium	Metabolite concentrations (nmol/mg of tissue N)						
		6PG	G6P	F6P	FDP	DHAP	G1P	3PG
1	Insulin	3.75 \pm 0.25	6.01 \pm 0.59	2.10 \pm 0.28	1.30 \pm 0.17	2.01 \pm 0.20	28.7 \pm 3.0	4.10 \pm 0.70
2	None	1.19 \pm 0.64†	3.35 \pm 0.72*	1.24 \pm 0.23*	0.64 \pm 0.17*	0.93 \pm 0.29*	14.1 \pm 3.4*	2.30 \pm 0.70
3	Anti-insulin serum	0.29 \pm 0.03†	2.55 \pm 0.23†	1.08 \pm 0.05†	0.39 \pm 0.01†	0.69 \pm 0.10†	11.2 \pm 1.1†	—
4	Insulin + acetate	7.22 \pm 1.04*	6.46 \pm 0.66	1.99 \pm 0.17	1.26 \pm 0.17	1.68 \pm 0.14	19.5 \pm 2.9	3.95 \pm 0.34
5	Insulin + pyruvate	7.49 \pm 1.21*	6.91 \pm 0.79	2.52 \pm 0.25	1.31 \pm 0.32	1.84 \pm 0.26	12.1 \pm 1.0†	7.30 \pm 0.49†
6	Insulin + lactate	8.36 \pm 0.91†	11.73 \pm 0.59†	3.63 \pm 0.09†	0.95 \pm 0.06	1.95 \pm 0.11	50.4 \pm 3.2†	2.72 \pm 0.27
7	Insulin + phenazine methosulphate	13.0 \pm 1.1†	4.18 \pm 0.29*	1.67 \pm 0.17	—	—	8.9 \pm 1.2†	—
8	Insulin + oleate + albumin	3.53 \pm 0.31	4.75 \pm 0.33	1.40 \pm 0.08*	1.25 \pm 0.14	1.73 \pm 0.16	17.5 \pm 1.2†	4.14 \pm 0.35
9	Insulin + adrenaline + albumin	2.03 \pm 0.42*	4.60 \pm 0.60	1.32 \pm 0.21	1.08 \pm 0.09	1.21 \pm 0.15*	20.9 \pm 1.6	3.13 \pm 0.43

sulphate. A significant decrease in the content of 6-phosphogluconate was observed in the presence of insulin + adrenaline. On the other hand, the contents of glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, dihydroxyacetone phosphate and 3-phosphoglycerate were not so responsive as that of 6-phosphogluconate to changes in incubation conditions in the presence of insulin. The content of glucose 6-phosphate was increased by incubation with lactate, and decreased by incubation with phenazine methosulphate, oleate or adrenaline. Changes in fructose 1,6-diphosphate content roughly followed those of glucose 6-phosphate. Except where insulin was excluded from the incubation medium, significant changes in fructose 6-phosphate content were not observed. Similarly, the dihydroxyacetone phosphate content was not greatly affected by changes in incubation conditions, except in the presence of adrenaline. Incubation of tissues with pyruvate significantly increased the concentration of 3-phosphoglycerate, and incubation with lactate caused a small decrease in the concentration of this metabolite. The concentration of glycerol 1-phosphate in the presence of insulin was particularly affected by the inclusion in the incubation medium of metabolites likely to change the redox state of the extramitochondrial NAD^+ - NADH couple of the tissues. Pyruvate and phenazine methosulphate produced highly significant decreases in the concentration of this metabolite, whereas lactate produced a considerable increase. The concentration of glycerol 1-phosphate was also decreased significantly in the presence of oleate + insulin, but was not decreased significantly by adrenaline in the presence of insulin, unlike the result of Denton *et al.* (1966).

Table 3 shows that the contents of adenine nucleotides were not greatly altered in most of the nine cases studied. A significant change in the total adenine nucleotide content ($\text{ATP} + \text{ADP} + \text{AMP}$) was only observed in tissues incubated in the presence of anti-insulin serum, and is attributable to decreases in the whole-tissue concentrations of ATP and AMP. Significant decreases in ATP were also found in tissues incubated with oleate or adrenaline in the presence of insulin. This was accompanied by a significant increase in the content of AMP in the case of incubation with oleate, but not in that of incubation with adrenaline. Appreciable changes in the $\text{ATP}:\text{ADP}:\text{AMP}$ ratio were only observed with tissues incubated with oleate or adrenaline. The recorded effect of adrenaline in the presence of insulin and albumin is similar to that reported by Denton *et al.* (1966).

Table 4 shows the effects of the nine experimental treatments on the whole-tissue concentrations of CoA derivatives and various carboxylic acids. Unlike the results of Denton & Halperin (1968),

Table 3. *Effects of insulin, adrenaline and various substrates on the whole-tissue concentrations of adenine nucleotides in incubated epididymal adipose tissues*

Details of incubation procedures are described in the Materials and Methods section. The results are given as mean values \pm S.E.M. * $P < 0.05$; † $P < 0.001$ (versus group 1). The tissues used were the same as those used in Table 1.

Expt. no.	Additions to incubation medium	Metabolite concentrations (nmol/mg of tissue N)				Ratio ATP:ADP:AMP
		ATP	ADP	AMP	(ATP + ADP + AMP)	
1	Insulin	89.8 ± 9.0	21.7 ± 2.4	22.2 ± 3.6	133.7 ± 13.7	10:2.4:2.5
2	None	84.7 ± 10.1	18.4 ± 0.8	19.5 ± 4.0	122.5 ± 13.4	10:2.2:2.3
3	Anti-insulin serum	63.8 ± 1.9*	18.4 ± 1.4	12.8 ± 1.0*	94.8 ± 2.9*	10:2.9:2.0
4	Insulin + acetate	70.4 ± 5.3	19.3 ± 1.4	13.2 ± 1.4	103.0 ± 7.3	10:2.7:1.9
5	Insulin + pyruvate	67.9 ± 5.3	—	—	—	—
6	Insulin + lactate	74.5 ± 3.2	19.5 ± 1.5	18.9 ± 1.9	112.7 ± 4.5	10:2.6:2.5
8	Insulin + oleate + albumin	64.5 ± 3.6*	22.1 ± 1.2	43.6 ± 2.3†	130.0 ± 5.9	10:3.4:6.8
9	Insulin + adrenaline + albumin	56.7 ± 6.1*	26.2 ± 2.8	19.3 ± 3.0	102.2 ± 8.5	10:4.6:3.4

Table 4. *Effects of insulin, adrenaline and various substrates on the whole-tissue concentrations of long-chain fatty acyl-CoA, acid-soluble CoA, malate, citrate, isocitrate and 2-oxoglutarate in incubated epididymal adipose tissues*

Details of incubation procedures are described in the Materials and Methods section. The results are given as mean values \pm S.E.M. * $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$ (versus group 1). The tissues used were the same as those used in Table 1. 'Total CoA' represents the sum of the measured long-chain fatty acyl-CoA and acid-soluble CoA.

Expt. no.	Additions to incubation medium	Long-chain fatty acyl-CoA	Metabolite concentrations (nmol/mg of tissue N)					
			Acid-soluble CoA	Total CoA	Malate	Citrate	Isocitrate	2-Oxoglutarate
1	Insulin	0.82 \pm 0.15	2.20 \pm 0.16	3.03 \pm 0.26	8.82 \pm 1.64	9.64 \pm 0.50	0.43 \pm 0.01	5.45 \pm 0.27
2	None	1.24 \pm 0.27	1.82 \pm 0.29	3.06 \pm 0.34	3.77 \pm 0.37*	7.33 \pm 0.97	—	—
3	Anti-insulin serum	1.24 \pm 0.11	1.82 \pm 0.14	3.07 \pm 0.13	3.36 \pm 0.29†	7.44 \pm 0.50*	—	—
4	Insulin + acetate	1.02 \pm 0.24	1.90 \pm 0.20	2.91 \pm 0.15	9.21 \pm 0.90	20.0 \pm 0.8†	0.70 \pm 0.01†	8.91 \pm 0.32†
5	Insulin + pyruvate	0.61 \pm 0.10	0.74 \pm 0.08†	1.35 \pm 0.17†	75.4 \pm 10.59†	69.9 \pm 5.3†	1.97 \pm 0.19†	—
6	Insulin + lactate	0.80 \pm 0.07	1.35 \pm 0.18†	2.43 \pm 0.25	40.4 \pm 3.1†	20.3 \pm 1.3†	0.85 \pm 0.06†	—
7	Insulin + phenazine methosulphate	—	—	—	23.5 \pm 0.9†	35.5 \pm 2.1†	—	—
8	Insulin + oleate + albumin	0.87 \pm 0.07	2.20 \pm 0.14	3.08 \pm 0.20	9.82 \pm 0.82	12.0 \pm 1.45	0.47 \pm 0.05	6.77 \pm 0.36*
9	Insulin + adrenaline + albumin	1.00 \pm 0.10	0.58 \pm 0.25†	1.59 \pm 0.26†	19.4 \pm 2.1†	46.2 \pm 2.3†	0.99 \pm 0.08†	11.59 \pm 0.91†

Table 5. *Effect of alkali on the concentration of acid-soluble CoA measurable in extracts obtained from epididymal adipose tissues incubated in the presence of insulin + adrenaline + albumin*

Incubation procedures were as described in the Materials and Methods section. Adrenaline was present at a concentration of $5 \mu\text{g/ml}$. Alkali treatment of extracts was effected by adjusting the pH of a sample of each extract to 12.0 and maintaining it at that pH for 10 min at 30°C . The extracts were then reacidified with 60% (v/v) HClO_4 and neutralized and freeze-dried as described in the Materials and Methods section. The results are given as mean values \pm S.E.M.

Acid-soluble CoA before alkali hydrolysis (nmol/mg of tissue N)	Acid-soluble CoA after alkali hydrolysis (nmol/mg of tissue N)	No. of determinations
0.96 ± 0.08	0.90 ± 0.10	6

Table 6. *Whole-tissue concentrations of metabolites in epididymal adipose tissues excised under Nembutal anaesthesia*

Results are mean values \pm S.E.M. of five determinations (fifteen animals were used in all, the pads of three animals being pooled) and are expressed as nmol of metabolite/mg of tissue N. Extraction and assay of metabolites was as described in the Materials and Methods section. Although precautions were taken to ensure that as little blood as possible was associated with the extracted tissues, no correction has been applied to the above figures to allow for a contribution from blood metabolites.

Long-chain fatty acyl-CoA	Acid-soluble CoA	Total CoA	ATP	ADP	AMP	(ATP + ADP + AMP)
0.88 ± 0.16	0.80 ± 0.11	1.68 ± 0.22	51.8 ± 3.2	17.1 ± 0.5	40.2 ± 3.7	109.0 ± 6.0
G6P	F6P	FDP	DHAP	G1P	6PG	
2.84 ± 0.26	0.86 ± 0.13	0.40 ± 0.07	0.68 ± 0.06	2.05 ± 0.50	0.61 ± 0.14	
	Malate	Citrate	Pyruvate	Lactate		
	7.82 ± 0.41	15.5 ± 1.64	80.9 ± 20.5	206.7 ± 15.9		

no significant changes in the concentrations of long-chain fatty acyl-CoA derivatives were observed in any of the treatments, although the observed alteration in the tissue concentration of long-chain fatty acyl-CoA between the insulin-treated and insulin-free states was qualitatively similar to that demonstrated by Denton & Halperin (1968). Surprising decreases in the tissue concentrations of acid-soluble CoA were encountered with tissues incubated with pyruvate, lactate or adrenaline + insulin. This resulted in significant decreases in the value of total CoA with tissues incubated with pyruvate or adrenaline. In an attempt to elucidate the reason for the observed decrease in acid-soluble CoA concentrations under these conditions, the experiment shown in Table 5 was performed. The results indicated that treatment with alkali of acid-soluble extracts prepared from tissues incubated in the presence of insulin + adrenaline does not increase the measurable acid-soluble CoA in these extracts. This would suggest that the observed decreases in acid-soluble CoA, and consequently in total CoA, with respect to insulin-treated controls, in tissues incubated with insulin + adrenaline (Table 4) cannot be accounted for by increases in the concentration of some acid-soluble, alkali-labile CoA

derivative. Table 4 shows that tissues incubated in the absence of insulin contained significantly lower concentrations of malate, but only slightly lower concentrations of citrate compared with the controls. In the presence of insulin, the tissue concentration of citrate was considerably increased by incubation with acetate, pyruvate, lactate, phenazine methosulphate and adrenaline. Increases in the concentration of malate were also observed under these conditions, except for tissues incubated with acetate. Where they were measured, the tissue concentrations of isocitrate and 2-oxoglutarate changed in response to altered incubation conditions in a similar fashion to that of citrate.

Measurements of metabolites in non-incubated adipose tissues. Measurements of the whole-tissue concentrations of a range of metabolites in non-incubated, directly freeze-clamped, epididymal fat pads are shown in Table 6. Tissue concentrations of lactate and pyruvate were determined in this experiment. In incubated fat pads reliable estimates of the tissue concentrations of lactate and pyruvate could not be made owing to the presence of considerable amounts of these metabolites in the incubation medium. Values of Fisher's 'P' were calculated for the metabolite concentrations mea-

sured in the non-incubated fat pads versus the results obtained with fat pads incubated in the presence of insulin and also versus the results obtained with fat pads incubated in the presence of anti-insulin serum. ($P < 0.05$ was taken to represent a significant difference.) The tissue contents of long-chain fatty acyl-CoA, ADP and (ATP + ADP + AMP) were the same in non-incubated tissues and in incubated tissues with and without insulin. The non-incubated tissues contained significantly higher contents of AMP and citrate, and significantly lower contents of acid-soluble CoA, total CoA, ATP and glycerol 1-phosphate than did tissues incubated with or without insulin. The concentration of malate in the non-incubated tissues was similar to that in tissues incubated in the presence of insulin, but significantly greater than that in tissues incubated in the absence of insulin. The concentrations in the non-incubated tissues of the phosphorylated sugars 6-phosphogluconate, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate and dihydroxyacetone phosphate were all similar to those in tissues incubated in the absence of insulin, but were significantly lower than those in tissues incubated in the presence of insulin.

Measurements of intracellular water spaces and glucose- ^{14}C sorbitol spaces in incubated adipose tissues. Measurements of total intracellular water spaces and glucose- ^{14}C sorbitol spaces are presented in Table 7. As reported by Denton *et al.* (1966) the intracellular water space was not significantly affected by the presence or absence of insulin; however the glucose- ^{14}C sorbitol space was significantly different in the treatments, being positive in the absence of insulin and negative in the presence of the hormone under the conditions employed. The overall mean value for the volume of intracellular water of $33.1 \pm 2.3 \mu\text{l/g}$ wet wt. was not greatly different from the value of $41 \mu\text{l/g}$ wet wt. reported by Crofford & Renold (1965) but was considerably greater than the value of $14 \pm 1.8 \mu\text{l/g}$ wet wt. found by Denton, *et al.* (1966).

Enzyme mass-action ratios and ratios of whole-tissue metabolite concentrations in incubated and non-incubated adipose tissues. Mass-action ratios (Γ) for the phosphofructokinase, adenylate kinase, phosphoglucose isomerase and aconitase reactions are shown in Table 8. Values for the combined mass-action ratio (aldolase \times triose phosphate isomerase) are also shown. Separate Γ values for aldolase and triose phosphate isomerase could not be determined since the concentration of glyceraldehyde 3-phosphate was generally too low to measure. Estimates were also made of the extramitochondrial free $[\text{NAD}^+]/[\text{NADH}]$ ratio in the tissues as calculated from the $[\text{lactate}]/[\text{pyruvate}]$ ratio in the incubation medium and from the $[\text{glycerol 1-phosphate}]/$

Table 7. *Measurements of intracellular water spaces and glucose- ^{14}C sorbitol spaces in epididymal adipose tissues in vitro in the presence and absence of insulin*

Additions to incubation medium	Mean body wt. of animals (g)	Mean wt. per pair of fat pads (mg)	Incubation procedures and calculation of tissue spaces were as described in the Materials and Methods section. All results are mean values \pm s.e.m. The first two rows represent an experiment with paired fat pads from the same animals incubated in the presence and absence of insulin. * $P < 0.001$ (versus the insulin-treated control). The third row represents a separate experiment involving a larger number of determinations made only in the presence of insulin.			No. of determinations
			^3H Water- ^{14}C sorbitol space/g of tissue (μl)	^3H Water- ^{14}C sorbitol space/mg of tissue N (μl)	Glucose- ^{14}C sorbitol space/mg of tissue N (μl)	
Anti-insulin serum + sorbitol	157 ± 3	1222 ± 54	24.0 ± 1.9	9.3 ± 1.0	$+4.0 \pm 0.9^*$	4
Insulin + sorbitol	163 ± 3	1232 ± 44	27.5 ± 2.3	10.6 ± 1.2	-14.4 ± 1.6	10
			33.1 ± 2.3	13.5 ± 1.0	-10.9 ± 1.5	

Table 8. *Enzyme mass-action ratios and ratios of whole-tissue metabolite concentrations measured in incubated epididymal adipose tissues and in non-incubated tissues excised under Nembutal anaesthesia*

The data on metabolite concentrations in incubated tissues were derived from Tables 1, 2, 3 and 4. The calculation of Γ for (aldolase \times triosephosphate isomerase) required the use of intracellular water space data of Table 7. The value of $13.5 \mu\text{l}$ of intracellular water/mg of tissue N was used throughout. \S The assumption was made that the non-incubated tissues had the same intracellular water spaces as tissues incubated for 1 h in the presence of insulin. The data on metabolite concentrations in non-incubated tissues were derived from Table 6. All results are given as mean values \pm S.E.M. except for the [DHAP]/[FDP] values. * $P < 0.05$; $\dagger P < 0.001$ (versus group 1). P values were not calculated for the data on non-incubated tissues. Extramitochondrial free [NAD $^{+}$]/[NADH] values were calculated, assuming an intracellular pH of 7.0, as described by Hohorst *et al.* (1961) assuming apparent equilibrium constants for lactate dehydrogenase and glyceral 1-phosphate dehydrogenase of 1.11×10^{-4} and 8.9×10^{-5} respectively (37°C, ionic strength 0.25) (Williamson *et al.* 1967; Hohorst *et al.* 1961).

Expt. no.	Additions to incubation medium	Enzyme mass-action ratios (Γ)										Calculated extramitochondrial free [NAD $^{+}$]/[NADH] ratios calculated from		
		Phospho-fructokinase: [FDP]/[ADP]	Adenylylate kinase: [ATP]/[AMP]	Phosphoglucose isomerase: [F6P] [G6P]		(Aldolase \times triose phosphate isomerase): [DHAP] † [FDP]		Aconitase: [citrate] [isocitrate]	[2-Oxoglutarate] ratio		[Lactate] [pyruvate]		[GIP] [DHAP]	
1	Insulin	0.16 \pm 0.03	4.66 \pm 1.19	0.35 \pm 0.04	2.3 $\times 10^{-4}$ M	12.7 \pm 0.7	22.6 \pm 1.5	—	—	1212 \pm 197	775 \pm 178	—	—	—
2	None	0.14 \pm 0.03	5.44 \pm 1.90	0.39 \pm 0.05	1.0 $\times 10^{-4}$ M	—	—	—	—	1542 \pm 366	819 \pm 93	—	—	—
3	Anti-insulin serum	0.11 \pm 0.01	2.55 \pm 0.33	0.44 \pm 0.04	9.1 $\times 10^{-5}$ M	—	—	—	—	1301 \pm 64	580 \pm 132	—	—	—
4	Insulin + acetate	0.19 \pm 0.05	2.60 \pm 0.43	0.31 \pm 0.02	1.7 $\times 10^{-4}$ M	26.8 \pm 1.1*	—	—	12.8 \pm 0.6	2053 \pm 415	957 \pm 119	—	—	—
5	Insulin + pyruvate	—	—	0.38 \pm 0.03	1.9 $\times 10^{-4}$ M	36.9 \pm 4.2*	—	—	—	—	1592 \pm 184*	—	—	—
6	Insulin + lactate	0.07 \pm 0.01*	3.96 \pm 0.64	0.31 \pm 0.01	3.0 $\times 10^{-4}$ M	24.2 \pm 0.8	—	—	—	—	431 \pm 19	—	—	—
7	Insulin + phenazine methosulphate	—	—	0.40 \pm 0.01	—	—	—	—	—	6806 \pm 833†	—	—	—	—
8	Insulin + oleate + albumin	0.31 \pm 0.04*	5.88 \pm 0.53	0.30 \pm 0.01	1.8 $\times 10^{-4}$ M	25.5 \pm 1.8	—	—	14.8 \pm 1.2	1644 \pm 62	1055 \pm 117	—	—	—
9	Insulin + adrenaline + albumin	0.44 \pm 0.10*	1.77 \pm 0.51	0.29 \pm 0.03	1.0 $\times 10^{-4}$ M	48.5 \pm 4.3†	—	—	12.1 \pm 1.2	2981 \pm 406†	626 \pm 59	—	—	—
Tissues removed under Nembutal anaesthesia; no incubation carried out		0.16 \pm 0.02	7.10 \pm 0.71	0.31 \pm 0.05	8.6 $\times 10^{-5}$ M§	—	—	—	—	3145 \pm 419	3636 \pm 826	—	—	—

[dihydroxyacetone phosphate] ratio in the tissues. For the purposes of this calculation, the arbitrary assumption was made that the medium [lactate]/[pyruvate] ratio was similar to that prevalent in the tissue at the time of sampling.

The Γ value for the phosphofructokinase reaction was found to be similar to that reported by Halperin & Denton (1969) for aerobic tissues *in vitro*, and to be far removed from the value of 1200 for the apparent equilibrium constant of phosphofructokinase (Hess, 1963). A significant decrease in this ratio was observed in the presence of lactate, whereas significant increases were observed in the presence of oleate or adrenaline.

The Γ value for the adenylate kinase reaction was somewhat larger than the published apparent equilibrium constant of 0.44 (Eggleston & Hems, 1952), but the Γ value calculated for the phosphoglucose isomerase reaction was always very close to the apparent equilibrium constant of 0.47 (Hess, 1963). The combined Γ value for (aldolase \times triosephosphate isomerase) was generally an order of magnitude smaller than the equilibrium values of [dihydroxyacetone phosphate]²/[fructose diphosphate] that can be ascertained from the literature (equilibrium values for this quotient range from $1.4 \times 10^{-3} \text{ M}$ to $3.7 \times 10^{-3} \text{ M}$: Herbert, Gordon, Sabrahmanyam & Green, 1940; Meyerhof & Junowicz-Kocholaty, 1943; Krebs & Kornberg, 1957; Hess, 1963; Lowry & Passonneau, 1964).

The calculated Γ values for the aconitase reaction were not greatly in excess of the apparent equilibrium constant of 15 (Krebs, 1953) for this reaction. However, significant increases in the Γ value were encountered in tissues incubated in the presence of insulin+acetate, pyruvate or adrenaline. Significant changes in the [2-oxoglutarate]/[isocitrate] ratio were not encountered under the same conditions.

It is noteworthy that the Γ values for the phosphofructokinase, adenylate kinase, phosphoglucose isomerase, and (aldolase \times triosephosphate isomerase) reactions were essentially the same in the non-incubated tissues as in the incubated tissues.

Appreciable changes in the extramitochondrial free $[\text{NAD}^+]/[\text{NADH}]$ ratio appeared to be caused by inclusion of pyruvate, lactate or phenazine methosulphate in the incubation medium. Changes in this ratio between other incubation conditions could not be shown to be significant owing to the large scatter between the individual determinations of metabolite ratios. In the case of the non-incubated tissues, where the tissue [lactate]/[pyruvate] ratio could be determined directly, quite good agreement was found between the two independent estimates of the extramitochondrial free $[\text{NAD}^+]/[\text{NADH}]$ ratio. However, in the case of incubated tissues, Table 8 shows that estimates

of the extramitochondrial free $[\text{NAD}^+]/[\text{NADH}]$ ratio from the medium [lactate]/[pyruvate] ratio and from the whole-tissue [glycerol 1-phosphate]/[dihydroxyacetone phosphate] ratio are not in such good agreement with each other.

Measurements of carbon fluxes in segments of incubated tissues. Tables 9 and 10 show measurements made of the fluxes of glucose-derived carbon through various metabolic pathways in the presence of various added hormones or metabolites. The results also indicate the magnitude of the fluxes of acetate-, lactate- or pyruvate-derived carbon through certain pathways in the presence of glucose.

In the absence of insulin (i.e. in the presence of anti-insulin serum) glucose-carbon flux through all pathways was far less than that found in the presence of insulin, although lactate+pyruvate formation and citric acid cycle flux appeared to be decreased to a lesser extent than other pathways. Qualitatively similar effects were observed by Flatt & Ball (1964).

In the presence of insulin+adrenaline, rates of glucose-carbon flux from hexose phosphate to triose phosphate, into glyceride glycerol, into lactate+pyruvate and into citric acid cycle CO_2 were greatly increased compared with the controls. These increases in flux rates were quantitatively far greater than those found by Flatt & Ball (1964). On the other hand, glucose-carbon fluxes into fatty acids and into hexose monophosphate pathway CO_2 were considerably decreased below the control values in this study, a finding not in accord with the observations of Flatt & Ball (1964). The differences between the present results and those of the latter authors may, however, be explained by the fact that these workers used adrenaline at a final concentration of $0.2 \mu\text{g/ml}$ in their incubations, which was only $\frac{1}{25}$ of that used in the present study. By using a final concentration of adrenaline of $5 \mu\text{g/ml}$, Denton & Randle (1967) have also found that rates of fatty acid synthesis from glucose carbon are decreased in the presence of insulin+adrenaline compared with the rates observed in the presence of insulin alone. Tables 9 and 10 show that incubation of tissues with dibutyryl 3':5'-cyclic AMP+insulin produced less pronounced, but basically similar, effects to those produced by adrenaline+insulin. Incubation of tissues with oleate in the presence of insulin led to increased fluxes of glucose carbon into glyceride glycerol and into citric acid cycle CO_2 , but did not mimic the effects of adrenaline or dibutyryl 3':5'-cyclic AMP in other respects.

Incubation of tissues with acetate produced essentially the same effects on glucose-carbon flux as those reported by Flatt & Ball (1966), with one exception. In the present study incubation with acetate led to considerable increases in measured

Table 9. *Measurements of fluxes of carbon derived from various substrates through metabolic pathways in incubated segments of epididymal adipose tissues*

The results for each experimental group are presented as percentage of the fluxes measured in control incubations (glucose + insulin present) with tissues from the same animals. To illustrate the magnitude of the fluxes, the means of all the control data are presented at the bottom of the table as μg -atoms of carbon/h per g wet wt. of tissue. Flux rates were calculated as described in the Materials and Methods section.

Expt. no.	Additions to incubation medium	No. of determinations	Hexose		Hexose phosphate to triose phosphate flux	Glyceride-glycerol formation from glucose	Glyceride-glycerol formation from all added substrates	Triose phosphate to pyruvate flux
			monophosphate pathway CO_2 production	production				
10	Anti-insulin serum	5	12		11	40	40	11
11	Insulin + acetate	8	156		133	120	120	137
12	Insulin + pyruvate	7	117		35	95	113	42
13	Insulin + lactate	8	118		36	90	115	40
14	Insulin + phenazine methosulphate	8	259		73	119	119	100
15	Insulin + oleate + albumin	11	116		119	250	250	110
16	Insulin + adrenaline + albumin	8	41		213	539	539	151
17	Insulin + dibutyl 3':5'-cyclic AMP + albumin	5	54		108	158	158	92
	Insulin (control)	45	10.01 \pm 0.71		68.02 \pm 4.82	6.12 \pm 0.27	6.12 \pm 0.27	71.30 \pm 5.24

Table 10. *Measurements of fluxes of carbon derived from various substrates through metabolic pathways in incubated segments of epididymal adipose tissues (continued)*

The results for each experimental group are presented as percentages of the fluxes measured in control incubations (glucose+insulin present) with tissues from the same animals. To illustrate the magnitude of the fluxes, the means of all the control data are presented at the bottom of the table as μg -atoms of carbon/h per g wet wt. of tissue. Flux rates were calculated as described in the Materials and Methods section.

Expt. no.	Additions to incubation medium	Lactate + pyruvate release (derived from glucose carbon)	CO ₂ produced by pyruvate decarboxylation		CO ₂ produced by citric acid cycle (derived from glucose carbon)		CO ₂ produced by citric acid cycle (derived from all added substrates)		Fatty acid synthesis from all added substrates
			7	7	81	81	81	81	
10	Anti-insulin serum	38	144	144	81	81	81	81	6
11	Insulin + acetate	110	144	144	324	490	139	167	167
12	Insulin + pyruvate	106	27	103	91	364	25	100	100
13	Insulin + lactate	95	28	125	34	162	28	125	125
14	Insulin + phenazine methosulphate	100	100	100	231	231	95	95	95
15	Insulin + oleate + albumin	118	108	108	342	342	103	103	103
16	Insulin + adrenaline + albumin	280	118	118	2300	2300	56	56	56
17	Insulin + dibutyl 3':5'-cyclic AMP + albumin	107	71	71	641	641	36	36	36
Insulin (control)		14.57 \pm 0.84	20.77 \pm 1.64	20.77 \pm 1.64	0.91 \pm 0.06	0.91 \pm 0.06	40.62 \pm 3.26	40.62 \pm 3.26	40.62 \pm 3.26

citric acid cycle flux. This effect was not found by Flatt & Ball (1966), but was noted by Rognstad & Katz (1966). It should be noted however, that different concentrations of glucose and acetate were used in each of the three studies.

Incubation of tissues with phenazine methosulphate in the presence of insulin did not appreciably alter the pattern of glucose metabolism compared with the controls, except that CO₂ production from glucose-derived carbon in the hexose monophosphate pathway and the citric acid cycle was considerably increased. These results agree with the observation of McLean (1960) that CO₂ production from [1-¹⁴C]glucose and from [6-¹⁴C]glucose by incubated adipose tissues is considerably increased in the presence of 0.1 mM-phenazine methosulphate.

Tables 9 and 10 show that in the presence of added lactate or pyruvate, metabolism of glucose to acetyl-CoA and hence to fatty acids was decreased compared with the controls, while utilization of glucose carbon by the hexose monophosphate pathway and for glyceride-glycerol synthesis was maintained. In most cases the pattern of glucose utilization in the presence of lactate was similar to that in the presence of pyruvate. Also, the pattern of lactate metabolism to fatty acids and to glyceride glycerol was mainly similar to that observed for pyruvate. However, it was observed that the appearance of glucose-derived carbon in citric acid cycle CO₂ was appreciably greater in the presence of pyruvate than in the presence of lactate. In addition, the production of CO₂ from pyruvate in this pathway was considerably greater than from lactate. It was found that a mean value of 41% of the CO₂ produced from [U-¹⁴C]pyruvate by tissues incubated with pyruvate+glucose could not be accounted for as CO₂ produced by oxidative decarboxylation of pyruvate or by the action of the citric acid cycle. A comparable mean value of 18% was found for CO₂ produced from [U-¹⁴C]lactate. The significance of these results is discussed below.

DISCUSSION

Measurements of tissue metabolite concentrations in vitro. Measurements of certain adipose tissue metabolites *in vitro* have been reported previously (Denton *et al.* 1966; Yorke, 1967; Denton & Halperin, 1968; Halperin & Denton, 1969). The effects of insulin and adrenaline on the concentrations of long-chain fatty acyl-CoA, acid-soluble CoA, adenine nucleotides, citrate, glycerol 1-phosphate, glucose 6-phosphate and fructose 1,6-diphosphate presented in the present study are in agreement with these. As far as can be ascertained, however, there have been no previous reports of measurements of dihydroxyacetone phosphate, malate, isocitrate,

Table 11. *Calculated concentrations of metabolites in incubated epididymal adipose tissues*

The data were calculated from the results of Tables 2, 3, 4 and 7. An intracellular water space of 13.5 μ l/mg of tissue N was assumed throughout. It was assumed that all the metabolites were evenly distributed throughout the entire intracellular water.

Additions to incubation medium	Long-chain fatty acyl-CoA	Concentrations of metabolites in intracellular water (μ M)									
		Acid-soluble CoA	ATP	ADP	AMP	GAP	FDP	DHAP	GIP	6PG	Citrate
Insulin	61	160	6700	1600	1700	450	97	150	2100	280	720
Anti-insulin serum	92	140	4700	1400	950	190	29	51	830	22	550

2-oxoglutarate, 3-phosphoglycerate or 6-phosphogluconate made in adipose tissues *in vitro*.

The measurements presented in Tables 2, 3 and 4 are expressed as nmol/mg of tissue N. To convert these measurements into nmol/g wet wt. it is necessary to multiply by a factor of approx. 3.5. On this basis the tissue contents of metabolites found in this study are approximately twice those found by Denton *et al.* (1966), Denton & Halperin (1968) and Halperin & Denton (1969). This difference can be explained by the fact that the intracellular water space in the tissues used in this study is approx. 2.5 times that found by Denton *et al.* (1966). The concentrations of intermediates, expressed as concentrations in the total intracellular water, found in this study (Table 11) are thus essentially in agreement with those recorded by Denton *et al.* (1966), although the concentrations of acid-soluble CoA and long-chain fatty acyl-CoA in this study appear to be generally lower than those reported by Denton & Halperin (1968). The discrepancy in intracellular water volume and hence in metabolite content/g wet wt. between this study and those of Denton & co-workers can probably be explained by the fact that rats of smaller body weight were used in this study. As discussed by Perry & Hales (1969), the intracellular water content/g wet wt. of rat epididymal adipose tissues is found to decrease with increasing body weight.

Assuming that the whole-tissue concentrations presented in Table 11 can be related to the relevant compartment(s) several metabolites exhibit intracellular concentrations that may have metabolic significance. The recorded concentrations of long-chain fatty acyl-CoA are sufficient to cause severe inhibition of liver acetyl-CoA carboxylase (Numa, Bortz & Lynen, 1964), liver citrate synthase (Tubbs, 1963) and liver or adipose tissue glucose 6-phosphate dehydrogenase (Eger-Neufeldt, Teinzer, Weiss & Wieland, 1965) *in vitro*, and also to produce various degrees of inhibition of many other enzymes (Taketa & Pogell, 1966). However, at intracellular protein concentrations, it is possible that inhibitory effects of long-chain fatty acyl-CoA might not be observed (Fang & Lowenstein, 1967).

Whole-tissue concentrations of glucose 6-phosphate in the presence of insulin were well above published K_i values for inhibition of adipose tissue hexokinases type I or II, by glucose 6-phosphate with respect to glucose or ATP (Grossbard & Schimke, 1966). Similarly, whole tissue concentrations of 6-phosphogluconate were in a range sufficient to cause appreciable inhibition *in vitro* of phosphoglucose isomerase from a number of tissues (Parr, 1956; Kahana, Lowry, Schulz, Passonneau & Crawford, 1960; Takeda, Hizukuri & Nikuni, 1967). However, since the activity of phosphoglucose

isomerase is relatively high in adipose tissue (Saggerson & Greenbaum, 1969), and this enzyme appears to maintain near equilibration of its reactants under all the conditions studied (Table 8), it would appear unlikely that 6-phosphogluconate concentrations are important in considerations of glycolytic control.

Concentrations of AMP of the magnitude shown in Table 11 produce near-maximal activation of semi-purified adipose tissue phosphofructokinase (Denton & Randle, 1966) whereas the whole-tissue concentrations of fructose 6-phosphate found in this study (ranging from 0.08 mM to 0.27 mM) would not produce maximal activities of phosphofructokinase in adipose tissue extracts in the presence of optimum concentrations of AMP (E. D. Saggerson & A. L. Greenbaum, unpublished work). The whole-tissue concentrations of fructose 1,6-diphosphate found in this study, except for those found in tissues incubated without insulin, were sufficient to activate fully the 'A' form of adipose tissue pyruvate kinase *in vitro* (Pogson, 1968). Also, the K_m of adipose tissue glycerol 1-phosphate dehydrogenase (8.6×10^{-5} M) determined by Halperin & Denton (1969) is intermediate between the whole-tissue concentrations of dihydroxyacetone phosphate found in tissues incubated with or without insulin. Glycerol 1-phosphate whole-tissue concentrations were high in the incubated tissues and were well above published K_m values of the adipose tissue esterifying system for this metabolite (Steinberg, Vaughan & Margolis, 1961; Angel & Roncari, 1967). It is therefore possible that the esterifying system of the tissues is saturated with this metabolite under the conditions employed.

Citrate concentrations varied considerably between the various incubation conditions (whole-tissue concentrations ranged from 0.55 mM to 5.18 mM). Inhibition of semi-purified adipose tissue phosphofructokinase was obtained over this concentration range (Denton & Randle, 1966); however it is not known to what extent high concentrations of citrate under some conditions might represent mitochondrial accumulations. The dependence of phosphofructokinase activity and fatty acid synthesis on the concentration of citrate is considered below.

It is noteworthy that concentrations of phosphorylated sugars in tissues incubated with anti-insulin serum were appreciably lower than those in tissues incubated solely in the absence of any added insulin (Table 2). These results probably indicate that, even after preincubation, some insulin is retained in association with the tissue, and that it is therefore necessary to employ anti-insulin serum to obtain a truly insulin-free state. The unusually high variability of the results obtained in the absence of insulin and anti-insulin serum (Table 2,

second row) also suggests that an uncertain proportion of associated insulin is removed by preincubation.

An unexpected result that cannot be explained readily was the finding that incubation with insulin and adrenaline or insulin+pyruvate led to considerable diminutions in the total amount of CoA measurable in the tissues. A similar, but smaller, effect due to incubation with adrenaline is noticeable in the data of Denton & Halperin (1968).

Measurements of tissue metabolite concentrations in vivo. As far as can be ascertained, the only previous measurements of adipose tissue metabolites *in vivo* are those of Ballard & Hanson (1969), who measured the concentrations of several metabolites in fat pads taken from ether-anaesthetized rats. In the present study no corrections were made for the presence of metabolites in the extracellular spaces. However, the results of Ballard & Hanson (1969) suggest that such a correction may be small except when measurements of lactate and pyruvate are made. It is noteworthy that, expressed as nmol/g wet wt., the concentrations of citrate, malate and lactate presented in Table 6 for normally fed rats were all approximately three times as high as those reported by Ballard & Hanson (1969). However, these workers used rats of double the body weight of those used in this study. The concentrations of pyruvate reported in Table 6 would be approx. 20 times those reported by Ballard & Hanson (1969) expressed as nmol/g wet wt. This high concentration of pyruvate leads to an unusually low value for the tissue [lactate]/[pyruvate] ratio. It is difficult to explain the reason for this low value.

The concentrations of phosphorylated sugar intermediates *in vivo* were all far lower than those found in tissues incubated with glucose and insulin, although the mass-action ratios of phosphofructokinase, phosphoglucose isomerase and aldolase \times triose phosphate isomerase were essentially the same as those found *in vitro*. The low concentrations of phosphorylated sugar intermediates *in vivo* could perhaps reflect the presence *in vivo* of lower concentrations of insulin, glucose, or both, than the unphysiologically high concentrations used *in vitro*, although the presence of other hormones *in vivo* may give rise to lower concentrations of phosphorylated intermediates. For example, Yorke (1967) showed that incubation with the corticosteroid analogue, dexamethasone, leads to a halving of the tissue concentrations of glucose 6-phosphate and glycerol 1-phosphate.

Enzyme mass-action ratios. (a) Phosphofructokinase. As was also found in adipose tissue by Halperin & Denton (1969) and in other tissues (Hess, 1963; Lowry & Passonneau, 1964; Williamson, 1965; Minakami & Yoshikawa, 1966;

Rolleston & Newsholme, 1967), the mass-action ratio for phosphofructokinase based on whole-tissue measurements was very much smaller than the K_a for this reaction. Phosphofructokinase in adipose tissue is therefore a potential site of control (Newsholme & Gevers, 1967) if the whole-tissue mass-action ratio accurately reflects the situation within the relevant cell compartment(s). The [fructose 1,6-diphosphate]/[fructose 6-phosphate] portion of the phosphofructokinase mass-action ratio may be a reasonable estimate of the ratio of these metabolites in the environment of phosphofructokinase. However, it is uncertain whether the whole tissue [ADP]/[ATP] ratio reflects the ratio in the cytoplasm.

(b) Phosphoglucose isomerase. As may be expected from the relatively high activity of this enzyme in adipose tissue (Saggerson & Greenbaum, 1969), it appears to maintain a near equilibration between its reactants under all the conditions studied. This situation is also encountered in other tissues.

(c) Aldolase and triose phosphate isomerase. The disequilibration of the reactants of the combined aldolase \times triose phosphate isomerase reaction was relatively small. In the one experimental case where it was possible to measure the concentration of glyceraldehyde 3-phosphate in adipose tissue (Saggerson & Greenbaum, 1970) it would appear that the disequilibration resides in the triose phosphate isomerase reaction.

(d) Hexokinase. Table 7 shows that values of the glucose-[^{14}C]sorbitol space were negative for tissues incubated in the presence of insulin, and therefore no estimate could be made of the concentration of intracellular glucose. However, in the case of tissues incubated with anti-insulin serum, the value of the glucose-[^{14}C]sorbitol space was positive and, with 20 mM-glucose in the medium, was of a magnitude that would indicate a minimal value for the intracellular glucose concentration of 80 nmol/mg of tissue N (Morgan, Henderson, Regen & Park, 1961). By using values from Tables 2 and 3, a maximal estimate of the hexokinase mass-action ratio may therefore be calculated as:

$$\Gamma = \frac{[\text{ADP}][\text{glucose 6-phosphate}]}{[\text{ATP}][\text{glucose}]} = 9.2 \times 10^{-3}$$

This may be compared with a value of 5.5×10^3 for the K_a of this reaction (Hess, 1963). For tissues incubated with 20 mM-glucose and anti-insulin serum therefore, the adipose tissue hexokinase reactants may be considered as being far from equilibrium and hexokinase may be a potential site of control (Newsholme & Gevers, 1967). However, as discussed for phosphofructokinase, there must be reservations concerning the ability of whole-

tissue [glucose 6-phosphate]/[glucose] ratios, and especially whole tissue [ADP]/[ATP] ratios to describe the situation existing in the relevant compartment(s). It is noteworthy that the apparent degree of disequilibrium observed in the hexokinase reactants in this study ($K_a/\Gamma = 6.0 \times 10^5$) is similar to that observed in the perfused rat heart (Williamson, 1965), in erythrocytes (Minakami & Yoshikawa, 1966) and in cerebral cortex slices (Rolleston & Newsholme, 1967).

(e) Aconitase. The aconitase mass-action ratio in the incubated tissues was generally higher than the K_a of 15 (Krebs, 1953). This may indicate a varying compartmentation of citrate and isocitrate in the tissues, or it may indicate a slight disequilibrium between the two metabolites. It is of note that the three significantly increased mass-action ratios compared with the control tissues were found in tissues incubated with insulin + acetate, pyruvate, or adrenaline. In all three cases considerable increases were observed in the measured rates of citric acid cycle flux (estimates of citric acid cycle flux did not include allowances for utilization of endogenous acetyl-CoA). Increased deviations from thermodynamic equilibrium might be expected under these conditions of increased flux (Bücher & Rüssman, 1964). Alternatively, observed alterations in the aconitase mass-action ratio could be explained by alterations in the concentration of metal ions such as Mg^{2+} and Ca^{2+} in the vicinity of aconitase within the tissues (England, Denton & Randle, 1967; Blair, 1969).

(f) Adenylate kinase. The whole-tissue $[ATP][AMP]/[ADP]^2$ ratio in all the incubated tissues and also in the tissues removed from anaesthetized animals was appreciably larger than the K_a for this reaction. Generally, in other tissues, whole-tissue contents of ATP, ADP and AMP are found to be very near to adenylate kinase mass-action equilibrium proportions (Williamson, 1967; Rolleston & Newsholme, 1967; Underwood & Newsholme, 1967; Start & Newsholme, 1968). Also, with the exception of anaerobic tissues, Denton *et al.* (1966) and Halperin & Denton (1969) have presented measurements of ATP, ADP and AMP that are near to mass-action equilibrium. The whole tissue $[ATP]/[ADP]$ ratios presented in this study are similar to those in other tissues and the high value of Γ for adenylate kinase is brought about by a low value for the $[ATP]/[AMP]$ ratio. Denton *et al.* (1966) also reported unusually low values for the $[ATP]/[AMP]$ ratio in incubated adipose tissues but this was compensated for by low values for the $[ATP]/[ADP]$ ratio. Halperin & Denton (1969) have since presented measurements indicating an $[ATP]/[AMP]$ ratio in aerobic adipose tissues incubated with insulin that is similar to that found in other tissues. At present there appears

to be no ready explanation for the discrepancy in adenine nucleotide measurements between various studies.

The value of K_a for the adenylate kinase reaction is dependent on the Mg^{2+} concentration (Bergmeyer, 1963). However, it is unlikely that this factor alone could account for the magnitude of the displacements from Mg^{2+} -free equilibrium found in this study. An apparent disequilibrium could be explained by a compartmentation of adenine nucleotides and adenylate kinase. There is evidence that in liver and muscle adenylate kinase is only found in the mitochondrial cristal spaces outside the 'attractylate-sensitive barrier' (Klingenberg & Pfaff, 1965; Heldt & Schwalbach, 1967). If, in adipose tissue, adenylate kinase maintained near equilibrium between ATP, ADP and AMP in the extramitochondrial spaces where the concentration of AMP may be higher and the $[ATP]/[AMP]$ ratio lower than in most other tissues because of the high rate of fatty acid activation and esterification, whole-tissue measurements of adenine nucleotides could possibly give rise to $[ATP][AMP]/[ADP]^2$ values higher than the K_a of adenylate kinase due to the compartmentation of this enzyme. In such a case whole-tissue $[ATP]/[AMP]$ and $[ATP]/[ADP]$ ratios could be higher than those found in the extramitochondrial spaces. These ratios have an important bearing on the control of phosphofructokinase since lower values for these ratios in the phosphofructokinase-containing spaces would suggest a fuller activation of the enzyme and a smaller displacement of its reactants from mass-action equilibrium, respectively.

Extramitochondrial free $[NAD^+]/[NADH]$ ratios. The values of this ratio in tissues incubated with and without insulin in the presence of glucose alone (Table 8) were essentially similar to those found in other tissues under normal conditions (Hohorst, Kreutz & Bücher, 1959; Williamson, 1965; Williamson, Lund & Krebs, 1967). In all cases where estimates of this ratio were made by using both the medium [lactate]/[pyruvate] ratio and the whole tissue [glycerol 1-phosphate]/[dihydroxyacetone phosphate] ratio, the results from the [glycerol 1-phosphate]/[dihydroxyacetone phosphate] ratio indicated a more negative redox state than that indicated by the [lactate]/[pyruvate] ratio. However, whereas the [glycerol 1-phosphate]/[dihydroxyacetone phosphate] ratio is representative of the redox state within the tissue at the time of sampling, the [lactate]/[pyruvate] ratio in the medium need not be strictly so. Denton *et al.* (1966) have shown that the medium [lactate]/[pyruvate] ratio increases during an incubation of fat pads in the presence of glucose + insulin. Presumably the interior of the fat-pad mass becomes progressively anaerobic during incubation thus causing the

medium [lactate]/[pyruvate] ratio to rise after a rise in the tissue [glycerol 1-phosphate]/[dihydroxyacetone phosphate] ratio. In the case of tissues taken directly from anaesthetized animals, the agreement between the two estimates of the extra-mitochondrial $[NAD^+]/[NADH]$ ratio was good. This finding, considered together with the fact that, in epididymal adipose tissues, the activities of both glycerol 1-phosphate dehydrogenase and lactate dehydrogenase are high (Shonk & Boxer, 1964; Saggerson & Greenbaum, 1969) would suggest that the concentrations of the reactants of both these enzymes in adipose tissue are maintained near to thermodynamic equilibrium.

The agreement between the estimates of the extramitochondrial $[NAD^+]/[NADH]$ ratios in this study calculated by using both the [lactate]/[pyruvate] and [glycerol 1-phosphate]/[dihydroxyacetone phosphate] ratios are as close as those found in other tissues. There are inconsistencies in the literature concerning the value of K_a for the lactate dehydrogenase reaction (Hakala, Glaid & Schwert, 1956; Hohorst, Kreutz & Reim, 1961; Williamson *et al.* 1967). The value found by the last-named workers was used in this study. By applying this value to the data of Hohorst *et al.* (1959), or to the data of Williamson (1965) values for the cytoplasmic free $[NAD^+]/[NADH]$ ratio estimated from the [lactate]/[pyruvate] ratio and from the [glycerol 1-phosphate]/[dihydroxyacetone phosphate] ratio were 818 and 1730 respectively for rat liver and 1695 and 2280 respectively for perfused rat hearts.

The results in Table 8 indicate that alterations in the [lactate]/[pyruvate] ratio produced by different conditions of incubation were followed by roughly parallel alterations in the [glycerol 1-phosphate]/[dihydroxyacetone phosphate] ratio except for tissues incubated with insulin + adrenaline, in which case there is a large unexplained discrepancy in the direction of change of the ratios. Halperin & Denton (1969) have also indicated that

changes in the [lactate]/[pyruvate] ratio should be paralleled by changes in the [glycerol 1-phosphate]/[dihydroxyacetone phosphate] ratio. It is interesting that certain incubation treatments in this study produced opposite changes in the cytoplasmic redox state to that encountered in other tissues. In the perfused rat heart, inclusion of acetate in the perfusate resulted in a more negative redox state (Williamson, 1965), whereas acetate had the opposite effect on adipose tissue in this study. Similarly, incubation with oleate in this study produced a slightly more positive redox state whereas infusion of oleate into perfused rat livers in the presence of various gluconeogenic precursors resulted in a more negative redox state (Williamson, Browning & Scholz, 1969).

It may be seen in Table 2 that the more oxidized cytoplasmic redox state found on incubation with pyruvate was accompanied by an increase in the tissue concentration of 3-phosphoglycerate whereas incubation with lactate lowered the concentration of this metabolite. These changes presumably reflect the effect of the altered free $[NAD^+]/[NADH]$ ratios on the concentrations of the reactants of glyceraldehyde 3-phosphate dehydrogenase and 3-phosphoglycerate kinase (Krebs & Veech, 1969). It is not known whether the reactants of these two enzymes are near to equilibrium in adipose tissue.

Phosphofructokinase and the regulation of glycolytic flux. In Table 12 attempts are made to relate alterations in glycolytic rate to alterations in the concentrations of the substrates and proposed allosteric effectors of adipose tissue phosphofructokinase. In the presence of insulin + oleate or adrenaline, increased rates of glycolytic flux were found while the concentration of fructose 6-phosphate fell. In the case of incubation with oleate this apparent stimulation of phosphofructokinase could be explained in terms of the increased concentration of AMP and the decreased concentration of ATP. The increase in the AMP concentration presumably arises from the increased rate of fatty acid activation

Table 12. *Correlation of changes in phosphofructokinase flux rates with changes in metabolite concentrations*

The results were taken from Tables 2, 3, 4 and 9 and are expressed as percentages of the measurements made in tissues incubated with insulin + glucose. The calculated flux from hexose phosphate to triose phosphate (Table 9) is taken as the phosphofructokinase flux rate.

Additions to incubation medium	Metabolite concentrations (% of controls)				Phosphofructokinase flux rate (% of control)
	F6P	AMP	ATP	Citrate	
Anti-insulin serum	51	58	71	77	11
Insulin + acetate	95	60	78	208	133
Insulin + pyruvate	120	—	—	727	35
Insulin + lactate	173	85	83	211	36
Insulin + oleate + albumin	67	196	72	125	119
Insulin + adrenaline + albumin	63	87	63	480	213

and esterification (Table 9). In the case of incubation with adrenaline, the apparent stimulation of phosphofructokinase could be explained in terms of a decrease in the concentration of ATP relative to that of AMP and of an increase in 3':5'-cyclic AMP concentration (Butcher, Ho, Meng & Sutherland, 1965). An increase in the concentration of 3':5'-cyclic AMP would be particularly effective in counteracting the increase in citrate concentrations observed on incubation with adrenaline (Denton & Randle, 1966). Halperin & Denton (1969) have also presented evidence that adipose tissue phosphofructokinase is stimulated in the presence of insulin+adrenaline and have demonstrated increased concentrations of AMP in these tissues. In the present study however, the increased rates of fatty acid activation and esterification found in the presence of adrenaline did not appear to be accompanied by increases in AMP concentration, a finding that is difficult to explain, although a similar observation was made by Denton *et al.* (1966).

In the presence of insulin+lactate or pyruvate glycolytic flux rates were equally decreased and were accompanied by a doubling of citrate concentrations and a significant increase in the concentration of fructose 6-phosphate in tissues incubated with lactate, but by sevenfold increases in citrate concentrations and only mild increases in those of fructose 6-phosphate in tissues incubated with pyruvate. These results would suggest that phosphofructokinase is inhibited by citrate under these conditions. However, there appears to be no meaningful correlation between the degree of fructose 6-phosphate accumulation, the whole-tissue concentration of citrate and the rate of glycolytic flux between the two incubation states. These represent two extremes of cytoplasmic redox state and the effect of this on factors such as citrate compartmentation and the [glucose 6-phosphate]/[6-phosphogluconate] ratio is not known.

In tissues incubated with insulin+acetate, increased rates of glycolytic flux were observed, as found by Flatt & Ball (1966). This change was not accompanied by meaningful changes in the concentrations of adenine nucleotides and the concentration of citrate was found to increase. These results are not consistent with the known regulatory properties of adipose tissue phosphofructokinase *in vitro* without a knowledge of the compartmentation of metabolites.

It would appear that a better understanding of the compartmentation of metabolites and of the kinetic properties of adipose tissue phosphofructokinase is required before the role of this enzyme in adipose tissue glycolytic regulation can be fully defined.

Correlations between the tissue concentrations of

long-chain fatty acyl-CoA, glycerol 1-phosphate and citrate and the rates of triglyceride and fatty acid synthesis. In a similar study, Denton & Halperin (1968) attempted to draw correlations between these parameters. They, however, concluded that neither the whole-tissue concentrations of glycerol 1-phosphate, long-chain fatty acyl-CoA nor of free fatty acids could have been the sole determinants of the rates of triglyceride synthesis under the conditions of their experiments. They also failed to find the expected relationships between the tissue contents of acetyl-CoA, citrate and long-chain fatty acyl-CoA and observed rates of fatty acid synthesis. The same conclusions must be drawn from the results of the present study.

Table 13 shows that inverse relationships between the whole-tissue concentrations of long-chain fatty acyl-CoA and the rate of fatty acid synthesis were only observed in tissues incubated with anti-insulin serum or with insulin+adrenaline. Similar findings were reported by Denton & Halperin (1968). On the other hand, long-chain fatty acyl-CoA concentrations were found to increase in tissues incubated with acetate, in which the rate of fatty acid synthesis was also increased. As found by Denton & Halperin (1968) and by Saggerson & Greenbaum (1970), alterations in whole-tissue concentrations of long-chain fatty acyl-CoA between various incubation conditions and physiological states are relatively slight, whereas alterations in fatty acid synthesis rates between these conditions are often large. Rates of triglyceride synthesis (as determined by ^{14}C -labelled substrate incorporation into glyceride glycerol) were also in no way related to the whole-tissue concentrations of long-chain fatty acyl-CoA.

In the present study wide alterations in the whole-tissue concentrations of citrate were produced by various treatments. Table 13 indicates that related alterations in the rate of fatty acid synthesis were not generally found. The alterations in the concentration of citrate did however appear to bear some relationship to the observed rates of citric acid cycle flux (the measurement of this is, however, inexact as mentioned above). The alterations in the concentration of citrate obviously need not refer to the cell compartment(s) containing acetyl-CoA carboxylase. Alternatively, it is conceivable that the adipose tissue acetyl-CoA carboxylase may be fully activated by citrate under the conditions observed and that fatty acid synthesis rates may be dependent on the supply of acetyl-CoA in the cytoplasm (Flatt & Ball, 1966; Saggerson & Greenbaum, 1970). In this respect it may be noted that Fang & Lowenstein (1967) have demonstrated that citrate activation of liver acetyl-CoA carboxylase is concentration- and time-dependent in an interdependent manner. Possibly extremely low

Table 13. Relationship between the rates of triglyceride synthesis and fatty acid synthesis and the concentrations of long-chain fatty acyl-CoA, citrate and glycerol phosphate

The results were taken from Tables 2, 4, 9 and 10. All values are expressed as percentages of the controls which were taken to be tissues incubated with insulin+glucose.

Additions to incubation medium	Metabolite concentrations (nmol/mg of tissue N) (% of controls)			Flux rates (% of controls)		
	Long-chain fatty acyl-CoA	Citrate	G1P	Triglyceride synthesis	Fatty acid synthesis	Total measured citric acid cycle flux
Anti-insulin serum	151	79	39	40	6	81
Insulin+acetate	124	211	68	120	167	490
Insulin+pyruvate	74	739	42	113	100	364
Insulin+lactate	98	215	176	115	125	162
Insulin+phenazine methosulphate	—	375	31	119	95	231
Insulin+oleate+albumin	106	127	61	250	103	342
Insulin+adrenaline+albumin	122	488	73	539	56	2300

steady-state concentrations of citrate in the cell could eventually lead to full activation of the enzyme. Also Gregolin *et al.* (1968) have demonstrated that binding of citrate by liver acetyl-CoA carboxylase is in fact surprisingly tight ($K_s = 2-3 \mu\text{M}$).

Alterations in the whole-tissue concentration of glycerol 1-phosphate in this study could be induced by incubation with substances likely to alter the cytoplasmic free $[\text{NAD}^+]/[\text{NADH}]$ ratio, such as acetate, pyruvate, lactate or phenazine methosulphate. These changes in glycerol 1-phosphate were not accompanied by changes in the rate of triglyceride synthesis. Conversely, alterations in the rate of triglyceride synthesis induced by supplying oleate to the tissues or by increasing the intracellular free fatty acid concentration with adrenaline were accompanied by only small alterations in the concentration of glycerol 1-phosphate. These findings, together with the fact that whole-tissue concentrations of glycerol 1-phosphate are high, as discussed above, would suggest that, under the conditions used, rates of triglyceride synthesis were not related to the concentrations of glycerol 1-phosphate and were solely dependent on the supply of free fatty acids or long-chain fatty acyl-CoA derivatives at the intracellular sites of esterification. *In vivo*, however, it is conceivable that the concentration of glycerol 1-phosphate might influence the rate of triglyceride synthesis since the concentration of this metabolite was far lower in tissues that were directly freeze-clamped (Table 6).

As suggested by Halperin & Denton (1969) it would appear that the concentration of glycerol 1-phosphate under the conditions employed is not

dependent on the rate of glycolysis in the tissue, but on the concentration of dihydroxyacetone phosphate and the free $[\text{NAD}^+]/[\text{NADH}]$ ratio in the cytoplasm. The latter factor would appear to be the more important in most cases studied, since a surprising constancy was observed in the concentration of dihydroxyacetone phosphate in all tissues incubated with insulin (Table 2). In the case of tissues incubated in the absence of insulin, however, the lower concentrations of glycerol 1-phosphate with respect to insulin-treated tissues would appear to result from the lower concentrations of dihydroxyacetone phosphate rather than from alteration of the redox state.

Generally, it would appear that the correlations attempted in the present study and in that of Denton & Halperin (1968) are unrewarding. It may be that effects of metabolites on enzymes in isolated systems may not occur within the intact tissue; or that, if such effects do occur, it may be impossible to make valid correlations because of unknown degrees of compartmentation. In certain cases it is also necessary to consider the possibility that intracellular metabolite concentrations are high enough to produce their effects to saturation.

Comparison of the effects of adrenaline and fatty acids on the metabolism of glucose. From the results of the present study and that of Denton & Halperin (1968), it is apparent that there are certain similarities between the effects of incubation with insulin+adrenaline and of incubation with insulin+oleate or palmitate. Under both conditions the following changes are produced with respect to control tissues incubated with insulin alone. Triglyceride synthesis, phosphofructokinase flux, production of

citric acid cycle CO_2 from glucose-derived carbon and tissue AMP concentrations with respect to those of ATP are all elevated, while [lactate]/[pyruvate] ratios in the incubation medium are lowered. Cahill, Le Boeuf & Flinn (1960) have also remarked on similarities in the metabolism of glucose in the two treatments. However, certain differences are also observed between the two treatments. Incubation with insulin+adrenaline results in elevated tissue concentrations of citrate and long-chain fatty acyl-CoA and in increased rates of lactate+pyruvate output while rates of fatty acid synthesis and CO_2 production by the hexose monophosphate pathway are decreased. These effects are not observed on incubation with insulin+oleate or palmitate. Cahill *et al.* (1960) suggested that the similarities between the effects of adrenaline and fatty acids on glucose metabolism might be accounted for solely by the lipolytic effects of adrenaline in producing free fatty acids. In this case the similarities between the two conditions could perhaps be explained as resulting from increased rates of fatty acid activation and esterification. The differences between the two treatments may result from the fact that in adrenaline-treated tissues free fatty acids are supplied directly to the tissues at an intracellular locus and may therefore produce other effects on glucose metabolism whereas externally supplied fatty acids in the presence of glucose and an active esterifying system may not be able to produce these effects. A fuller discussion of these considerations is presented elsewhere (Saggerson & Greenbaum, 1970).

Relationship between the rate of fatty acid synthesis and the production of cytoplasmic NADPH by the hexose monophosphate pathway and by the 'malic enzyme'. Table 14 shows results taken from this study. Results are also included from a further study (Saggerson & Greenbaum, 1970) since they are relevant to this discussion. The results suggest that there is a parallelism between observed rates of fatty acid synthesis and calculated rates of CO_2 (and therefore also NADPH) production by the hexose monophosphate pathway for a wide range of incubation conditions for normal tissues and also for tissues taken from animals of varied dietary status. A close linking between the hexose monophosphate pathway flux and the rate of fatty acid synthesis is even more evident in the results (Table V) of Katz *et al.* (1966) where a plot of their calculated rates of glucose 6-phosphate dehydrogenation versus observed rates of fatty acid synthesis for six experimental conditions gives an excellent linear relationship with a small intercept at a positive value of glucose 6-phosphate dehydrogenation for zero fatty acid synthesis. Further, the likelihood of such a relationship between hexose

monophosphate pathway NADPH production and fatty acid synthesis is supported by the conclusion of Katz & Rognstad (1966) that NADPH is used solely for reductive synthesis of fatty acids.

It is noteworthy that hexose monophosphate pathway rates were doubled in tissues incubated with phenazine methosulphate, a finding in agreement with that of McLean (1960). It is therefore unlikely that the hexose monophosphate pathway is operating near to maximum capacity, at least in normal tissues. This conclusion supports the suggestion that hexose monophosphate pathway operation passively follows NADPH utilization and is limited by the supply of NADP^+ (McLean, 1960).

The concentration of 6-phosphogluconate (per mg of tissue N) varied considerably, depending on the experimental conditions (Table 14). Alterations in the concentration of this metabolite did not, however, necessarily parallel alterations in fatty acid synthesis rate or in calculated rates of hexose monophosphate pathway flux. On the other hand, a more meaningful correlation can be shown between the calculated rates of hexose monophosphate pathway flux and the whole-tissue [6-phosphogluconate]/[glucose 6-phosphate] ratios. This ratio can, however only be correlated with changes in hexose monophosphate pathway flux for tissues of the same dietary status since it must depend to some extent on the absolute and the relative activities, and on the distribution of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, which may change with changes in dietary status. As discussed by Veech, Eggleston & Krebs (1969), it is not possible at present to establish whether the reactants of the reaction catalysed by glucose 6-phosphate dehydrogenase or of the hydrolysis of 6-phosphogluconolactone are near to mass-action equilibrium. However, if near-equilibrium existed within the adipose tissues used in this study, then:

$$\frac{[\text{NADP}^+]}{[\text{NADPH}]} = \frac{1}{K_1 K_2} \cdot \frac{1}{[\text{H}_2\text{O}]} \cdot \frac{[\text{6-phosphogluconate}][\text{H}^+]}{[\text{glucose 6-phosphate}]}$$

where $[\text{NADP}^+]$ and $[\text{NADPH}]$ represent the free concentrations of these metabolites in the cytoplasm and K_1 and K_2 are the equilibrium constants for the glucose 6-phosphate dehydrogenase and 'lactonase' reactions. At constant pH and in an aqueous environment it would then be reasonable to suggest that the whole-tissue [6-phosphogluconate]/[glucose 6-phosphate] ratio is proportional to the cytoplasmic free $[\text{NADP}^+]/[\text{NADPH}]$ ratio provided near-equilibrium is maintained. It is also

Table 14. *Relationships between rates of hexose monophosphate pathway flux, rates of fatty acid synthesis, whole-tissue concentrations of 6-phosphogluconate and whole-tissue [6-phosphogluconate]/[glucose 6-phosphate] ratios in incubated adipose tissues*

The results were taken from Tables 2, 9 and 10 of this study and from Tables 2, 9 and 10 of Saggerson & Greenbaum (1970). The values used were flux rates or metabolite concentrations expressed/mg of tissue N and are percentages of the values found in normal tissues incubated with glucose+insulin.

Dietary status of animals	Additions to incubation medium	Total fatty acid synthesis (% of control)	Hexose monophosphate CO ₂ production (% of control)	6-phosphogluconate concentration (% of control)	$\frac{[6PG]}{[G6P]}$ ratio
Normal	Anti-insulin serum	6	12	8	0.11
	Insulin + dibutyl 3':5'-cyclic AMP + albumin	36	54	—	—
	Insulin + adrenaline + albumin	56	41	54	0.44
	Insulin (control)	100	100	100	0.63
	Insulin + pyruvate	100	117	200	1.09
	Insulin + oleate + albumin	103	116	94	0.74
	Insulin + lactate	125	118	223	0.71
	Insulin + acetate	167	156	193	1.12
Starved for 72 h	Insulin	12	24	35	0.29
	Insulin + acetate	29	33	39	0.30
Starved for 72 h and refed high-fat diet for 72 h	Insulin	12	38	129	0.67
	Insulin + acetate	20	44	142	0.76
Starved for 72 h and refed bread for 144 h	Insulin	183	205	275	1.25
	Insulin + acetate	248	338	396	1.92
Normal	Insulin + phenazine methosulphate	95	259	347	3.13

Table 15. *¹⁴CO₂ Production from [U-¹⁴C]- and [1-¹⁴C]pyruvate in the presence of glucose + insulin*

Tissues were incubated with 20 mM-glucose + 20 mM-[1-¹⁴C]pyruvate in the presence of insulin. Tissues from the same animals were also incubated with 20 mM-glucose + 20 mM-[U-¹⁴C]pyruvate, 20 mM-[U-¹⁴C]glucose + 20 mM-[6-¹⁴C]glucose + 20 mM-pyruvate in the presence of insulin. Measurements of citric acid cycle CO₂ and pyruvate dehydrogenase CO₂ were made as described in the Materials and Methods section. The results are expressed as μ g-atoms of carbon/h per mg of tissue N and are mean values \pm S.E.M. of five determinations.

Citric acid cycle CO ₂ from [U- ¹⁴ C]pyruvate	CO ₂ from [U- ¹⁴ C]-pyruvate produced by pyruvate dehydrogenase	Total yield of ¹⁴ CO ₂ from [U- ¹⁴ C]pyruvate	Unexplained ¹⁴ CO ₂ production from [U- ¹⁴ C]pyruvate	Pyruvate dehydrogenase CO ₂ + unexplained CO ₂ (A)	Total yield of ¹⁴ CO ₂ from [1- ¹⁴ C]pyruvate (B)	ratio (A)/(B)
1.19 \pm 0.37	5.23 \pm 1.67	9.00 \pm 1.75	2.60 \pm 0.53	7.80 \pm 2.10	8.80 \pm 1.50	0.89

necessary to assume that, in the intact cell, the concentration of 6-phosphogluconate predominates over that of 6-phosphogluconolactone since, under the extraction conditions used in this study, the measurement of 6-phosphogluconate represents the sum of these two metabolites.

Table 14 shows that the [6-phosphogluconate]/[glucose 6-phosphate] ratio increases in tissues from normal, starved, starved and fat-refed and starved and bread-refed rats as the rate of fatty acid synthesis and, hence of hexose monophosphate pathway flux, is increased. Considering all of the results in Table 14, approx. 40-fold variations in fatty acid synthesis rates (per mg of tissue N) are reported and these are paralleled by 17-fold changes in the [6-phosphogluconate]/[glucose 6-phosphate] ratios which may reflect similar changes in the cytoplasmic NADP redox state. In the case of tissues incubated with insulin + pyruvate observed values for the [6-phosphogluconate]/[glucose 6-phosphate] ratio were unusually high. However, the unusually high concentrations of pyruvate may have affected the $[NADP^+]/[NADPH]$ ratio, and hence the [6-phosphogluconate]/[glucose 6-phosphate] ratio, through adjustment of the 'malic enzyme' equilibrium (Veech *et al.* 1969).

Table 14 also shows that percentage changes with respect to the control of calculated hexose monophosphate pathway rates did not always correlate well with observed alterations in the rate of fatty acid synthesis, particularly at low rates of flux. This suggests that hexose monophosphate pathway NADPH production need not always parallel fatty acid synthesis, or that the calculations of hexose monophosphate pathway CO_2 production may be inexact, or that estimates of fatty acid synthesis may be underestimated. Under conditions of starvation, starvation and fat refeeding or of treatment with lipolytic agents or in the absence of insulin, appreciable dilution of the fatty acid precursor pool of acetyl-CoA may arise from endogenous sources, thus leading to underestimation of the rate of fatty acid synthesis. In this study hexose monophosphate pathway CO_2 production was determined by the method of Flatt & Ball (1964), i.e. by subtracting the estimated rates of production of glucose-derived CO_2 by the citric acid cycle and pyruvate dehydrogenase from the total production of $^{14}CO_2$ from $[U-^{14}C]$ glucose. However, it is likely that $^{14}CO_2$ is produced from $[U-^{14}C]$ glucose by reactions other than the three routes considered. Kneer & Ball (1968) have demonstrated, in the presence of adipose tissues, an exchange reaction between C-1 of pyruvate and $NaH^{14}CO_3$ which is most probably dependent on the operation of the dicarboxylic acid shuttle. The results of Leveille (1967), Ballard, Hanson & Leveille (1967) and Chakrabarty & Leveille (1968)

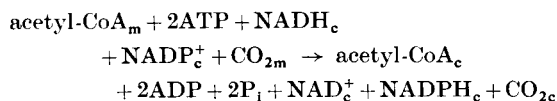
also suggest that $^{14}CO_2$ could be produced in this way. It is also known that phosphoenolpyruvate carboxykinase alone catalyses an exchange between $H^{14}CO_3^-$ and oxaloacetate (Utter & Kurahashi, 1954; Chang & Lane, 1966). In the present study, 41% of the $^{14}CO_2$ derived from $[U-^{14}C]$ pyruvate and 18% of that derived from $[U-^{14}C]$ lactate could not be accounted for as pyruvate dehydrogenase or citric acid cycle CO_2 . In a supplementary experiment to ascertain the source of this extra CO_2 there was found to be quite good agreement between the amount of this unexplained $^{14}CO_2$ produced from $[U-^{14}C]$ pyruvate and that produced from $[1-^{14}C]$ pyruvate that cannot be accounted for as arising from pyruvate dehydrogenation. The results of this experiment are summarized in Table 15.

From these considerations it is evident that the method of calculation of hexose monophosphate pathway CO_2 production used in this study (Flatt & Ball, 1964) is likely to lead to overestimations under certain conditions. At least three reaction sequences could lead to 'extra' $^{14}CO_2$ production from $[U-^{14}C]$ glucose, namely glyceride glycerol production from glucose-derived $[U-^{14}C]$ pyruvate, the operation of the 'pyruvate cycle' (Rognstad & Katz, 1966) for fatty acid synthesis or possibly, the operation of an energy consuming 'futile' cycle of the type pyruvate \rightarrow oxaloacetate \rightarrow phosphoenolpyruvate \rightarrow pyruvate (Gevers, 1967).

The extent of pyruvate carboxylation and the degree of involvement of oxaloacetate in these pathways under the various incubation conditions presented in Table 14 is unknown and therefore the degree of overestimation of the hexose monophosphate pathway flux must remain unknown. Errors of the type considered above are unlikely to affect the determination of hexose monophosphate pathway contribution as described by Katz *et al.* (1966) and it may be that the apparent increasing contribution of the hexose monophosphate pathway to the supply of NADPH for lipogenesis at lower rates of flux in their study may be solely due to dilution of the fatty acid precursor acetyl-CoA pool.

It remains to consider the sources of NADPH used for fatty acid synthesis. Flatt & Ball (1964) and Katz *et al.* (1966), on the basis of ^{14}C balances concluded that only 50–60% of the NADPH required to maintain high rates of fatty acid synthesis from glucose as sole substrate is derived from the hexose monophosphate pathway. It is presumed that the rest of the required NADPH is obtained from cytoplasmic NADH through the action of the 'pyruvate cycle' (Rognstad & Katz, 1966). This cyclic pathway serves not only to carry out a transhydrogenation from cytoplasmic NADH to NADPH, but also to transfer acetyl-CoA from

the mitochondria to the cytoplasm with an overall stoichiometry:



where subscripts m and c denote mitochondrial and cytoplasmic respectively.

At low rates of lipogenesis, on the other hand, the results of Flatt & Ball (1964), Katz *et al.* (1966) and of Table 14 of this study, indicate that calculated hexose monophosphate pathway rates are sufficient to supply all, or even more than, the NADPH apparently required for fatty acid synthesis. Under these conditions therefore, there is no need to invoke the transhydrogenation functions of the pyruvate cycle, unless fatty acid synthesis rates are underestimated and calculated hexose monophosphate pathway flux rates are overestimated for reasons discussed above. In fact the transhydrogenation role of the pyruvate cycle cannot be easily dissociated from its role in the transport of acetyl units since the production of acetyl-CoA in the cytoplasm by cleavage of citrate yields corresponding equimolar quantities of oxaloacetate which must be disposed of. Normally it is envisaged that oxaloacetate is reduced to malate and then oxidatively decarboxylated to pyruvate, yielding NADPH. Flatt & Ball (1964), on the basis of their nicotinamide nucleotide balances, suggested that some cytoplasmically generated H-equivalents are reoxidized by oxygen under the agency of the respiratory chain. Transport of malate into the mitochondria from the cytoplasm would facilitate this reoxidation of cytoplasmic NADH and also remove cytoplasmic oxaloacetate, thus short-circuiting the transhydrogenation function of the pyruvate cycle. On the other hand, Katz *et al.* (1966), and Rognstad & Katz (1966, 1969) have shown that there is a near balance between the production of cytoplasmic reducing equivalents and their utilization in the cytoplasm for reductive syntheses, suggesting that there should be little transport of reducing equivalents into the mitochondria. Rognstad & Katz (1969) and Schmidt & Katz (1969) have in fact proposed that transport of reducing equivalents from cytoplasm to mitochondria via malate transport or via a glycerol phosphate shuttle does not occur under normal conditions. This is perhaps not surprising if the mitochondrial NAD^+/NADH couple of adipose tissue is far more negative than that of the cytoplasm as found in liver (Williamson *et al.* 1967). Also, Halperin & Robinson (1970) have demonstrated that lactate metabolism by adipose tissues may be limited by the rate at which NADH is removed in the cytoplasm. It is only in the special

case of tissues incubated with dinitrophenol that Rognstad & Katz (1969) have suggested the possibility of malate transport into the mitochondria. Schmidt & Katz (1969) have also demonstrated that, for tissues from starved rats at least, it is unlikely that cytoplasmic oxaloacetate could be removed into the mitochondria as aspartate. By using mitochondria obtained from adipocytes, Robinson & Halperin (1970) have shown that neither a glycerol phosphate shuttle nor a Borst (1962) cycle appears to operate to any significant degree in white adipose tissue. Cytoplasmic oxaloacetate could presumably be disposed of by conversion into phosphoenolpyruvate however (Kneer & Ball, 1968), although this is energetically expensive.

The considerations outlined above would suggest that, under normal conditions, the transhydrogenation of a molecule of cytoplasmic NADH to NADPH may be an obligatory accompaniment to the cytoplasmic cleavage of a molecule of citrate. However, this would mean that exactly 50% of the NADPH for fatty acid synthesis should be produced by the hexose monophosphate pathway and 50% by the action of the 'malic enzyme' under all conditions. This is not observed although, allowing for endogenous dilution of acetyl-CoA pools at low rates of lipogenesis, as discussed above, the results of Katz *et al.* (1966) might suggest an approximately constant proportion between the hexose monophosphate pathway rate and the rate of fatty acid synthesis. However, in this case the hexose monophosphate pathway appeared to supply on average 65% of the required NADPH.

Srere (1965) has discussed the possibility of transfer of acetyl units across the mitochondrial membrane by three methods that do not involve the formation of citrate. The NADPH required for fatty acid synthesis from cytoplasmic acetate units supplied by these methods could all be obtained from the hexose monophosphate pathway thus leading to a hexose monophosphate pathway NADPH contribution of greater than 50%. Alternatively, Madsen, Abraham & Chaikoff (1964) and D'Adamo & Haft (1965) have proposed that citrate may be generated in the cytoplasm by a 'reverse citric acid cycle'. The operation of this pathway would require the transhydrogenation of a molecule of cytoplasmic NADH to remove oxaloacetate but would also require the expenditure of an extra molecule of cytoplasmic NADPH per acetyl unit to reduce 2-oxoglutarate to isocitrate. In this case also the hexose monophosphate pathway NADPH contribution to fatty acid synthesis from glucose would appear to be greater than 50%. Leveille & Hanson (1966) have shown that the activity of this pathway increases on carbohydrate feeding. In this respect it is noteworthy that calculated rates

of hexose monophosphate pathway flux supplied 72% of the NADPH apparently required for fatty acid synthesis from glucose by tissues from starved, bread-refed rats (Saggerson & Greenbaum, 1970) whereas the hexose monophosphate pathway NADPH-contribution to fatty acid synthesis for normal tissues in the presence of insulin in this study was 59% (assuming an average fatty acid chain-length of 17 carbon atoms). Utilization of cytoplasmic NADPH for fatty acid desaturation would also increase the contribution of hexose monophosphate pathway NADPH to above 50%.

Assuming the absence of mitochondrial oxidation of reducing equivalents derived from oxidation of triose phosphates, and assuming that adipose tissue fatty acid synthesis from glucose (but not necessarily from acetate) always involves the derivation of cytoplasmic acetate units from citrate (which may be directly transported to the cytoplasm or formed from glutamate), an obligatory association of NADH transhydrogenation with oxaloacetate disposal would mean that the production of reducing equivalents by oxidation of triose phosphates should correspond closely to the utilization of cytoplasmic reducing equivalents for reductive synthesis of glycerol phosphate, reductive

synthesis of lactate and in supplying one half of the reducing equivalents needed for fatty acid synthesis from glucose. A very close correspondence of this kind is in fact found for the experimental groups examined in this study and elsewhere (Saggerson & Greenbaum, 1970). These results are summarized in Table 16. A similar close correspondence between these parameters can also be discerned in the data of Flatt & Ball (1964, 1966).

As might be expected, the agreement between cytoplasmic NADH production and proposed utilization is not so exact in tissues incubated with phenazine methosulphate. Table 16 shows, however, that only 19% of the cytoplasmic NADH production in the presence of this artificial acceptor is unaccounted for. This may be compared with the more considerable increase in hexose monophosphate pathway NADP turnover in the presence of phenazine methosulphate. Presumably this either reflects a greater efficiency of the NADH-utilizing processes to compete with phenazine methosulphate for available hydrogen or reflects a more negative redox state of the cytoplasmic NADP⁺/NADPH couple.

A complete tabulation of NADH utilization and production was not possible for tissues incubated

Table 16. *Comparison between observed production and proposed utilization of cytoplasmic NADH*

The results were obtained from Tables 9 and 10 of this study and from Tables 9 and 10 of Saggerson & Greenbaum (1970). The rates of production/utilization are μmol of NADH/h per mg of tissue N. For calculation of H-equivalents needed for fatty acid synthesis it was assumed that 0.837 μmol of NADH are required for the incorporation of 1 μg -atom of carbon into fatty acids (Flatt & Ball, 1964). For tissues incubated in the presence of acetate, alternative values (in parentheses) are presented for NADH utilization rates expected if acetyl-CoA is generated directly from acetate in the cytoplasm.

Dietary status of animals	Additions to incubation medium	NADH production by triose phosphate oxidation	Cytoplasmic NADH utilization (lactate synthesis + glycerol phosphate synthesis + $\frac{1}{2}$ fatty acid synthesis)	$\frac{(A)}{(B)}$ ratio
		(A)	(B)	
Normal	Insulin	4.80	5.11	0.96
	Anti-insulin serum	0.94	0.94	1.00
	Insulin + acetate	9.85	9.94 (8.56)	0.99 (1.15)
	Insulin + phenazine methosulphate	5.03	4.08	1.23
	Insulin + oleate + albumin	7.85	7.42	1.06
	Insulin + adrenaline + albumin	7.71	7.19	1.07
	Insulin + dibutyryl 3':5'- cyclic AMP + albumin	6.13	5.38	1.14
	Insulin	3.68	3.65	1.01
Starved for 72 h	Insulin + acetate	4.03	4.05 (3.79)	1.00 (1.06)
Starved for 72 h and refed high- fat diet for 72 h	Insulin	3.42	3.36	1.02
	Insulin + acetate	2.48	2.69 (2.39)	0.92 (1.04)
Starved for 72 h and refed bread for 144 h	Insulin	13.70	12.00	1.14
	Insulin + acetate	16.80	16.08 (14.47)	1.04 (1.16)

in the presence of insulin + pyruvate or lactate since the formation of lactate from glucose-derived pyruvate was not monitored in these experiments, nor were total utilizations of lactate and pyruvate determined. The fact that measured citric acid cycle flux rates and pyruvate- CO_2 exchange rates were greater in the presence of pyruvate than in the presence of lactate suggests that ATP utilization for pyruvate carboxylation or perhaps also for phosphoenolpyruvate formation from oxaloacetate may be greater in the presence of pyruvate than in the presence of lactate. There is a near proportionality between the production of 'unexplained $^{14}\text{CO}_2$ ' from $[\text{U-}^{14}\text{C}]$ pyruvate or $[\text{U-}^{14}\text{C}]$ lactate, the total measured rates of citric acid cycle flux, and the whole-tissue contents of malate (which are probably proportional to the concentration of oxaloacetate).

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