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Measurement of Flow of Carbon Atoms from Glucose and Glycogen Glucose to Glyceride Glycerol and Glycerol in Rat Heart and Epididymal Adipose Tissue

EFFECTS OF INSULIN, ADRENALINE AND ALLOXAN-DIABETES

BY R. M. DENTON AND P. J. RANDLE
Department of Biochemistry, University of Bristol

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1. Flow of carbon atoms from glucose and glycogen glucose to glyceride glycerol, glyceride fatty acids and glycerol was calculated in the perfused rat heart and incubated epididymal adipose tissue from the incorporation of ^{14}C from [U-^{14}C]-glucose (into glyceride glycerol, glyceride fatty acids and glycerol in the medium), and from measurements of the specific activity of L-glycerol 3-phosphate, and the effects of insulin, adrenaline and alloxan-diabetes were studied. Measurements were also made of the uptake of glucose and the outputs of lactate, pyruvate and glycerol. 2. New methods are described for the measurement of radioactivity in small amounts of metabolites (glycerol, glucose 6-phosphate and fructose 6-phosphate and L-glycerol 3-phosphate) in which use has been made of alterations in charge induced by enzymic conversions to effect resolution by ion-exchange chromatography. 3. In hearts the specific activity of L-glycerol 3-phosphate was less than that of glucose in the medium but similar to that of lactate released during perfusion. Because repeated measurements of the specific activity of L-glycerol 3-phosphate was impracticable, the specific activity of lactate has been used as an indirect measurement of glycerol phosphate specific activity. 4. In fat pads, specific activity of lactate was the same as that of glucose in the medium and thus the specific activity of L-glycerol 3-phosphate was taken to be the same as that of medium glucose. 5. In hearts from alloxan-diabetic rats, despite decreased glucose uptake and L-glycerol 3-phosphate concentration, flow of carbon atoms through L-glycerol 3-phosphate to glyceride glycerol was increased about threefold. 6. In fat pads, flow of carbon atoms through L-glycerol 3-phosphate to glyceride glycerol was increased by insulin (twofold), by adrenaline in the presence of insulin (fivefold) and by diabetes in pads incubated with insulin (1·5-fold). These increases could not be correlated either with increases in glucose uptake, which was unchanged by adrenaline and decreased in diabetes, or with the concentration of L-glycerol 3-phosphate, which was decreased by adrenaline and unchanged in diabetes. 7. These results are discussed in relation to the control of glyceride synthesis in heart and adipose tissue and to the regulation of glyceride fatty acid oxidation in the perfused rat heart.

The term glycerides is used in this paper to refer collectively to mono-, di- and tri-glycerides and the term glyceride synthesis to refer to the synthesis of glycerides from L-glycerol 3-phosphate (glycerol phosphate).

Little is known of the factors which may control glyceride synthesis in muscle and adipose tissue. In liver and adipose tissue glycerol phosphate and acyl-CoA have been identified as precursors of glyceride glycerol and glyceride fatty acids (Weiss & Kennedy, 1956; Smith, Weiss & Kennedy, 1957; Steinberg, Vaughan & Margolis, 1961) and a similar pathway is presumed to exist in muscle. In rat heart and epididymal adipose tissue glycerol phosphate appears to be formed mainly by glycolysis since glycerol kinase activity is low and glycerol is poorly utilized (Wieland & Suyter, 1957; Shapiro, Chowers & Rose, 1957; Kreisberg, 1966). The suggestion has been made by a number of workers that the supply of glycerol phosphate by glycolysis may control glyceride synthesis in muscle and adipose tissue (for reviews see Fritz, 1961; Vaughan, 1961). Two recent observations have suggested that factors other than the concentration of glycerol

phosphate may be important. In rat epididymal adipose tissue incubated *in vitro* with glucose and insulin the concentration of glycerol phosphate is markedly reduced by adrenaline (Denton, Yorke & Randle, 1966), whereas radioisotope data have shown an increased flow of glucose carbon atoms to glyceride glycerol (Cahill, Leboeuf & Flinn, 1960; Flatt & Ball, 1964). In alloxan-diabetes in the rat the concentration of glycerol phosphate in the heart, and the rate of glycolysis, are reduced *in vivo* and after perfusion *in vitro* with glucose and insulin (Garland & Randle, 1964; Newsholme & Randle, 1964; P. J. Randle, unpublished work). Nevertheless the concentration of glycerides is increased in the heart in diabetes, suggesting that their synthesis may be enhanced (Denton & Randle, 1967). In the present investigation rates of glyceride synthesis calculated from radioactive data have been correlated with tissue concentrations of glycerol phosphate in experiments with rat heart and rat fat pad *in vitro*.

The rate of glyceride synthesis may be calculated from the incorporation of radioactivity from [^{14}C]-glucose into glyceride glycerol if the specific activity of glycerol phosphate and the radioactivity in glycerol formed by lipolysis are known. The incorporation of radioactivity from [^{14}C]glucose into glyceride glycerol has been measured under many conditions in epididymal adipose tissue (for review see Vaughan, 1961) and shown to occur in heart muscle (Opie, Evans & Shipp, 1963); but measurements of glycerol radioactivity appear to be lacking, except for the data given by Katz, Landau & Bartsch (1966). Measurements of glycerol phosphate specific activity do not appear to have been made and the use of glucose specific activity for the calculation of rates of glyceride synthesis has been criticized because of the possibility of glycerol phosphate formation from unlabelled glycogen (Vaughan, 1961). In the present investigation glycerol radioactivity has been measured but it was impracticable to make repeated measurements of glycerol phosphate specific activity because of the low concentration and the difficulty of separation from other radioactive metabolites. Since glycerol phosphate and lactate are both formed by glycolysis the possibility of using lactate specific activity as an indirect measurement of glycerol phosphate specific activity has been explored. Because the triose phosphates which give rise to lactate and glycerol phosphate may be incompletely equilibrated (Cahill, Leboeuf & Renold, 1959; Katz *et al.* 1966) we have used [U-^{14}C]glucose. The present studies have shown that in adipose tissue the specific activities of lactate and glucose are the same and that the loss of radioactivity in glycerol is small. Earlier measurements of rates of glyceride synthesis based on glucose specific activity and incorporation of radioactivity into glyceride glycerol may thus be substantially correct. In muscle the specific activity of lactate was found to be lower than that of glucose. Methods have therefore been developed for the measurement in muscle of the specific activities of glucose 6-phosphate and glycerol phosphate, to define the relationship between lactate, glucose and glycerol phosphate specific activities.

MATERIALS

Rats. Epididymal fat pads were obtained from male albino Wistar rats of 200–250g. and hearts from rats of 250–350g. fed on diet 41B (Short & Parkes, 1949) and not deprived of food beforehand. Alloxan-diabetes was induced 48hr. before use by intravenous administration (60mg./kg.) under ether anaesthesia. The animals had blood glucose concentrations in excess of 3mg./ml.; this was ascertained with Dextrostix (Ames Co. Ltd., Stoke Poges, Slough, Bucks.) before incubation of pads or perfusion of hearts.

Chemicals. Glycolytic intermediates, adenine nucleotides, coenzymes, triethanolamine hydrochloride and enzymes were from Boehringer Corporation (London) Ltd., London, W.5; alloxan was from Eastman Kodak Ltd., Rochester, N.Y., U.S.A. Adrenaline was obtained from British Drug Houses Ltd., Poole, Dorset, and a freshly prepared stock solution (6mg./ml.) made in 0·1N-HCl; crystalline insulin was given by Boots Pure Drug Co. Ltd., Nottingham, or Burroughs Wellcome, Beckenham, Kent, and a stock solution (20units/ml.) made in 3mN-HCl; heparin was obtained from Evans Medical Ltd., Liverpool, and veterinary Nembutal from Abbott Laboratories Ltd., Queenborough, Kent. Bovina plasma albumin (fraction V) from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, was purified as described by Denton *et al.* (1966). Silicic acid (100-mesh powder), analytical reagent from Mallinckrodt Chemical Works, New York, N.Y., U.S.A., was activated by heating at 105° for 24hr. Dowex 1 (AG1-X4; Cl^- form; 200–400 mesh) obtained from BioRad Laboratories, Richmond, Calif., U.S.A., was converted into the formate form by washing with 3M-sodium formate until the washings were chloride-free and with water until no longer alkaline. Glycerol phosphate was prepared from glycerol, ATP and glycerokinase and purified by chromatography on Dowex 1 (Cl^- form) followed by freeze-drying.

[U-^{14}C]Glucose (approx. 4mc/m-mole) was from The Radiochemical Centre, Amersham, Bucks. For the purposes of the present experiments the radiochemical purity (99%) was not sufficiently high and it was further purified as described in the Procedure section. On the basis of experiments conducted by The Radiochemical Centre labelling was uniform to within 3–4%.

Media. Fat pads were incubated, and hearts perfused, with a bicarbonate-buffered medium (Krebs & Henseleit, 1932) gassed with O_2+CO_2 (95:5). The concentrations of the various additions are given in the text or Tables. Media containing albumin were dialysed as described by Denton *et al.* (1966).

METHODS

Glucose. Glucose was assayed spectrophotometrically in incubation or perfusion media with glucose oxidase (Huggett & Nixon, 1957) or with glucose 6-phosphate dehydrogenase

and NADP after reaction with ATP and yeast hexokinase.

Glycerol. Glycerol was assayed spectrophotometrically by the method of Garland & Randle (1962).

Lactate, pyruvate, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate and L-glycerol 3-phosphate. These were assayed spectrophotometrically by methods given in Bergmeyer (1963), with hydrazine as a trapping agent for lactate and glycerol phosphate assays. Fructose 1,6-diphosphate was assayed with glycerol phosphate dehydrogenase after conversion into dihydroxyacetone phosphate with aldolase and triose phosphate isomerase.

Radioactivity assay. ^{14}C-labelled compounds were assayed in a Nuclear–Chicago Liquid Scintillation System (model 725). For aqueous solutions the scintillator was 1,4-dioxan containing naphthalene, 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene (see Butler, 1961). For solutions in organic solvents (*n*-heptane) the scintillator was toluene containing 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene (Synder, 1961). At very low count rates (<50% above background) the background count rate was ascertained for each container with scintillator before addition of the sample. Quenching corrections were calculated by the channel ratio method or, where the count rate was less than 500 counts/min., by measurement of the recovery of radioactivity after addition of a known quantity of [U-^{14}C]glucose. The largest correction was 40%. All samples were counted for 40 min. or until 4000 counts were recorded.

Ion-exchange chromatography. For purification of radioactive glucose and separation of glycerol from glucose 6-phosphate (see the Procedure section) 10 cm. × 1 cm. columns of Dowex 1 (Cl^- form) were used. Glucose and glycerol were washed through the column with water under pressure of filtered compressed air. For separations of lactate, glycerol phosphate, (glucose + fructose) 6-phosphates and fructose 1,6-diphosphate by gradient elution, 20 cm. × 1 cm. columns of Dowex 1 (formate form) were used. Increasing exponential gradients of formic acid or of ammonium formate–formic acid were applied under gravity (200 cm. water head) by addition of M-formic acid to a closed reservoir containing 250 ml. of water or by addition of M-ammonium formate to 250 ml. of 0·63 M-formic acid. Fractions of approx. 10 ml. were collected with a drop-counting fraction collector.

For assays of lactate, glucose 6-phosphate and glycerol phosphate neutralization of fractions was unnecessary. For assay of fructose 1,6-diphosphate fractions were brought to pH 7·0–8·0 (glass electrode) with 50% (w/v) KOH. For assays of radioactivity, fractions containing formic acid mixed satisfactorily with scintillator based on 1,4-dioxan. Turbidity was avoided with fractions containing ammonium formate by mixing fractions, 6 M-formic acid and 1,4-dioxan-based scintillator in the proportions (<1):2:15 (by vol.).

PROCEDURE

Purification of [U-^{14}C]*glucose and preparation of incubation and perfusion media.* Experiments in which [U-^{14}C]-glucose was put through the procedure for separation of radioactive glycerol showed that [U-^{14}C]glucose may contain up to 0·1% of [^{14}C]glycerol. This degree of contamination can seriously interfere with the measurement of [^{14}C]glycerol production from [U-^{14}C]glucose by rat heart or epididymal adipose tissue. The following procedure was used to remove radioactive glycerol from radioactive glucose immediately before each experiment (within 1 hr. of each incubation or perfusion).

The required amount of [U-^{14}C]glucose (20–50 μC, depending on the experiment) was dissolved in 25 ml. of 10 mM-sodium phosphate, pH 7·5, containing 25 μmoles of $MgCl_2$, 25 μmoles of ATP and 10 μmoles of glycerol. The glycerol was then converted into glycerol phosphate by addition of glycerokinase (50 μg.) and incubation at room temperature for 15 min. (shown by assay of glycerol phosphate formed). The reaction mixture was then poured on to a column of Dowex 1 (Cl^- form), the glucose washed through with water and NaCl added to the glucose solution to 0·154 M. The NaCl solution containing glucose was then used in place of 0·154 M-NaCl in the preparation of the bicarbonate medium (Krebs & Henseleit, 1932). When the medium was to contain bovine plasma albumin a solution of 4% albumin was prepared and dialysed and mixed in equal proportion with medium containing radioactive glucose. Other additions were then made as given in the text or Tables.

Incubation of epididymal fat pads. Rats were killed by decapitation and the pads removed as described by Winegrad & Renold (1958). The pads were then rinsed and preincubated at 37° for 30 min. in medium containing glucose (3 mg./ml.) in groups of eight pads (from four rats) in 25 ml. After preincubation each group of eight pads was lightly blotted and transferred to flasks containing 25 ml. of medium for incubation with radioactive glucose. Further details are given in the text or Tables.

Heart perfusion. Rats were killed and hearts removed as described by Newsholme & Randle (1964). They were then perfused by drip through for 5 min. with media containing glucose (1 mg./ml.) and insulin (0·1 unit/ml.) through a cannula fitted with a two-way tap. They were then switched to perfusion by recirculation with 15 ml. of medium containing radioactive glucose in the apparatus of Morgan, Henderson, Regen & Park (1961). Further details are given in the text or Tables.

Extraction of fat pads. At the end of incubation the pads were rapidly and lightly blotted and immediately frozen at the temperature of acetone–solid CO_2 with the tissue clamp of Wollenberger, Ristau & Schoffa (1960). The frozen pads in each group were weighed and reduced to a fine powder with a pestle (in a plastic beaker) under liquid N_2. Samples of powder (0·5 g.) were then extracted with 25 ml. of chloroform–methanol (2:1, v/v) in a manually operated Potter–Elvehjem homogenizer and allowed to stand overnight at 0°. The extract containing lipid was then analysed as described in a later section.

Extraction of rat heart. In experiments in which measurements were confined to cardiac lipids the procedure was as follows. At the end of perfusion the heart was transected at the auriculo-ventricular junction and the ventricular muscle (free of obvious adipose tissue) rapidly and lightly blotted and frozen with the tissue clamp of Wollenberger *et al.* (1960). The frozen muscle was weighed and then powdered in a percussion mortar at the temperature of solid CO_2. The powders from four hearts in each group were pooled and mixed thoroughly. Samples (1 g.) of frozen heart powder were extracted with 20 ml. of chloroform–methanol (2:1, v/v) in a hand-operated Potter–Elvehjem homogenizer, and allowed to stand overnight at 0°. This extract containing lipid was then analysed as described below.

For measurement of the specific activities of muscle glucose 6-phosphate and fructose 6-phosphate, glycerol phosphate, lactate and pyruvate, the hearts were frozen on the cannula with the tissue clamp. They were then powdered and extracted with 5% perchloric acid (2ml./g.) and the supernatant was separated by centrifugation, neutralized with, and perchlorate ion removed by, addition of saturated $KHCO_3$ at 0°. The extract was then analysed as described in a later section.

Analysis of tissue extracts

Assay of ^{14}C in glyceride glycerol and fatty acids in epididymal fat pads and hearts. The chloroform–methanol extract of fat pads was separated by centrifugation and shaken with 5ml. of 4mM-$MgCl_2$. After standing for 10–15min. phases were separated by centrifugation and the upper phase was aspirated. The interphase was carefully washed (three times) with 5ml. of the aqueous layer separated after shaking 4 parts of water and 20 parts of 4mM-$MgCl_2$ with 100 parts of chloroform–methanol (2:1, v/v). No radioactivity was detected in the final wash. A sample (10ml.) of the organic layer was then evaporated to dryness, redissolved in 8ml. of chloroform and phospholipid removed by shaking with 0·5g. of silicic acid (Carlson, 1963). A sample (6ml.) was then evaporated to dryness and the residue saponified with 4ml. of 4% KOH in 95% ethanol (prepared by adding 5 parts of aq. 80%, w/v, KOH to 95 parts of ethanol). After acidification with 4ml. of 10% perchloric acid the fatty acids released by saponification were extracted with four washes of 5ml. of light petroleum (b.p. 40–60°). The last wash was free of radioactivity. The combined light-petroleum fraction was evaporated to dryness, the fatty acids were redissolved in 5ml. of heptane, washed once with 5ml. of aq. 20mN-HCl and radioactivity was assayed with 0·5ml. added to 3ml. of toluene-based scintillator. The aqueous layer, after extraction of fatty acids with light petroleum, was centrifuged at 0° to remove potassium perchlorate, and [^{14}C]glycerol was assayed with 1ml. added to 15ml. of dioxan-based scintillator.

The same procedure was applied to chloroform–methanol extracts of rat heart with the following modifications. The volume of 4mM-$MgCl_2$ was 3·6ml.; the volume of ethanolic KOH for saponification was 1ml. Since no radioactivity was found in fatty acids released by saponification the perchloric acid extract was only extracted twice with 3ml. of light petroleum. [^{14}C]Glycerol was assayed in 0·2–1·0ml. samples in 15ml. of dioxan-based scintillator.

To exclude the possibility of contamination of glyceride glycerol and fatty acid fractions with radioactivity from glucose, control experiments were performed as follows. Hearts were perfused with non-radioactive glucose, frozen and powdered and radioactive glucose was added (0·4μc/g. of powder). The glyceride glycerol and fatty acid fractions were then separated and found to be free of detectable radioactivity.

Measurement of specific activities of glucose 6-phosphate and fructose 6-phosphate and of lactate and pyruvate in extracts of rat heart. The amounts of lactate, pyruvate, glucose 6-phosphate and fructose 6-phosphate in the perchloric acid extracts were assayed spectrophotometrically. Assay of radioactivity in lactate and pyruvate and in glucose and fructose 6-phosphates was effected by their isolation on Dowex 1 (formate form). To identify the peaks, non-radioactive pyruvate and glucose 6-phosphate were added to the perchloric acid extract and pyruvate was then converted into lactate by treatment with lactate dehydrogenase and NADH. Lactate is apparently well separated from other intermediates in tissue extracts on columns of Dowex 1 (formate form) but glucose 6-phosphate and fructose 6-phosphate are poorly separated from citrate and glycerol phosphate (Hoberman, 1965). Glucose 6-phosphate and fructose 6-phosphate were purified together in fractions containing them by conversion into fructose 1,6-diphosphate under the influence of phosphofructokinase and separation of the latter by a further column of Dowex 1 (formate form). The procedure was as follows.

Perchloric acid extract equivalent to approx. 2–3g. wet wt. of heart was diluted with 10mM-triethanolamine buffer, pH7·2, to 20ml. and 5μmoles each of sodium pyruvate and glucose 6-phosphate were added. Lactate dehydrogenase (100μg.) and excess of NADH were then added and the conversion of pyruvate into lactate was confirmed by spectrophotometric assay. The extract was then applied to a column of Dowex 1 (formate form), the lactate eluted with a gradient of formic acid (elution volume 100–140ml.) and the glucose 6-phosphate and fructose 6-phosphate eluted with a second gradient of ammonium formate (elution volume 70–120ml.) (see the Methods section). The specific activity of lactate in the peak tube was assayed and the specific activity of lactate and pyruvate in the original heart extract determined from the known dilution.

The fractions containing glucose 6-phosphate and fructose 6-phosphate were combined and an equal volume of 10mM-triethanolamine buffer, pH7·0, containing 10μmoles of $MgSO_4$ and 4μmoles of ATP was added and the pH adjusted to 7·8 with KOH (glass electrode). Glucose 6-phosphate and fructose 6-phosphate were then converted into fructose 1,6-diphosphate by addition of 0·2mg. of glucose 6-phosphate isomerase and 0·5mg. of phosphofructokinase (the preparation of phosphofructokinase obtained from Boehringer was apparently desensitized, for it failed to show inhibition by excess of ATP; this greatly facilitated quantitative conversion into fructose 1,6-diphosphate). The completeness of the reaction was confirmed by following the disappearance of glucose 6-phosphate and the appearance of fructose 1,6-diphosphate, which took about 30min. at room temperature. The mixture was then applied to a Dowex 1 (formate form) column and the fructose 1,6-diphosphate eluted with a gradient of M-ammonium formate into 0·63M-formic acid (elution volume 200–250ml.) (see the Methods section). The specific activity of fructose 1,6-diphosphate was then determined in the peak tube and the specific activity of the glucose 6-phosphate and fructose 6-phosphate in the original heart extracts determined from the known dilution.

Measurement of specific activity of L-glycerol 3-phosphate in extracts of rat heart. The amount of glycerol phosphate in the perchloric acid extracts was assayed spectrophotometrically and radioactivity was assayed after separation by ion-exchange chromatography. Glycerol phosphate is poorly separated from glucose 6-phosphate, fructose 6-phosphate and citrate on columns of Dowex 1 (formate form). Satisfactory chromatographic resolution was achieved by enzymic conversion of glucose 6-phosphate and fructose 6-phosphate into fructose 1,6-diphosphate with phosphofructokinase and of glycerol phosphate into glycerol with acid phosphatase after addition of carrier

glucose 6-phosphate and glycerol phosphate. The procedure was as follows.

Perchloric acid extract equivalent to 2–3g. wet wt. of heart was diluted with 10mM-triethanolamine buffer, pH7·6, to 25ml. and 100μmoles of $MgSO_4$, 5μmoles of glucose 6-phosphate, 10μmoles of ATP and 10μmoles of glycerol phosphate were added. The glucose 6-phosphate and fructose 6-phosphate were converted into fructose 1,6-diphosphate by addition of 0·2mg. of glucose 6-phosphate isomerase and 0·5mg. of phosphofructokinase as described previously. This was then applied to a 20cm. × 1cm. column of Dowex 1 (formate form) and the fractions containing glycerol phosphate were eluted (by using the gradients described for elution of glucose and fructose 6-phosphates). The fractions were pooled, an equal amount of 4mM-$MgCl_2$ was added, the pH was adjusted to 4·6 with 50% KOH and 10mg. of acid phosphatase was added (Boehringer preparation SP-11, 15439). Conversion of glycerol phosphate into glycerol at room temperature was complete in 15min. (shown by spectrophotometric assay of glycerol). Phosphatase was destroyed by incubation at 100° for 10min. and the reaction mixture was then applied to a 20cm. × 1cm. column of Dowex 1 (Cl^- form) and glycerol washed through with water (60ml.). The glycerol was rephosphorylated by addition of 5ml. of 0·1M-triethanolamine, 20μmoles of ATP and, after adjusting to pH7·6, addition of 0·2mg. of glycerokinase. The glycerol phosphate was then separated by elution from a 20cm. × 1cm. column of Dowex 1 (formate form) with M-ammonium formate–formic acid buffer, pH4·0. The specific activity of glycerol phosphate was determined in the peak tube and the specific activity in the original heart extract determined from the known dilution.

Analysis of incubation and perfusion media

Glucose specific activity. The initial incubation media were assayed for glucose concentration with glucose oxidase against a solution of glucose in saturated benzoic acid standardized by polarimetry. Radioactivity was assayed as described in the Methods section. A small correction (3–4%) was made for the quantity of preincubation or pre-perfusion medium which was carried over by the fat pads or hearts into incubation or perfusion media.

Lactate specific activity. A portion (10–15ml.) of medium was treated with 10μmoles of NADH and 100μg. of lactate dehydrogenase for 30min. at room temperature to convert pyruvate into lactate and deproteinized by addition of perchloric acid (70%) to give a final concentration of 5%. Carbon dioxide was removed *in vacuo* and potassium perchlorate by centrifugation at 0° after neutralization with

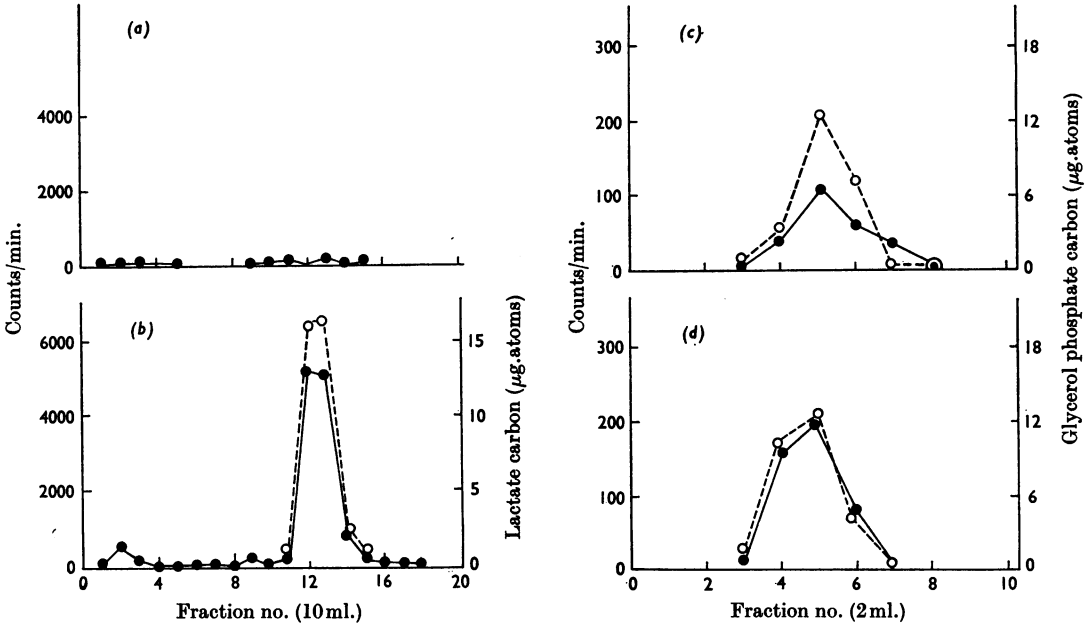

Fig. 1. Elution patterns of [^{14}C]lactate and L-[^{14}C]glycerol 3-phosphate from Dowex 1 (formate form) columns. ●, Counts/min.; ○, μg.atoms of lactate or of glycerol phosphate carbon. (*a*) and (*b*) Ion-exchange chromatography of lactate in medium on Dowex 1 (formate form) with an exponential gradient of M-formic acid in water (see the Methods section). (*a*) Initial medium. (*b*) Medium after perfusion of normal heart. Medium contained [U-^{14}C]glucose (1mg./ml. and 0·01μc/ml.) and insulin (0·1unit/ml.). (*c*) and (*d*) Glycerol in the medium after removal of glucose and phosphorylation with glycerokinase (see the Procedure section). Chromatography on Dowex 1 (formate form) with M-ammonium formate–formic acid, pH4·0. (*c*) Initial perfusion medium to which non-radioactive glycerol was added. (*d*) Medium after perfusion of normal hearts with addition of non-radioactive glycerol (see the Procedure section). Medium contained [U-^{14}C]glucose (1mg./ml. and 0·3μc/ml.) and insulin (0·1unit/ml.).

saturated $KHCO_3$. Lactate was then assayed in the supernatant and the remainder applied to a column of Dowex 1 (formate form). Radioactive glucose was eluted with 100 ml. of water and lactate eluted with a gradient of formic acid (see the Methods section). The specific activity of the lactate was assayed in the peak tube. A sample of the initial perfusing medium was also put through the procedure for the separation of lactate; radioactivity in the vicinity of the lactate peak was less than 1% of that found after fat-pad incubation or heart perfusion (see Fig. 1). Details of the elution pattern are given in Fig. 1.

Output of glycerol and glycerol radioactivity. The quantity of glycerol in incubation media was assayed after deproteinizing with perchloric acid by the method of Garland & Randle (1962).

The proportion of glucose carbon atoms which are converted into glycerol by rat heart and rat epididymal fat tissue was small. For example, in experiments with the perfused heart the ratio of radioactivity in glucose to that in glycerol in the perfusion medium was as high as 20000:1. Complete separation of glycerol from glucose by direct methods was difficult because of their similar chemical and physical properties. The method to be used had to separate glycerol and glucose completely and at the same time be capable of handling large amounts of medium to obtain measurable quantities of radioactivity in glycerol. Paper chromatography or thin-layer chromatography were thus not readily applicable. The possibility of separation by ion-exchange chromatography after phosphorylation with glycerokinase was thus explored. It was found, however, that a small amount of glucose was phosphorylated under these conditions and glucose 6-phosphate and glycerol phosphate were not readily separated by ion-exchange chromatography. Because of this, glucose was first removed by conversion into glucose 6-phosphate and treatment with Dowex 1 (Cl^- form). The glycerol was then phosphorylated and chromatographed on Dowex 1 (formate form). The procedure was as follows.

Heart perfusion media (15 ml.) or fat-pad incubation media (7 ml.) were diluted with about 60 ml. of 10 mM-triethanolamine buffer, pH 7·5, containing 300 μmoles of $MgSO_4$, 100–150 μmoles of ATP (dependent on the total amount of glucose present) and 10 μmoles of glycerol. Yeast hexokinase (0·5 mg.) was added and the formation of glucose 6-phosphate followed by spectrophotometric assay on 50–200 μl. samples with glucose 6-phosphate dehydrogenase and NADP. Phosphorylation was complete in less than 15 min.

The reaction mixture was then applied to a column of Dowex 1 (Cl^- form) and glycerol separated from glucose 6-phosphate and other anions by washing through with distilled water (total volume of eluate 100 ml.). ATP was then added to the eluate (30 μmoles), the pH adjusted to 7·5 (glass electrode), 50 μg. of glycerokinase added and the formation of glycerol phosphate followed by spectrophotometric assay of this compound. Phosphorylation was 95–100% complete after about 20 min. and the mixture was then freeze-dried. The residue from freeze-drying was taken up in 20 ml. of water, applied to a small column (10 cm. × 1 cm.) of Dowex 1 (formate form) and the column washed with 50 ml. of water followed by 50–100 ml. of M-formic acid until the washings were free of detectable radioactivity. The glycerol phosphate was then eluted with M-ammonium formate–formic acid buffer, pH 4·0, fractions of 2 ml. being collected. The fractions containing glycerol phosphate were identified by spectrophotometric assay (by using glycerol phosphate dehydrogenase) and glycerol radioactivity was assayed in three peak tubes. The specific activity of glycerol present in incubation or perfusion media was then calculated from the known dilution. In each experiment a sample of the initial incubation or perfusion medium was put through the entire procedure and the radioactivity in the glycerol phosphate peak applied as a correction to that obtained after fat-pad incubations or heart perfusions. The correction was about 50%. Details of the elution pattern are given in Fig. 1.

Recoveries

The recovery of glyceride glycerol was found by triglyceride addition to heart powder to be substantially complete (Denton & Randle, 1967). It was confirmed that the radioactivity attributed to glyceride glycerol in the heart was in glycerol by conversion into glycerol phosphate with ATP and glycerokinase followed by chromatography on Dowex 1 (formate form). The recovery of fatty acid was not ascertained but it was found that extraction of radioactivity by light petroleum after saponification was complete. The recovery of lactate in perfusion media after ion-exchange chromatography was over 95%. The removal of glucose after conversion into glucose 6-phosphate and treatment with Dowex 1 (Cl^- form) was apparently complete. The recovery of glycerol as glycerol phosphate after ion-exchange chromatography was 85% or better; recovery in the three peak tubes taken for assay of radioactivity was 75% or better.

The recovery of glucose 6-phosphate and fructose 6-phosphate from extracts of rat heart after separation together as fructose 1,6-diphosphate by ion-exchange chromatography was 80% or better. The recovery of lactate from extracts of rat heart after ion-exchange chromatography was about 85%. The recovery of glycerol phosphate radioactivity in measurements of specific activity was 75% or better.

Calculations and expression of results

Carbon atom flows. The carbon atom flows measured (glucose uptake, lactate and pyruvate output, glycerol output and flow of glucose carbon atoms into glyceride glycerol, glycerol and fatty acids) have been calculated and expressed as μg.atoms of carbon/g. wet wt. of fat pad or g. dry wt. of ventricular muscle/hr. Since the results with heart were referred to weights of ventricular muscle the values for glucose uptake and lactate output are somewhat higher than those given previously, which were based on the weight of the whole heart (ventricular muscle accounts for 75% of the weight of the whole heart). In calculating carbon atom flows to glyceride glycerol and glycerol the contribution of glycerol in fat pad or heart was ignored. Analyses of these tissues showed that this was less than 10% of the glycerol found in the medium. It was not practicable to assay radioactivity in this small amount of glycerol.

In experiments with the perfused heart, concentrations of glucose, glycerol and lactate in the medium and ^{14}C in glyceride glycerol in the tissue and in glycerol in the medium have been measured after 7·5 min. and 15 min. of perfusion. From these data rates of glucose uptake, lactate

and glycerol output and carbon atom flow from glucose and glycogen glucose to glyceride glycerol and glycerol have been calculated for the two periods of perfusion. Since the rates measured over these two periods of perfusion were similar it has seemed reasonable to assume that they are representative of rates which obtain at any time during 15 min. of perfusion. In experiments with adipose tissue, measurements were made after 60 min. of incubation. Evidence that rates of glucose uptake, lactate output, glycerol output and incorporation of ^{14}C from [^{14}C]glucose into tissue lipids are linear during this period of incubation has been summarized in an earlier paper (Denton *et al.* 1966).

Specific activities. Specific activities of glucose, lactate and pyruvate and glycerol in the medium, and muscle lactate and pyruvate and muscle glucose 6-phosphate and fructose 6-phosphate, were calculated as counts/min./μg.atom of carbon. In calculating the specific activities of glucose 6-phosphate and fructose 6-phosphate and glycerol phosphate in rat heart it has been assumed that these are confined to intracellular water. The specific activity of intracellular lactate in the heart was calculated from the total muscle lactate concentration and radioactivity, the perfusate lactate concentration and radioactivity, and known values for the volumes of intracellular and extracellular water (see Newsholme & Randle, 1964). This calculation assumes that the concentration and specific activity of extracellular lactate in the heart is the same as that in the perfusate.

RESULTS

Effects of insulin, adrenaline and alloxan-diabetes on carbon atom flow from glucose to lactate plus pyruvate, glyceride glycerol, glyceride fatty acids and glycerol in the medium in rat epididymal fat pads. Tables 1 and 2 give data for rat epididymal adipose tissue incubated for 1 hr. in medium containing [U-^{14}C]glucose and effects of insulin, adrenaline (with insulin) and alloxan-diabetes. Table 1 records the incorporations of ^{14}C into glyceride glycerol, glyceride fatty acids and glycerol in the medium, the specific activities of lactate and glucose in the medium and their ratio. Table 2 gives the flow of carbon atoms from glucose to glyceride glycerol, glyceride fatty acids and glycerol in the medium calculated from incorporation of ^{14}C and the specific activity of glucose in the medium; the Table also shows the uptake of glucose carbon atoms and the outputs of lactate and pyruvate and of glycerol carbon atoms measured by direct assay.

The specific activities of lactate and glucose in the medium were very close. The mean ratio was 0·99 and no change was detected with insulin, adrenaline (with insulin) or alloxan-diabetes. Insulin decreased the flow of glucose carbon atoms to glyceride glycerol without changing either the flow to glycerol in the medium or the output of glycerol; the hormone produced the expected increases in glucose uptake and in the flow of glucose carbon atoms to lactate and pyruvate and to glyceride fatty acids. These particular products of glucose metabolism accounted for 52% of the uptake of glucose carbon atoms with insulin and for 135% without insulin (the recovery in excess of 100% is presumably due to the errors inherent in the measurement of very small glucose uptakes). Adrenaline (with insulin) markedly increased the

Table 1. *Effects of insulin, adrenaline and alloxan-diabetes on the radioactivity of lactate, glyceride glycerol and fatty acids and glycerol in the medium in rat epididymal fat pads incubated with* [U-^{14}C]*glucose*

The medium contained glucose (3 mg./ml.) and bovine plasma albumin (20 mg./ml.). Where added, the concentrations were: adrenaline, 5 μg./ml., and insulin, 0·01 unit/ml. Fat pads were preincubated for 30 min. in medium containing only [^{12}C]glucose and then incubated for 60 min. in media containing [U-^{14}C]glucose, albumin and other additions as shown. Each value is a single observation for eight fat pads incubated together in 25 ml. of medium with two experiments under each set of conditions.

State of animal	Hormone(s)	Sp. activity (counts/min./μg.atom of carbon)		Ratio of sp. activities	Incorporation of ^{14}C (counts/min./g. wet wt. of tissue)		
		Glucose in medium	Lactate in medium	(lactate/glucose)	Into glyceride glycerol	Into glyceride fatty acids	Into glycerol in medium
Normal	None	2285	2321	1·02	5070	9800	0
		2815	2700	0·96	4000	3066	160
	Insulin	2310	2243	0·97	9827	55630	0
		2740	2633	0·96	9480	48250	450
	Insulin + adrenaline	2290	2217	0·97	48660	18840	3470
		2790	2866	1·03	44420	28460	3570
Normal	Insulin	2430	2666	1·09	10030	50348	680
		2538	2366	0·93	10030	38320	150
Diabetic	Insulin	2630	2500	0·95	16080	6038	350
		2666	2620	0·98	15390	7060	20

Table 2. *Effects of insulin, adrenaline and alloxan-diabetes on flow of carbon atoms from glucose to lactate plus pyruvate, glyceride glycerol and fatty acids, and glycerol in the medium, in rat epididymal fat pads*

Composition of media and conditions of incubation are as given in Table 1. Carbon atom flow by radioactive assay was calculated from: radioactivity in product/sp. activity of glucose or lactate (shown to be the same: see Table 1). Values are means of two experiments, in each of which eight fat pads were incubated together in 25 ml. of medium. Radioactive data are given in Table 1.

		Carbon flow (μg.atoms of carbon/g. wet wt. of tissue/hr.)						
					Flow of glucose carbon (calc. from radioactive data)			
State of animal	Hormone(s)	Glucose uptake	Lactate+ pyruvate output	Glycerol output	To glyceride glycerol (*A*)	To glyceride fatty acids	To glycerol in medium (*B*)	(*A*+*B*)
Normal	None	6	3·7	1·2	1·8	2·6	0·1	1·9
	Insulin	58	4·8	1·1	4·0	20·8	0·1	4·1
	Insulin+adrenaline	61	8·7	19·9	18·7	9·2	1·4	20·1
Normal	Insulin	75	5·4	1·1	4·0	17·9	0·2	4·2
Diabetic	Insulin	28	16·1	2·9	6·1	2·5	0·1	6·2

Table 3. *Effect of alloxan-diabetes on the radioactivity of lactate, glyceride glycerol and glycerol in the medium in rat hearts perfused with* [U-^{14}C]*glucose*

The medium contained glucose (1 mg./ml.) and insulin (0·1 unit/ml.). Hearts were pre-perfused for 5 min. with medium lacking [^{14}C]glucose and then perfused for either 7·5 min. with medium containing 0·3 μc of [U-^{14}C]glucose/mg./ml. or for 15 min. with medium containing 0·15 μc of [U-^{14}C]glucose/mg./ml. Each value is a single observation for a pool of four hearts. No radioactivity was detected in glyceride fatty acids and this column is omitted (cf. Table 1).

		Sp. activity (counts/min./μg.atom of carbon)			Incorporation of ^{14}C (counts/min./g. dry wt. of ventricle)	
State of animal	Time of perfusion (min.)	Glucose in medium	Lactate in medium	Ratio of sp. activities (lactate/glucose)	Into glyceride glycerol	Into glycerol in medium
Normal	7·5	17100	8600	0·50	2584	1099
Diabetic			3160	0·18	2671	40
Normal	7·5	19500	9920	0·51	3177	1485
Diabetic			3530	0·18	5887	306
Normal	15	6720	4660	0·70	1998	1026
Diabetic			2300	0·34	3863	679
Normal	15	8420	5460	0·65	3829	1665
Diabetic			2230	0·26	5428	866

flow of glucose carbon atoms to glyceride glycerol and glycerol in the medium and the output of glycerol. This was accompanied by a diminution in the flow of carbon atoms from glucose to glyceride fatty acid and by a small increase in the output of lactate and pyruvate. The uptake of glucose was unchanged. These metabolites accounted for 62% of the glucose carbon atoms taken up with adrenaline. In pads from alloxan-diabetic rats incubated with insulin the flow of glucose carbon atoms to glyceride glycerol was increased; the flow to glycerol in the medium was not increased though the output of glycerol was enhanced. The output of lactate and pyruvate was increased in the diabetic pads and the expected diminutions in glucose uptake and in the flow of glucose carbon atoms to glyceride fatty acids were observed. These products accounted for 87% of the glucose carbon atoms taken up by the diabetic tissue.

Effects of alloxan-diabetes on the flow of carbon atoms from glucose and glycogen to lactate and pyruvate, glyceride glycerol and glycerol in the medium in the perfused rat heart. In Tables 3, 4 and 5 are given data for hearts from normal and alloxan-diabetic rats perfused for 7·5 or 15 min. with medium containing [U-^{14}C]glucose. Table 3 shows the specific activities of glucose and lactate in the medium and their ratio and the incorporation of radioactivity

Table 4. *Comparison of the specific activities of intracellular lactate plus pyruvate, L-glycerol 3-phosphate and (glucose plus fructose) 6-phosphates in hearts from normal and diabetic rats after perfusion for 7·5 min. with [U-^{14}C]glucose and insulin*

Hearts were pre-perfused with medium containing glucose (1 mg./ml.) and insulin (0·1 unit/ml.) for 5 min. and then perfused for 7·5 min. by recirculation of 15 ml. of medium containing [U-^{14}C]glucose (1 mg./ml. and 0·3 μc/ml.) and insulin (0·1 unit/ml.). Results shown are determinations made with pooled medium or frozen heart powder from eight hearts from either normal or diabetic rats. Sp. activity of medium [U-^{14}C]glucose was 13700 counts/min./μg.atom of carbon. Other details are given in the text.

	Normal			Alloxan-diabetic		
		Sp. activity			Sp. activity	
Metabolites	Concn. (mM)	(counts/min./ μg.atom of carbon	(% of that of glucose)	Concn. (mM)	(counts/min./ μg.atom of carbon)	(% of that of glucose)
Lactate+pyruvate						
Perfusate	0·68	6700	49	0·77	3200	23
Whole heart	0·89	8000	58	1·21	3800	27
Intracellular	1·24	9300	68	1·92	4600	34
L-Glycerol 3-phosphate	0·89	9500	70	0·42	3900	28
(Glucose+fructose) 6-phosphates	0·68	10500	77	0·93	5800	42

Table 5. *Effect of alloxan-diabetes on flow of carbon atoms from glucose and glycogen to lactate plus pyruvate, glyceride glycerol and glycerol in the medium in perfused rat heart*

Composition of media and conditions of perfusion are as given in Table 3. Carbon atom flow by radioactive assay was calculated from: radioactivity in product/sp. activity of lactate (glucose and glycogen) or glucose. Radioactive data are given in Table 3. No radioactivity was detected in glyceride fatty acids and this column is omitted (cf. Table 1).

		Carbon flow (μg.atoms of carbon/g. dry wt. of ventricle/hr.)						
					Flow of glucose and glycogen carbon (from radioactive data)			Flow of glucose carbon to glyceride glycerol and glycerol
State of animal	Time of perfusion (min.)	Glucose uptake	Lactate+ pyruvate output	Glycerol output	To glyceride glycerol (*A*)	To glycerol in medium (*B*)	Total flow (*A*+*B*)	
Normal	7·5	2165	1039	10	2·4	1·0	3·4	1·7
Diabetic		1272	739	64	6·8	0	6·8	1·2
Normal	7·5	2897	839	14	2·6	1·2	3·8	1·9
Diabetic		1073	1019	70	13·4	0·8	14·2	2·6
Normal	15	1732	—	12	1·7	1·0	2·7	1·9
Diabetic		606	—	50	6·8	1·2	8·0	2·7
Normal	15	3466	1459	12	2·8	1·2	4·0	2·6
Diabetic		1346	1199	56	9·8	1·6	11·4	3·0
Overall mean:								
Normal	7·5+15	2564	1112	12	2·4	1·1	3·5	2·0
Diabetic		1073	986	60	9·2	0·9	10·1	2·4

into glyceride glycerol in the tissue and glycerol in the medium. Table 4 compares the specific activities of glucose, lactate and pyruvate in the medium, intracellular lactate and pyruvate, intracellular glucose 6-phosphate and fructose 6-phosphate and intracellular glycerol phosphate. Table 5 gives the calculated flows of carbon atoms from glucose in the medium and glycogen glucose in the muscle to glyceride glycerol in the muscle and glycerol in the medium and the uptake of glucose carbon atoms and the outputs of glycerol carbon atoms and lactate and pyruvate carbon atoms.

The specific activity of lactate in the medium was lower than that of glucose under all experimental conditions investigated. In the normal heart the ratio of specific activities was 0·5 after 7·5 min. of

perfusion and 0·7 after 15 min. The ratios of specific activities in the diabetic tissue were lower than in the normal, being 0·18 after 7·5 min. and 0·30 after 15 min. The incorporation of ^{14}C into glyceride glycerol was greater in the diabetic heart than in the normal at both time-intervals whereas the incorporation into glycerol in the medium was lower in the diabetic tissue. The overall incorporation into glyceride glycerol and into glycerol in the medium was very similar in normal and diabetic hearts. No radioactivity was detected in glyceride fatty acids in any of these experiments.

The specific activity of intracellular lactate and pyruvate after 7·5 min. of perfusion in both normal and diabetic muscle was greater than that of lactate and pyruvate accumulated in the medium during this period and lower than that of glucose in the medium. The specific activity of intracellular glucose 6-phosphate and fructose 6-phosphate was lower than that of glucose in the medium and greater than that of intracellular lactate and pyruvate. The specific activity of glycerol phosphate was comparable with that of intracellular lactate and pyruvate. In the diabetic heart the specific activities of intracellular lactate and pyruvate, glucose 6-phosphate and fructose 6-phosphate and glycerol phosphate were lower than in the normal tissue.

The flows of carbon atoms from glucose to glyceride glycerol in the tissue and glycerol in the medium calculated from incorporation of ^{14}C and the specific activity of glucose in the medium were very similar in normal and diabetic hearts. The total flow of carbon atoms from glucose in the medium and glycogen in the muscle to glyceride glycerol in the muscle and glycerol in the medium calculated from ^{14}C incorporation and the specific activity of lactate in the medium was greater in the diabetic tissue than in the normal. The uptake of glucose carbon atoms was diminished in the diabetic tissue whereas the output of glycerol carbon atoms was increased and that of lactate and pyruvate unchanged.

DISCUSSION

Use of enzymic conversion to effect chromatographic resolution of metabolites. The methods that have been developed for the separation of glucose and glycerol (by selective enzymic conversion into their phosphorylated derivatives) and for the separation together of glucose 6-phosphate and fructose 6-phosphate from citrate and glycerol phosphate (by enzymic conversion into fructose 1,6-diphosphate) and for the separation of glycerol phosphate (by enzymic conversion into glycerol) are capable of more widespread application. Thus enzymes may be used with a variety of metabolites to add or remove amino, phosphate or carboxyl groups and thus to effect their separation by ion-exchange chromatography. This method which uses the known specificity of enzymes to improve chromatographic resolution may be particularly useful for measuring the incorporation of radioactivity into metabolites. Under these circumstances carrier may be added to raise their concentration and thus facilitate the spectrophotometric identification of peaks of radioactivity.

Errors in the calculation of rates of glyceride synthesis. (*a*) Adipose tissue. In experiments with adipose tissue the specific activities of glucose and lactate were indistinguishable. The specific activity of glycerol phosphate is thus likely to be similar to that of glucose. The results with adipose tissue were different from those obtained with the perfused heart where the specific activity of lactate was lower than that of glucose. The pool of non-radioactive precursors of lactate in relation to the rate of glycolysis may be much smaller in adipose tissue in view of the small volume of intracellular water (Denton *et al.* 1966). The rates of glycolysis in fat pad and heart as μmoles of glucose/ml. of intracellular water with insulin were 700 and 140 respectively.

One possible source of error not excluded by these measurements is the formation of glycerol by hydrolysis of glycerol phosphate (Vaughan, 1961). The contribution of glycerol radioactivity to the calculated rates of glyceride synthesis was, however, small ($< 8\%$).

(*b*) Heart muscle. In experiments with the perfused heart a number of differences in specific activity were observed which may call for further comment. In both normal and diabetic hearts the specific activities of lactate in the medium and of muscle lactate, glucose 6-phosphate and fructose 6-phosphate and glycerol phosphate were lower than that of glucose. Moreover the specific activities of these metabolites were lower in the diabetic tissue than in the normal. The specific activities of muscle glycerol phosphate and muscle lactate were, however, similar in both the normal and the diabetic heart. The specific activity of lactate in the medium would appear to provide a satisfactory index of glycerol phosphate specific activity for the calculation of rates of glyceride synthesis.

The differences in specific activities in hexose monophosphates, lactate and glycerol phosphate between normal and diabetic hearts were not unexpected. Glycogen breakdown occurs during perfusion of diabetic hearts at a rate which is approximately equal to that of glucose uptake. In the normal heart there is no change of glycogen concentration during perfusion under these conditions (Randle, Newsholme & Garland, 1964). The ratios of specific activities observed in normal and diabetic tissues are consistent with these differences in glycogen breakdown.

In the normal heart the specific activities of

intracellular glucose 6-phosphate and fructose 6-phosphate were lower than that of glucose in the medium. This may be accounted for by the turnover of glycogen which incorporates radioactive glucose into glycogen without change of concentration (Randle *et al.* 1964). The reason for the difference in specific activity between muscle glucose 6-phosphate and fructose 6-phosphate, and muscle lactate, and lactate in the medium, was not ascertained. It may result from isotope equilibration between lactate formed by glycolysis and unlabelled alanine. The alanine pool in the perfused heart is apparently large enough to account for this difference (see Scharff & Wool, 1965). The increase in lactate specific activity observed when the period of perfusion was extended from 7·5 to 15 min. could also be consistent with this interpretation.

L-Glycerol 3-phosphate concentration and the rate of glyceride synthesis from glycerol phosphate in rat heart and epididymal adipose tissue. The relationship between rates of glyceride synthesis and tissue concentrations of glycerol phosphate in heart and adipose tissue is shown in Table 6. In adipose tissue insulin increased the concentration of glycerol phosphate and the rate of glyceride synthesis. In this instance glycerol phosphate concentration may be a factor in the control of glyceride synthesis. In adipose tissue incubated with insulin and adrenaline a substantial fall in glycerol phosphate concentration (to approx. 15%) was associated with a fivefold increase in the rate of glyceride synthesis. Similarly in the diabetic rat heart *in vitro* an increased rate of glyceride synthesis was associated with a lowered concentration of glycerol phosphate. Moreover in the heart *in vivo* in alloxan-diabetes increased deposition of glyceride, which may be due to an increased rate of glyceride synthesis, was associated with a diminished concentration of glycerol phosphate. In these instances factors other than the concentration of glycerol phosphate apparently determined the rate of glyceride synthesis. One possibility is the concentration of fatty acyl-CoA which is increased in the heart in alloxan-diabetes (Garland & Randle, 1964).

In the perfused heart no incorporation of radioactivity from [U-^{14}C]glucose into glyceride fatty acids could be detected. In adipose tissue on the other hand a substantial proportion of the glucose radioactivity taken up was incorporated into glyceride fatty acids. In view of this it seems unlikely that glyceride synthesis in adipose tissue cells associated with the heart could have made a significant contribution to the incorporation of radioactivity into glyceride glycerol in the heart.

Lipolysis and the regulation of glyceride fatty acid oxidation in the perfused rat heart. Measurements of glycerol output by Garland & Randle (1964) suggested that an increased rate of hydrolysis of muscle glycerides was an important factor in the increased rate of oxidation of fatty acids in the diabetic

Table 6. *Relationship between L-glycerol 3-phosphate concentration and rate of esterification of fatty acids to triglyceride in rat epididymal adipose tissue and heart muscle*

The glycerol phosphate concentrations in adipose tissue are taken from Denton *et al.* (1966) and were measured after 60 min. of incubation; similar values were obtained with shorter periods of incubation (see Denton *et al.* 1966). The glycerol phosphate concentrations in heart are taken from Garland & Randle (1964) and were measured after 15 min. of perfusion. Similar differences in concentration between normal and diabetic hearts are seen after 7·5 min. of perfusion (see Table 4). Rates of esterification are the values for flow of carbon atoms from glucose and glycogen glucose to glyceride glycerol in the tissue and glycerol in the medium taken from Tables 2 and 5. The values for heart are pooled values for 7·5 and 15 min. of perfusion.

Tissue	State of animal	Hormone(s)	Concn. of L-glycerol 3-phosphate (μmoles/ml. of intracellular water)	Rate of esterification (μg.atoms of glycerol carbon/g. wet. wt. of pad or g. dry wt. of ventricle/hr.)	Rate of glucose uptake (μg.atoms of glucose carbon/g. wet wt. of pad or g. dry wt. of ventricle/hr.)
in vitro					
Fat pad	Normal	None	0·23	1·9	6
		Insulin	1·1	4·1	58
		Insulin + adrenaline	0·16	20·1	61
	Normal	Insulin	1·9	4·2	75
	Diabetic	Insulin	2·3	6·2	28
Heart	Normal	Insulin	0·57	3·5	2564
	Diabetic	Insulin	0·22	10·1	1073
in vivo					
Heart	Normal	—	0·25	—	—
	Diabetic	—	0·04	—	—

heart *in vitro*. The increased glycerol output by the diabetic heart has been confirmed in the present study; the conclusion that this is an important factor in accelerated fatty acid oxidation has been reinforced by the finding that the rate of re-esterification of fatty acids is increased rather than diminished in the diabetic tissue. Furthermore, radioactive measurements in the present study have shown that the flow of carbon atoms from glucose and glycogen glucose to glycerol is small in relation to the glycerol output of the normal heart (less than 10%), and that the flow to glycerol is not increased in the diabetic tissue although the glycerol output and the flow to glyceride glycerol are accelerated. This would appear to show that the amount of glycerol which may be formed by direct hydrolysis of glycerol phosphate is small compared with the glycerol formed by hydrolysis of glyceride. This finding confirms the use of glycerol output as a measurement of lipolysis rate. The findings with the diabetic heart suggest that the pool of non-radioactive glyceride from which glycerol is formed by lipolysis is increased in the diabetic tissue. In keeping with this suggestion it has been found that the concentration of glyceride is increased in diabetic muscle (Denton & Randle, 1967).

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