Biochem. J. (1967) **104**, 416

Concentrations of Glycerides and Phospholipids in Rat Heart and Gastrocnemius Muscles

EFFECTS OF ALLOXAN-DIABETES AND PERFUSION

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(*Received* 14 *November* 1966)

1. Methods are described for the extraction of lipid and assay of mono-, di- and triglyceride glycerol and phospholipid phosphorus in rat heart and gastrocnemius muscles. 2. In hearts from normal animals, concentrations found were: monoglyceride, 0·6; diglyceride, 0·1; triglyceride, 12·6 μmoles of glyceride glycerol/g. of dry muscle; phospholipid, 171 μg.atoms of phospholipid phosphorus/g. of dry muscle. Concentrations of glycerides in gastrocnemius muscle were similar to heart muscle but those of phospholipids were lower (64 μg.atoms of phospholipid phosphorus/g. of dry muscle). 3. Alloxan-diabetes increased the concentration of triglyceride in the muscles twofold. This increase was shown to be dependent in the heart on the availability of growth hormone and cortisol but not on the availability of dietary lipid. Total glyceride in the heart was increased after 48 and 72hr. starvation but not after 96hr. Changes in glyceride concentration seen in starvation and diabetes were not associated with significant changes in phospholipid concentration. It is suggested that mobilization of free fatty acids in diabetes leads to the synthesis of additional glyceride in muscle. 4. The possible contribution of glyceride fatty acid in the heart to respiration during perfusion has been calculated from the net loss of glyceride during perfusion, and also from the relative rates of lipolysis and esterification and compared with oxidation of fatty acid required for the balance of oxygen consumption (oxygen not utilized in the oxidation of glucose or glycogen glucose). In the normal or diabetic heart perfused with glucose and insulin the breakdown of glyceride can account for the balance of oxygen consumption. In the normal heart perfused without substrate the balance of oxygen consumption is not entirely accounted for by the breakdown of glyceride.

The term glycerides is used in this paper to refer collectively to mono-, di- and tri-glycerides.

The oxidation of glucose or glycogen glucose may account for only part of the oxygen consumption of the perfused rat heart. The fraction has been found to be 30–40% in the normal heart perfused without substrate, 82% in the normal heart perfused with glucose and insulin and 50% in the alloxan-diabetic rat heart perfused with glucose and insulin (Williamson & Krebs, 1961; Randle *et al.* 1966). The balance has been attributed to the oxidation of fatty acids released by the breakdown of muscle glycerides and evidence that this is accelerated in the diabetic tissue has been obtained from measurements of the concentrations of free fatty acids and fatty acyl-CoA and the output of glycerol (Garland & Randle, 1964). In the present paper direct evidence for the breakdown of muscle glycerides in the perfused heart has been obtained by measurement of the concentrations of glycerides and phospholipids before and after perfusion.

These measurements have shown that the concentration of glycerides in the heart is increased in alloxan-diabetes. Similar changes were detected in gastrocnemius muscle. To define the mechanism for this increase in muscle glyceride concentration the importance of dietary lipids has been investigated with low fat diet and with total starvation. The possibility that increased mobilization of lipids from adipose tissue in diabetes leads to increased deposition of glycerides in muscle has been explored in further experiments with hypophysectomized-diabetic rats with and without injections of growth hormone and cortisol. Mobilization of lipid from adipose tissue in diabetes is apparently dependent upon growth hormone and corticosteroids (Garland & Randle, 1964). A preliminary account of some of these findings has been published (Denton & Randle, 1965).

MATERIALS

Rats and diets. Except as stated below, hearts and gastrocnemius muscles were obtained from male albino

Wistar rats (250–300g.) fed on a stock laboratory diet 41B (Short & Parkes, 1949) with free access to food and water at all times. Rats described as starved were deprived of food but not water for the stated number of hours. Rats fed on 'low fat diet' were given only boiled polished rice for 48hr. before use. This contains 74% water, 2·2% protein, 22% carbohydrate and 0·1% fat. Alloxan-diabetes was induced as described by Denton & Randle (1967). Hypophysectomized rats (200–250g.) were obtained from Charles River Breeding Laboratories, North Wilmington, Mass., U.S.A.; in experiments involving hypophysectomized animals, control rats of the same strain and similar weight were obtained from the same source.

Chemicals. Triethanolamine hydrochloride was from Boehringer Corporation (London) Ltd., London, W.5. Silicic acid (100 mesh) was from Mallinckrodt Chemical Works, New York, N.Y., U.S.A., and activated before use by heating at 110° for 8–12hr. Silica gel G (for thin-layer chromatography) was from E. Merck A.-G. through Anderman and Co. Ltd., London, S.E. 1. Heparin (from Evans Medical Ltd., Liverpool) was dissolved in 0·9% NaCl to 2500 units/ml. and diluted with an equal volume of veterinary Nembutal solution (Abbott Laboratories Ltd., Queenborough, Kent) (60mg./ml.). Alloxan was obtained from Eastman Kodak Co., Rochester, N.Y., U.S.A.

Crystalline ox insulin (given by Boots Pure Drug Co. Ltd., Nottingham, or Burroughs Wellcome, Beckenham, Kent) was dissolved in 3·3mN-HCl to yield a stock solution of 20units/ml. Growth hormone was prepared in the Department of Biochemistry, University of Cambridge, by Mr B. R. Slater from ox anterior pituitary lobes by the method of Wilhelmi, Fishman & Russell (1948) and injected as a solution in distilled water (2mg./ml.). Cortisol as the sodium monosuccinate ester (Efcortelan Soluble) was obtained from Glaxo Laboratories Ltd., Greenford, Middlesex, and injected as a solution in distilled water (50mg./ml.). Butoxamine was a gift from Burroughs Wellcome Ltd., Tuckahoe, N.Y., U.S.A.

Glycerol tri[1-^{14}C]palmitate was from The Radiochemical Centre, Amersham, Bucks. Glycerol mono-oleate (purity >99%) and glycerol tripalmitate (purity 99%) were obtained from the Hormel Institute, University of Minnesota, St Paul, Minn., U.S.A. Glycerol trioleate (from British Drug Houses Ltd., Poole, Dorset) was found by thin-layer chromatography to contain di- and mono-oleate in sufficient quantity to provide a reference marker for all three classes. It is referred to as oleate (mono-+di-+tri-) glycerides.

Enzymes. Lactate dehydrogenase, pyruvate kinase and glycerokinase were from Boehringer Corporation (London) Ltd.

Media. Hearts and hind limbs were perfused with bicarbonate-buffered medium (Krebs & Henseleit, 1932) gassed with O_2+CO_2 (95:5). Concentrations of glucose and insulin are given in the text and Tables.

PROCEDURE AND METHODS

Hearts. Rats were injected with Nembutal and heparin and hearts removed as described by Newsholme & Randle (1964). For measurement of phospholipid and glyceride concentrations *in vivo* the hearts were perfused by drip through for up to 5min. to remove blood and blood lipids with medium containing glucose (1mg./ml.) and insulin (0·1unit/ml.). In experiments in which the disappearance of triglyceride and phospholipid during perfusion was investigated, initial concentrations were measured after 5min. of perfusion to remove blood and blood lipids and the final concentrations after 60min. of perfusion. At the end of perfusion the heart was transected at the auriculo-ventricular junction and the ventricular muscle, free of obvious adipose tissue, was rapidly and lightly blotted and then frozen with the tissue clamp of Wollenberger, Ristau & Schoffa (1960), which had been previously cooled in acetone–solid CO_2 mixture. The frozen hearts were powdered in a percussion mortar kept at the temperature of solid CO_2.

Gastrocnemius. Rats were injected intraperitoneally with butoxamine (1ml. of 5mg./ml. in 0·9% NaCl) to prevent possible stimulation of lipolysis by catecholamine release (Burns & Lemberger, 1965). Rats were then injected intraperitoneally with Nembutal (18mg.) and heparin (600units). After 15min., a cannula was inserted into the abdominal aorta just above its bifurcation, the inferior vena cava divided and blood washed out of the hind limb by 10min. of perfusion with bicarbonate-buffered medium containing glucose (2mg./ml.). The gastrocnemius muscles were excised from both legs and frozen in acetone–solid CO_2 mixture. The frozen gastrocnemius muscles were powdered in the percussion mortar.

Extraction of lipids from powdered frozen muscle. Weighed quantities of powdered frozen muscle were extracted with approx. 20vol. of chloroform–methanol (2:1, v/v) in a hand-operated glass Potter–Elvehjem homogenizer and allowed to stand for at least 2hr. at 0°. The supernatant, after brief centrifugation, was shaken with 0·3vol. of 4mM-$MgCl_2$ and, after 30min., separation of phases was completed by further centrifugation.

The upper phase was removed by aspiration and discarded. The total volume of the lower phase at this point was two-thirds of the volume of original chloroform–methanol used. A known volume of the lower phase (50–75%) was carefully removed, evaporated to dryness in a water bath at between 65° and 80°, and the residue dissolved in 7ml. of chloroform. Samples of this solution were then used for the assay of muscle phospholipid phosphorus and muscle glycerides. To assay glycerides as glyceride glycerol it was necessary to remove phospholipids, since glycerophosphatides may yield glycerol on saponification. The procedure was as follows.

Removal of phospholipid. Chloroform solutions (6ml.) of muscle lipid were shaken with 0·5g. of silicic acid (Carlson, 1963). The silicic acid was allowed to settle for about 5min. and packed by centrifugation. Great care was taken that no silicic acid was left adhering to the sides or stopper of the tube. It was found essential to carry out this procedure on solutions of muscle lipid in pure chloroform, because, in the presence of small amounts of methanol, glycerophosphatides (e.g. cardiolipin) were not fully adsorbed on to silicic acid. In all cases the efficiency of the removal of phospholipid from the solution was tested by assay of phospholipid phosphorus. The residual phospholipid was usually less than 0·05% of the original. Tubes containing more than 0·1% of the original phospholipid were either re-treated with silicic acid or rejected. The phospholipid-free supernatant was used for the assay of glycerides (as glycerol after saponification; see below) or where mono-, di- and triglycerides were determined separately was treated as follows.

Separation of mono-, di- and tri-glycerides. A sample (usually 4ml.) of phospholipid-free supernatant was evaporated to dryness at 65–80°. Mono-, di- and tri-glycerides were separated by thin-layer chromatography on 20cm. × 20cm. plates coated with silica gel G (0·25mm. thick) with light petroleum (b.p. 40–60°)–diethyl ether–methanol–acetic acid (90:20:2:3, by vol.). Six samples were run on each plate together with markers of oleate (mono-+di-+tri-)glycerides and pure glycerol tripalmitate and glycerol mono-oleate (about 0·5 μmole). The lipid residue in each tube was transferred in a total volume of 75 μl. of chloroform and evaporated on to the plate by a draught of cold air in two small adjacent spots about 5mm. apart. The plates were run until the solvent front had reached about 10cm. above the origin. The R_F values of the separated classes were: monoglycerides, 0·05; diglycerides, 0·45; triglycerides, 0·90. The spots located by markers were scraped from the plate and extracted with chloroform–methanol (2:1, v/v). Spots containing the triglycerides from the muscle of a single animal were each extracted twice with 2ml. of chloroform–methanol. Spots containing mono- or di-glycerides from muscles of up to six animals were combined and extracted twice with 4ml. of chloroform–methanol. Mono-, di- and tri-glycerides were then assayed as glycerol after saponification as described below.

Assay of phospholipid phosphorus. A suitable volume of chloroform solutions of muscle lipids (containing up to 0·1 μg.atom of phospholipid phosphorus) was evaporated to dryness at 65–80°. The lipid residue was then digested with 0·15ml. of nitrogen-free conc. H_2SO_4 and 0·05ml. of 70% perchloric acid and assayed for phosphate by the method of Lindberg & Ernster (1956).

Assay of glyceride glycerol. A suitable volume of chloroform–methanol solution of glycerides (containing up to 1 μmole) was evaporated to dryness at 65–80° and the glyceride saponified for 30min. at 60° with 0·5ml. of 4% KOH in 95% ethanol (prepared by adding 5 parts of aq. 80% KOH to 95 parts of ethanol). Saponification was terminated by adding 0·5ml. of 10% perchloric acid. After cooling to 0°, potassium perchlorate and co-precipitated fatty acids were removed by centrifugation. The solution was then neutralized with saturated $KHCO_3$ (approx. 0·25ml.), the potassium perchlorate removed by centrifugation at 0° and glycerol assayed in samples of supernatant (up to 0·5ml.) by the method of Garland & Randle (1962).

Recovery of added glycerides. Recovery of glycerol tri[1-^{14}C]palmitate added at the time of extraction of frozen heart powder and taken through the procedures for removal of phospholipid and separation of glycerides by thin-layer chromatography was better than 90%. Recovery of glycerol mono-oleate added at the time of extraction of frozen heart powder and taken through the entire procedure used for determination of monoglycerides was in excess of 80%.

Oxygen uptake. Oxygen uptake of the heart was measured in the apparatus described by Randle, Newsholme & Garland (1964). The medium issuing from the heart in the 5–60min. period of perfusion was collected under heptane. The oxygen concentration in the medium before and after passage through the heart was then measured with an oxygen electrode calibrated with perfusion medium saturated at 37° with O_2+CO_2 (95:5).

Heart glycogen. Glycogen in a sample of frozen heart powder was assayed as described by Randle *et al.* (1964) by using the extraction procedure of Walaas & Walaas (1950).

Expression of results. Glyceride concentration (as μmoles of glyceride glycerol), phospholipid concentration (as μg.atoms of phospholipid phosphorus), glycogen concentration (as μmoles of glycogen glucose) and oxygen uptake (as μmoles) were expressed in terms of dry weight of muscle. The dry weight of muscle was ascertained by weighing a sample of muscle powder before and after drying to constant weight in a vacuum desiccator containing P_2O_5. Ventricular muscle only was taken for analysis in the heart experiments because of the adherence of pericardial adipose tissue to the atria. Measurements showed that the weight of ventricular muscle was 75·5±0·5% (mean±S.E.M.) of the weight of the whole heart.

RESULTS

Effect of diabetes, hypophysectomy and starvation on glyceride and phospholipid concentrations in rat heart and gastrocnemius. Table 1 shows the concentrations of mono-, di- and tri-glycerides and phospholipids in rat heart and gastrocnemius and effects of diabetes and starvation. In the normal heart the concentration of phospholipids (approx. 170 μg.atoms of phospholipid phosphorus/g. dry wt. of ventricle) was much greater than that of glycerides (approx. 14 μmoles/g. dry wt. of ventricle). In gastrocnemius muscle the concentration of phospholipid was much lower (approx. 60 μg.atoms of phospholipid phosphorus/g. dry wt. of muscle) but the concentration of glycerides was similar to that in the heart. In both tissues the concentrations of mono- and di-glycerides were very small (< 10%) in relation to that of triglyceride.

Alloxan-diabetes increased the concentration of glycerides in both heart and gastrocnemius muscles. It was shown in further experiments that the increase in glyceride concentration in the heart in diabetes is dependent upon growth hormone and cortisol and not dependent upon dietary lipid. Thus the increase in glyceride concentration in the heart was seen in diabetic rats fed on a fat-free diet; but it was not seen in hypophysectomized-diabetic rats unless they were injected with growth hormone and cortisol. This change in total glyceride in diabetes was due to an increase in triglyceride concentration. Any changes which may have occurred in the concentrations of mono- and di-glycerides were too small for conclusions to be drawn. The concentration of total glyceride in the heart was also increased after 48hr. and 72hr. of starvation but returned to normal after 96hr. No significant change in phospholipid concentration accompanied these changes in total glyceride.

Hypophysectomy led to a significant fall in the concentration of phospholipid and induction of alloxan-diabetes in the hypophysectomized rat heart to a significant increase in phospholipid

Table 1. *Effects of diabetes, hypophysectomy and starvation on glyceride and phospholipid concentrations in rat heart and gastrocnemius*

Hearts and gastrocnemius muscles were washed free of blood by perfusion (see the Procedure and Methods section). In treatment with growth hormone the dose by injection was 0·1 mg./100 g. at 24, 12 and 4 hr. beforehand and with cortisol the dose by injection was 2·5 mg./100 g. at 24 hr. and 1·25 mg./100 g. at 12 and 4 hr. beforehand. Results are given as mean values for the numbers of animals given in parentheses in the phospholipid column, with standard errors where analyses were made on individual muscle samples. $*P<0·001$; $**P<0·02$; $***P<0·05$; for other differences $P>0·05$ (versus appropriate control).

Rat	Tissue	Concn. (μmoles of glyceride glycerol or μg.atoms of phospholipid P/g. dry wt. of muscle) Mono-glyceride	Di-glyceride	Tri-glyceride	Total glyceride	Phospholipid	
Normal	Heart	0·6	0·1	12·6±1·3	13·3	171±8	(6)
Alloxan-diabetic		0·7	0·6	26·3±1·7*	27·6	152±8	(6)
Normal (fat-free diet)		0·9	0·1	13·1±1·2	14·1	175±8	(6)
Alloxan-diabetic (fat-free diet)		0·8	0·1	24·1±1·0*	25·0	177±8	(6)
Hypophysectomized		0·7	0·3	9·6±1·3	10·6	133±4**	(6)
Hypophysectomized-diabetic		0·5	0·1	9·7±0·6	10·3	148±3***	(12)
Hypophysectomized-diabetic, treated with growth hormone and cortisol		0·6	0·1	21·9±1·1*	22·6	152±4	(10)
Normal	Gastrocnemius	0·4	0·1	12·0±1·5	12·5	64±2	(8)
Alloxan-diabetic		0·5	0·4	26·0±2·7*	26·9	63±1	(8)
Normal	Heart	—	—	—	15·6±1·1	158±5	(10)
Normal, starved 48 hr.		—	—	—	21·1±1·8**	157±7	(10)
Normal, starved 72 hr.		—	—	—	22·6±3·3***	148±5	(6)
Normal, starved 96 hr.		—	—	—	16·6±0·8	151±3	(6)

Table 2. *Change in glyceride and phospholipid concentrations during perfusion of rat heart*

Hearts were perfused for the time stated with bicarbonate-buffered medium lacking substrate or containing glucose (1 mg./ml.) and insulin (0·1 unit/ml.) as stated. The number of hearts perfused is given in parentheses. Results are given as mean ± S.E.M. except where hearts or media were pooled for analyses, where the mean only is given. $*P<0·001$ vs. 5 min. value. For other differences $P>0·05$.

Rat and perfusion	Time of perfusion (min.)	Concn. (μmoles of glyceride glycerol or μg.atoms of phospholipid P/g. dry wt. of ventricle) Glyceride	Phospholipid
Normal (no substrate) (8)	5	16·7±0·7	166±5
	60	8·2±0·6*	154±4
Alloxan-diabetic (no substrate) (6)	5	37·2±1·6	157±4
	60	21·7±1·5*	148±6
Alloxan-diabetic (glucose and insulin) (5)	5	29·1	157
	60	16·4	152

concentration. This change in phospholipid concentration in the hypophysectomized-diabetic rat was not associated with an increase in triglyceride concentration. The concentrations of triglyceride and total glyceride were somewhat lower in the hypophysectomized rat heart than in the normal but the statistical significance of this change was not established.

Changes in glyceride and phospholipid concentrations during perfusion of rat heart. These results are shown in Table 2. There was a significant diminution of glyceride concentration when hearts from normal or diabetic rats were perfused without substrate for 60 min. The loss of glyceride was approx. 50% and it was greater in the diabetic than in the normal. Similarly when hearts from diabetic rats were perfused for 60 min. with medium containing glucose and insulin the concentration of

glyceride fell by about 50%. No significant change in phospholipid concentration during perfusion was detected.

DISCUSSION

Muscle glycerides and phospholipids as sources of fatty acids for oxidation in the perfused rat heart. The concentrations of glycerides and phospholipids found in heart and gastrocnemius muscles of normal rats in the present study agree well with those obtained by Shipp, Thomas & Crevasse (1964) and Masoro & Spitzer (1966). Before discussing the extent to which these two classes of lipid may furnish fatty acids for respiration in the perfused rat heart and effects of endocrine manipulations on their concentrations, it may be useful to summarize evidence which appears to show that the glycerides which have been estimated may be present in muscle cells and not in adipose tissue cells in the preparations.

Sections of heart and gastrocnemius muscles, cut with a freezing microtome and stained for lipid with Sudan III or Sudan Black, were examined by light-microscopy. No adipose tissue cells were seen amongst the muscle fibres of heart or gastrocnemius and none was seen around the periphery of sections of gastrocnemius. In sections of ventricular muscle adipose tissue cells were seen very occasionally (< six per section) in connexion with the pericardium. Orth & Morgan (1962) have observed lipid droplets in muscle cells in sections of heart muscle by light-microscopy (after staining by Sudan IV) and by electron microscopy. We have been able to confirm their findings in sections of ventricular muscle examined by light-microscopy after staining by Sudan III and in unstained sections examined with the phase-contrast microscope. Lipid droplets which were, however, less numerous than in heart muscle could also be detected in the gastrocnemius by the same methods. Measurements of ^{14}C incorporation into glycerides of rat heart after perfusion with [U-^{14}C]glucose showed that ^{14}C was present in glyceride glycerol but not in glyceride fatty acids. This pattern of labelling is different from that found in adipose tissue, where, as many workers have shown, a substantial proportion of the ^{14}C incorporated into glyceride was in fatty acid (Denton & Randle, 1967). Measurements of glycerol output have shown that the rate of lipolysis in the perfused heart is at least as great per unit weight as that of epididymal adipose tissue (Garland & Randle, 1964; Denton & Randle, 1967). It seems unlikely that such a high rate of lipolysis in the perfused heart could be accounted for solely by lipolysis in adipose tissue cells associated with the preparation.

In the normal heart and the diabetic heart perfused without substrate, disappearance of glycerides from the muscle has been demonstrated. Loss of glyceride from the diabetic heart could also be demonstrated after perfusion with glucose and insulin. We conclude that breakdown of glycerides furnishes fatty acids for oxidation under these conditions. No significant diminution of concentration of phospholipid was detected. It should be pointed out, however, that loss of phospholipid equivalent to that of glycerides would be difficult to detect on account of the much higher concentration of phospholipid in the heart. The question whether net breakdown of phospholipid may contribute some fatty acids for oxidation remains open (see also following paragraph). Shipp *et al.* (1964) have also measured the concentrations of glycerides and phospholipids before and after perfusion of the heart without substrate. They have concluded that fatty acids for respiration were derived from the breakdown of phospholipid and were unable to detect a significant fall in the concentration of glycerides. It is questionable, however, whether this conclusion was justifiable because of radioactive measurements made by Shipp *et al.* (1964) in the same investigation. In these experiments [^{14}C]palmitate was introduced into glyceride and phospholipid fractions *in vivo* before removal of the heart for perfusion. After perfusion it was found that ^{14}C-labelled fatty acids had disappeared from the glyceride fraction and $^{14}CO_2$ had been produced; there was no loss of radioactive fatty acids from phospholipid. These radioactive measurements are in keeping with our conclusion that breakdown of glyceride furnishes fatty acids for respiration. The failure of Shipp *et al.* (1964) to demonstrate a statistically significant fall in glyceride concentration during perfusion could be explained by the restricted number of observations.

Further evidence bearing on this point has been obtained by considering the extent to which oxidation of glyceride fatty acids may account for the balance of oxygen consumption in the heart (i.e. the oxygen which is not accounted for by the metabolism of glucose or glycogen). These data are given in Table 3. Three independent methods of calculation have been used and these are detailed at the head of the Table. Method (A) is based on measurements of oxygen consumption, lipolysis rate and re-esterification rate; method (B) on measurements of oxygen consumption and net glyceride loss; method (C) on measurements of oxygen consumption, glucose uptake, glycogen concentration, lactate output and pyruvate output. In methods (A) and (B) the possible contribution of intracellular fatty acid has been ignored because of its low concentration ($< 0{\cdot}5\,\mu$mole/g. dry wt. of ventricle; Garland & Randle, 1964). The possibility that some fatty acids may be lost to the perfusion medium

Table 3. *Contributions of carbohydrate and lipid to oxygen consumption in the perfused rat heart*

Methods of calculation used: (A) Rate of oxidation of glyceride fatty acid (μmoles of palmitate/g. dry wt. of ventricle/hr.)$=L-E$, where L is glycerol output in μg.atoms of glycerol carbon/g. dry wt. of ventricle and E is rate of re-esterification in μg.atoms of glycerol carbon/g. dry wt. of ventricle. Residual fuel (calculated as μmoles of glucose/g. dry wt. of ventricle/hr.)$=[O-23(L-E)]/6$, where O is oxygen consumption in μmoles of oxygen/g. dry wt. of ventricle/hr. The data are taken from Denton & Randle (1967). Period of perfusion was 15 min. (B) Rate of oxidation of glyceride fatty acid (μmoles of palmitate/g. dry wt. of ventricle/hr.)$=3(T_1-T_2)$, where T_1 is initial glyceride concentration and T_2 glyceride concentration after perfusion, in μmoles of triglyceride/g. Residual fuel is calculated as in method (A). The data are taken from Table 2. Period of perfusion was 55 min. (C) Rate of oxidation of glucose or glycogen glucose or both (μmoles of glucose/g. dry wt. of ventricle/hr.)$=A+B-2(C+D)$, where A is glucose uptake, B is change in glycogen concentration, C is lactate output and D is pyruvate output, expressed as μmoles/g. dry wt. of ventricle/hr. Residual fuel is calculated as μmoles of palmitate/g. dry wt. of ventricle/hr. $=\{O-6[A+B-2(C+D)]\}/23$. The data are taken from Randle *et al.* (1966). Period of perfusion was 15 min. The concentrations of glucose and insulin, where present, were 1 mg./ml. and 0·1 unit/ml.

Rat and perfusion	Method of calculation	Oxygen consumption (μmoles of O_2/g. dry wt. of ventricle/hr.)	Rate of oxidation (μmoles of glucose or palmitate/g. dry wt. of ventricle/hr.)		
			Glucose and/or glycogen glucose	Glyceride fatty acid	Residual fuel calc. as glucose or palmitate
Diabetic (glucose+insulin)	(A)	2000	—	49·9	142 (glucose)
	(B)	1850	—	41·6	148 (glucose)
	(C)	2000	173	—	41·6 (palmitate)
Normal (glucose+insulin)	(A)	1800	—	8·5	267 (glucose)
	(C)	1800	247	—	14 (palmitate)
Normal (no substrate)	(B)	1360	—	27·8	120 (glucose)
	(C)	1800	120	—	47 (palmitate)

would appear unlikely since no loss was detected in hearts perfused with albumin to trap fatty acids which might be released (Garland & Randle, 1964). The period of perfusion with method (B) was longer than that used in methods (A) and (C), to obtain a measurable fall in glyceride concentration.

In the diabetic heart perfused with glucose and insulin the rates of glyceride fatty acid oxidation calculated by methods (A) and (B) were comparable. They were, moreover, close to those predicted on the basis of the balance of oxygen consumption in method (C). In this instance the rate of glyceride fatty acid oxidation was sufficient to account for the balance of oxygen consumption. The results obtained with methods (A) and (C) suggest a similar conclusion for the normal heart perfused with glucose and insulin. In the normal heart perfused without substrate the oxidation of glyceride fatty acid may not account for the balance of oxygen consumption. In particular, it was found with method (B) that 120 μmoles of glycogen glucose/g. dry wt. of ventricle/hr. would need to be oxidized to balance the oxygen uptake. Measurements showed that the loss of glycogen during perfusion amounted to only 82·5 μmoles of glycogen glucose. It is thus possible that phospholipid contributed some fatty acid to respiration under these conditions. When normal hearts were perfused without substrate for 55 min. the oxygen consumption fell sharply after about 40 min. and cardiac contraction was almost completely arrested. Since no significant fall in phospholipid concentration was detected after 55 min. of perfusion, it seems unlikely that phospholipid fatty acids are readily available for oxidation. There were, moreover, measurable quantities of glyceride in the heart even after 55 min. of perfusion. In normal and diabetic hearts perfused for 55 min. with glucose and insulin, cardiac contraction and oxygen consumption were well maintained for the whole period of perfusion.

The change in glyceride concentration observed during starvation provides some evidence for the utilization of muscle glyceride in the heart *in vivo*. In particular, the fall in glyceride concentration found between 72 and 96 hr. of starvation would indicate utilization of glyceride stores by the heart. Carlson, Fröberg & Nye (1966) have measured the concentration of triglycerides and phospholipids in heart and gastrocnemius muscles of starved rats in which the mobilization of free fatty acids from adipose tissue was inhibited by injection with nicotinic acid. After the injection of nicotinic acid

the concentration of triglyceride fell but there was no significant change in phospholipid concentration. These findings *in vivo* are consistent with our conclusions from studies *in vitro* that it is the triglyceride fraction and not the phospholipid fraction which is readily utilized by the rat heart muscle.

Effects of starvation, diabetes, hypophysectomy and treatment with growth hormone and cortisol on muscle glyceride and phospholipid concentrations. The conditions in which an increase in triglyceride and total glyceride concentrations have been shown in heart and gastrocnemius muscles in the present investigation are conditions in which the mobilization of free fatty acids from adipose tissue glycerides is augmented. Thus glycerides were found to be increased in muscle by diabetes and starvation and the increase was dependent upon the availability of growth hormone and corticosteroid but not upon the availability of dietary lipid. Similarly the mobilization of free fatty acids from adipose tissue in diabetes may depend upon the availability of growth hormone and corticosteroid (Fain, Kovacev & Scow, 1965; Garland & Randle, 1964). The sequence of events in diabetes may thus be mobilization of free fatty acids from adipose tissue with an increase in the plasma concentration of free fatty acids and in the rate of glyceride synthesis in muscle. The demonstration of increased rates of glyceride synthesis in the diabetic heart *in vitro* could be consistent with this proposal (Denton & Randle, 1967). Moreover, Carlson, Liljedahl & Wirsén (1965) find in the dog that mobilization of free fatty acids induced by noradrenaline infusion leads to an increase in number of Sudan-positive droplets in histological sections of the myocardium. The alternative possibility of uptake of plasma triglyceride into the muscle glyceride fraction seems less likely in view of the failure to detect uptake of triglyceride by the heart (Kreisberg, 1966).

Orth & Morgan (1962) have observed an increase in the number of lipid droplets in the heart in diabetes in sections examined by light- and electron-microscopy. The histological appearance of sections of normal and diabetic hearts which we have examined by techniques described in the preceding section is consistent with their observations. Orth & Morgan (1962) also observed an increase in the number of lipid droplets in the normal heart after a short period of anaerobic perfusion. This might suggest that triglyceride is normally present as submicroscopic droplets in the heart of the normal rat. Alloxan-diabetes in the rat may thus lead both to an increase in the concentration of triglyceride in cardiac muscle and to an increase in the size of triglyceride droplets. It would be of interest to know whether either of these changes in muscle triglyceride is responsible for the increased rate of lipolysis which develops in the diabetic heart.

We thank Dr J. M. Boss and Mr B. A. Bees of the Department of Physiology, University of Bristol, for providing facilities and assistance in the histological examination of muscle, Dr R. O. Law for assistance with hind-limb perfusion and Miss J. E. Sanders and Mrs I. M. Harris for skilled technical assistance. The cost of these investigations was defrayed in part by grants from the British Diabetic Association, the Medical Research Council and The Royal Society.

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