Measurement of Concentrations of Metabolites in Adipose Tissue and Effects of Insulin, Alloxan-Diabetes and Adrenaline

By R. M. DENTON, R. E. YORKE* AND P. J. RANDLE

Department of Biochemistry, University of Bristol

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1. Methods are described for the extraction and assay of ATP, ADP, AMP, glucose 6-phosphate, L-glycerol 3-phosphate and citrate in rat epididymal adipose tissue incubated *in vitro* for 1 hr. At this time of incubation rates of glucose uptake and outputs of glycerol, free fatty acids, lactate and pyruvate were shown to be constant. 2. In fat pads incubated in medium containing glucose (3mg./ml.) and albumin (20mg./ml.) the concentrations (in μ moles/g. wet wt.) were: ATP, 70; ADP, 36; AMP, 9.0; glucose 6-phosphate, 3.0; L-glycerol 3-phosphate, 3.3; citrate, 8.1. 3. The volume of intracellular water calculated from ($[^3\text{H}]$ water space - $[^{14}\text{C}]$ -sorbitol space), ($[^{14}\text{C}]$ urea space - inulin space) and (weight loss on drying - $[^{14}\text{C}]$ -sorbitol space) was 1.4 ml./100 g. wet wt. of tissue. The intracellular volume was not changed by insulin, alloxan-diabetes or adrenaline. 4. When compared in terms of μ moles/ml. of intracellular water the concentration of ATP in adipose tissue was less than in heart and diaphragm muscles. The concentrations of ADP and AMP were greater both in absolute terms and relative to ATP. Insulin, alloxan-diabetes and adrenaline had no significant effects on the concentrations of the adenine nucleotides in adipose tissue. 5. The concentration of glucose 6-phosphate was increased by insulin and lowered by alloxan-diabetes and adrenaline. The concentration of L-glycerol 3-phosphate was increased by insulin, unchanged by alloxan-diabetes and lowered by adrenaline. The concentration of citrate was increased by adrenaline and alloxan-diabetes and unchanged by insulin. 6. The effect of glucose concentration in the medium on rates of glucose uptake in adipose tissue from normal rats and alloxan-diabetic rats was investigated. The K_m of glucose uptake was 29-44 mg./100 ml. and the V_{max} was 0.77 mg./g. wet wt. of tissue/hr. Insulin increased the V_{max} and alloxan-diabetes diminished it, but neither agent significantly altered the K_m . 7. The significance of these results in relation to control of metabolism of adipose tissue is discussed.

Measurements of rates of flow through metabolic pathways coupled with assay of the steady-state concentrations of intermediates have yielded valuable information about enzymes of regulatory significance in a number of tissues (e.g. muscle, liver and brain). Although methods have recently been described for calculating rates of flow in pathways of glucose and lipid metabolism in rat epididymal adipose tissue (Flatt & Ball, 1964; Landau, Bartsch, Katz & Wood, 1964), measurements of steady-state concentrations of intermediates are lacking. In the present paper, methods are described for the extraction and assay of adenine nucleotides, glucose 6-phosphate, L-glycerol 3-phosphate and citrate in rat epididymal adipose tissue and the effects of

insulin, alloxan-diabetes and adrenaline on their concentrations. A preliminary account of some of these findings has been given by Denton & Randle (1965).

MATERIALS

Rats. Epididymal fat pads were obtained from male albino Wistar rats (100-250 g.) fed on a stock laboratory diet (Short & Parkes, 1949). Unless indicated otherwise the animals were not deprived of food before use. Alloxan-diabetes was induced in rats by the intravenous administration of alloxan (60 mg./kg. body wt.) under ether anaesthesia. The animals were used 48 hr. later, when blood glucose concentrations were in excess of 300 mg./100 ml.

Chemicals. Hexose phosphates, ATP, ADP, 3',5'-(cyclic)-AMP, phosphoenolpyruvate and 1,3-diphosphoglycerate (tricyclohexylammonium salts), NAD, NADH₂, NADP, tris (Sigma 121) and triethanolamine hydrochloride were obtained from the Sigma Chemical Co. (through

* Present address: St Paul's School, West Kensington, London, W.14.

George T. Gurr Ltd., London, S.W. 6) or C. F. Boehringer und Soehne G.m.b.H. (through Courtin and Warner Ltd., Lewes, Sussex); alloxan was from Eastman-Kodak Co., Rochester, N.Y., U.S.A.; adrenaline was from British Drug Houses Ltd., Poole, Dorset. Crystalline insulin was given by Boots Pure Drug Co. Ltd., Nottingham, or Burroughs Wellcome, Beckenham, Kent, dissolved in 3M-NaCl to yield a stock solution of 20 units/ml., which was diluted to the required concentration with incubation medium. Bovine plasma albumin (fraction V) from the Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, was purified as described by Garland, Newsholme & Randle (1964) including passage through powdered cellulose. The resulting preparation was free of both insulin by immunoassay (Hales & Randle, 1963) and citrate by assay with citrate lyase containing oxaloacetate decarboxylase (Dagley, 1963). Citrate was present in all albumin preparations before purification and unless removed is a serious source of error in citrate assays in adipose tissue because of the high ratio of extracellular to intracellular water. Inulin was obtained from T. Kerfoot and Co. Ltd., Vale of Bardsey, Lancs.; D-[14 C]-sorbitol, [3 H]water and [14 C]urea were from The Radiochemical Centre, Amersham, Bucks.

Enzymes. Glucose 6-phosphate dehydrogenase, glucose 6-phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, pyruvate kinase, lactate dehydrogenase, myokinase, glycerol kinase, glycerol phosphate dehydrogenase and hexokinase were from Boehringer.

Preparation of citrate lyase containing oxaloacetate decarboxylase. Citrate lyase and oxaloacetate decarboxylase were purified together from a culture of *Aerobacter aerogenes* grown under the conditions given by Dagley (1963). The *Aerobacter* were harvested, suspended in 3 vol. of 30 mM-potassium phosphate buffer, pH 7.4, containing MgSO_4 (1.6 mM), and treated ultrasonically at 20 kcyc./sec. for 5 min. After centrifugation at 38000g for 1 hr. the supernatant was treated with 0.2 vol. of alumina C₇ gel (21 mg./ml.). The two enzymes were then precipitated together from solution by adding solid $(\text{NH}_4)_2\text{SO}_4$ to give 45% saturation. The precipitate was dissolved in 50 mM-potassium phosphate buffer, pH 7.4, containing MgSO_4 (1.6 mM).

The NADH₂-oxidase activity of the preparation was less than 3% of the citrate-lyase activity. The preparation was stored at -15° . Activity after preparation was 0.6 unit/mg. This fell to about 0.1 unit/mg. during 6 months of storage at -15° .

Media. Fat pads were incubated in a bicarbonate-buffered medium (Krebs & Henseleit, 1932) gassed with $\text{O}_2 + \text{CO}_2$ (95:5). The concentrations of the various additions are given in the text or Tables.

When albumin was added to the medium, the bicarbonate buffer containing the albumin but no other additions was dialysed at 0° overnight against 20 vol. of bicarbonate buffer in Visking seamless cellulose tubing treated to remove plasticizing substances as described by Garland *et al.* (1964).

PROCEDURE

Incubation of fat pads. Rats were killed by decapitation and the fat pads rapidly excised, rinsed briefly in the medium for subsequent incubation, blotted lightly and transferred to stoppered flasks gassed with $\text{O}_2 + \text{CO}_2$ (95:5) and incubated with shaking at 37° . The pads were weighed before

incubation when glucose uptakes were measured and after incubation when substances were assayed in the pads. Pairs of fat pads from individual animals were distributed equally between control and experimental groups except (a) in experiments in which glucose uptake was measured at different medium glucose concentrations, when they were distributed at random between media of different glucose concentrations within a single experimental group, and (b) in experiments in which pads from normal and diabetic animals were compared. The volume of incubation medium was 3 ml./fat pad of 500–700 mg. in experiments in which metabolic intermediates were assayed in fat pads. In glucose-uptake experiments the volume of medium was adjusted so that no more than 10–20% of the glucose in the medium was utilized during the period of incubation. Other details of incubation in individual experiments are given in the text, Tables or Figures.

Extraction of fat pads. The procedure at the end of incubation depended on the measurements to be made. For the assay of metabolic intermediates the pads were lightly and rapidly blotted and immediately frozen between two blocks of aluminium (7.5 cm. diam. $\times$ 3 cm. thick) previously cooled in liquid N₂ (Wollenberger, Ristau & Schoffa, 1960). The frozen pads were weighed and three to six pads within single experimental groups were pooled and extracted with 5 ml. of 5% (v/v) HClO_4 and 4 ml. of diethyl ether in a hand-operated Potter-Elvehjem homogenizer at 0° . The contents were transferred to a 15 ml. centrifuge tube with one rinse of 2 ml. of 5% HClO_4 and centrifuged for 5 min. in an MSE Minor centrifuge. The ether layer was removed by suction, the aqueous layer was carefully decanted (from fat plug and precipitated protein) into a small beaker and 2 ml. of 0.1 M-triethanolamine hydrochloride and 3 ml. of water were added. The extract was then brought to pH 7 (glass electrode) with successive use of 50% (w/v) KOH (to pH 2), 5% KOH (to pH 5) and 0.5% KOH (to pH 7). The neutralized extract was transferred to a 50 ml. centrifuge tube and KClO_4 separated after cooling to 0° . The supernatant was freeze-dried and the solids were taken up in 2 ml. of water, transferred to a 15 ml. centrifuge tube and KClO_4 was again separated after cooling to 0° . Metabolites were assayed in this extract (pH 7.0–7.4) either immediately or after storage at -15° . To assess recoveries, citrate, glucose 6-phosphate and ATP were added at the point of extraction of a sample of pooled fat pads with HClO_4 -ether and taken through the entire procedure.

For the assay of glucose single pads were rapidly frozen and then extracted with 3 ml. of 5% HClO_4 and 2.5 ml. of diethyl ether (as above) and the aqueous layer after centrifugation was separated and neutralized with 0.3 ml. of saturated KHCO_3 .

For the assay of [3 H]water, [14 C]urea, [14 C]sorbitol or inulin the pads were blended with 3 ml. of hot water in a hand-operated Potter-Elvehjem homogenizer, and the mixture was heated at 80° for 10 min., cooled and deproteinized with $\text{Zn}(\text{OH})_2$ (Somogyi, 1945). The aqueous extract was separated either by centrifugation or by filtration through Whatman no. 1 filter paper.

For the assay of Na⁺ and K⁺ the pads (in pairs) were dried at 80° for 60 min., extracted (three times) with 5 ml. of ether, dissolved with warming in 0.5 ml. of conc. HNO_3 and diluted with water to 8 ml. for the assay of K⁺. For the assay of Na⁺ further dilution (20-fold) was appropriate. Recoveries of added Na⁺ and K⁺ were in excess of 90%.

METHODS

Metabolic intermediates. Assays were made by the production or disappearance of NADH_2 or NADPH_2 as appropriate measured either by changes in extinction or in fluorescence. ATP, ADP and AMP were assayed spectrophotometrically in an Eppendorf photometer fitted with a mercury-vapour lamp and a filter transmitting at $366\text{ m}\mu$; the assays were performed in a final volume of 2 ml. in a 4 cm. light-path cell. Glucose 6-phosphate, glycerol phosphate and citrate were assayed fluorimetrically in an Eppendorf fluorimeter fitted with a mercury-vapour lamp, a primary filter transmitting at $366\text{ m}\mu$ and a secondary filter at $420\text{--}3000\text{ m}\mu$. The assays were performed in a total volume of 3 ml.; each assay was calibrated by the addition of freshly prepared solution of NADH_2 , standardized by extinction at 340 or $366\text{ m}\mu$ in a spectrophotometer.

Glucose 6-phosphate, ATP, ADP, AMP and glycerol phosphate were assayed by the methods described by Newsholme & Randle (1964), Garland *et al.* (1964) and Garland & Randle (1964). Citrate was assayed by the method of Dagley (1963).

Inulin. This was assayed spectrophotometrically in fat-pad extracts or incubation media deproteinized with $\text{Zn}(\text{OH})_2$ (Somogyi, 1945) by the method of Roe, Epstein & Goldstein (1949). In some experiments the inulin was hydrolysed at 100° with $0.5\text{ N-H}_2\text{SO}_4$ ($0.5\text{ ml. of } 1.5\text{ N-H}_2\text{SO}_4$ plus 1 ml. of extract) and the fructose formed assayed enzymically with hexokinase, phosphoglucose isomerase and glucose 6-phosphate dehydrogenase by the method of Klotzsch & Bergmeyer (1963). With both methods assays were performed with reference to standard inulin solutions.

Total water. This was assayed by the loss of weight on drying frozen pads *in vacuo* over P_2O_5 to constant weight.

$[^{14}\text{C}]\text{sorbitol}$, $[^{14}\text{C}]\text{urea}$ and $[^3\text{H}]\text{water}$. These were assayed in fat-pad extracts or incubation media, deproteinized with $\text{Zn}(\text{OH})_2$ (Somogyi, 1945), in a Nuclear-Chicago liquid-scintillation system with a scintillator that will accept up to 20% (v/v) of aqueous extracts [naphthalene (100 g./l.), 2,5-diphenyloxazole (7 g./l.) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.03 g./l.) in 1,4-dioxan (Butler, 1961)]. Simultaneous assays of $[^3\text{H}]\text{water}$ and $[^{14}\text{C}]\text{sorbitol}$ were made as described by Hendler (1963). The amount of radioactive substances used was such that the final volume of aqueous extract added to $15\text{ ml. of scintillator}$ was approx. 0.2 ml. Corrections for quenching were small and always less than 5%.

Glycerol, pyruvate and lactate. Glycerol, pyruvate and lactate were assayed in incubation media deproteinized with 5% HClO_4 and neutralized with saturated KHCO_3 . Glycerol was estimated by the method of Garland & Randle (1962) and lactate and pyruvate were determined as described by Garland *et al.* (1964).

Free fatty acids. Free fatty acids in incubation media were extracted by the method of Dole & Meinertz (1960). A sample of the upper phase (containing $50\text{--}500\text{ m}\mu\text{moles}$ of free fatty acid and not washed with blank lower phase) was evaporated to dryness at 37° in a stream of N_2 . The residue was dissolved in $5\text{ ml. of chloroform}$ and the free fatty acids were assayed by the method of Duncombe (1963).

Glucose. Glucose was assayed by the glucose-oxidase method (Huggett & Nixon, 1957) in $10\text{--}100\text{ }\mu\text{l.}$ samples of incubation media without deproteinization or in extracts of fat pads deproteinized with $\text{Zn}(\text{OH})_2$.

DNA. DNA content was measured by the method of Burton (1956) on the combined fat plug and precipitate formed by HClO_4 -ether extraction of pads. The fat plug and precipitate were defatted by treatment with 5 ml. of acetone (once) and $5\text{ ml. of diethyl ether}$ (twice or three times). The recovery of DNA added at the stage of extraction was over 85%.

Tissue glycogen. Glycogen was assayed as described for heart by Randle, Newsholme & Garland (1964) by using the extraction procedure of Walaas & Walaas (1950).

Sodium and potassium. Sodium and potassium were estimated in tissue extracts and in incubation media (diluted to give concentrations comparable with those in tissue extracts) with an EEL flame photometer, standard solutions of NaCl and KCl being used. NaCl was added to KCl standards to a concentration equivalent to those found in tissue extracts.

Calculations. Glucose uptake was calculated from the disappearance of glucose from medium as $\text{mg. of glucose/g. wet wt. or mg. of pad DNA/hr. of incubation}$. The concentrations of metabolic intermediates were calculated as $\text{m}\mu\text{moles/g. wet wt. of pad or mg. of pad DNA}$. Total water, $[^3\text{H}]\text{water}$, $[^{14}\text{C}]\text{urea}$, $[^{14}\text{C}]\text{sorbitol}$, inulin and glucose spaces were calculated as described by Randle & Smith (1958).

RESULTS

Effects of time of incubation on the uptake of glucose and outputs of glycerol, free fatty acids, lactate and pyruvate by rat epididymal fat pad in vitro

The experiments described in this and the following section were carried out to define conditions for the assay of metabolic intermediates. Because the extraction and assay of intermediates is a lengthy process requiring a considerable number of animals it was impracticable to measure their concentrations at a number of periods of incubation. In view of this it seemed important to attempt to define steady-state conditions by using parameters of metabolism that are more easily determined. For this purpose we have measured uptake of glucose, outputs of glycerol, free fatty acids, lactate and pyruvate and the lactate/pyruvate concentration ratio in the medium at 30 or 60 min. intervals during a total period of incubation of 3 or 4 hr. These results are given in Tables 1 and 2.

Hourly rates of uptake of glucose by pads from normal rats incubated with or without insulin or from alloxan-diabetic rats incubated with insulin showed no significant difference over a total period of incubation of 4 hr. The results, however, did suggest that the rate of uptake during the first hour might be different from that in subsequent hours (Table 1). Epididymal adipose tissue from normal rats incubated in medium containing insulin showed little variation in the rate of glycerol output during the 30–150 min. period of incubation though the output may have been somewhat higher in the first 30 min. The outputs of free fatty acids during the

Table 1. *Effect of the time of incubation on the glucose uptake by the rat epididymal fat pad in vitro*

Fat pads were incubated in 2 ml. of buffer (Expt. 1) or 3 ml. of buffer (Expts. 2 and 3) containing albumin (4 mg./ml.), penicillin (200 units/ml.), streptomycin (0.22 mg./ml.) and glucose (0.5 mg./ml.), and were transferred to fresh medium at the end of each hour. In Expt. 1 glucose uptakes are expressed per g. wet wt. of tissue and in Expts. 2 and 3 per mg. of pad DNA. The initial glucose concentration in the pad was determined in one member of each pair and the other incubated for the determination of glucose uptake. The glucose present in the pad was taken into account in computing glucose uptakes. Rats were starved for 16–18 hr. before use. Results are given as means $\pm$ S.E.M. of four observations.

Expt. no.	Rat	Insulin (milliunit/ml.)	Period ...	Glucose uptake (mg./mg. of pad DNA or g. wet wt. of tissue/hr.)			
				... 0–1 hr.	1–2 hr.	2–3 hr.	3–4 hr.
1	Normal	0		0.46 $\pm$ 0.08	0.28 $\pm$ 0.06	0.24 $\pm$ 0.06	0.23 $\pm$ 0.07
2	Normal	0.5		3.9 $\pm$ 0.48	4.6 $\pm$ 0.65	5.2 $\pm$ 0.58	5.0 $\pm$ 0.53
3	Alloxan-diabetic	0.5		1.3 $\pm$ 0.16	1.8 $\pm$ 0.13	1.9 $\pm$ 0.21	1.9 $\pm$ 0.15

Table 2. *Effect of the time of incubation of rat epididymal adipose tissue on the glycerol output, free fatty acid output, lactate output, pyruvate output and the lactate/pyruvate concentration ratio in the incubation medium*

A pool of six fat pads was incubated for 180 min. in 4 ml. of medium containing glucose (3 mg./ml.), insulin (10 milliunits/ml.) and albumin (20 mg./ml.). Samples of medium were taken for analysis (with regassing) at the times shown. Results are given as means $\pm$ S.E.M. for four observations in each group.

	Period ...	Output (μ moles/g./30 min.) or concentration ratio				
		... 0–30 min.	30–60 min.	60–90 min.	90–120 min.	120–180 min.
Glycerol output		0.3 $\pm$ 0.15	0.19 $\pm$ 0.04	0.16 $\pm$ 0.01	0.27 $\pm$ 0.03	0.15 $\pm$ 0.04
Free fatty acid output		0.7 $\pm$ 0.2	0.5 $\pm$ 0.45	0.0 $\pm$ 0.15	0.1 $\pm$ 0.4	–0.5 $\pm$ 0.2
Pyruvate output		0.15 $\pm$ 0.02	0.10 $\pm$ 0.02	0.03 $\pm$ 0.03	0.11 $\pm$ 0.01	–0.02 $\pm$ 0.01
Lactate output		1.0 $\pm$ 0.2	0.9 $\pm$ 0.4	1.1 $\pm$ 0.4	0.6 $\pm$ 0.2	0.6 $\pm$ 0.05
Lactate/pyruvate concn. ratio		7.8 $\pm$ 2.4	8.3 $\pm$ 1.9	10.8 $\pm$ 1.3	9.6 $\pm$ 1.2	13.8 $\pm$ 1.3

0–30 and 30–60 min. periods of incubation were similar. After 60 min. the rate declined and in the third hour there was a net uptake of fatty acid from the medium. Rates of lactate and pyruvate output were similar during the first two 30 min. periods of incubation but declined somewhat thereafter. The lactate/pyruvate concentration ratio, which was constant during the first 60 min. of incubation, subsequently tended to increase (Table 2.)

On the basis of these measurements the tissue appeared to show a steady state of metabolism at 60 min. of incubation and this period was chosen for the assay of metabolites. Previous studies by Winegrad & Renold (1958) and Ball, Martin & Cooper (1959) had indicated that the rate of conversion of [U- 14 C]glucose into carbon dioxide and lipid and the rate of net gas exchange in rat epididymal adipose tissue are also constant during this period of incubation.

Effect of the glucose concentration in the medium on the rate of uptake of glucose by rat epididymal fat pad in vitro

In these experiments the rate of uptake of glucose

by fat pads was measured over a period of 90 min. after 160 min. of preincubation at different concentrations of glucose in the medium ranging from 25 to 200 mg./100 ml. The longer period of incubation was used because these experiments formed part of an investigation into effects of steroids on adipose-tissue metabolism, which are of long latency. Since uptake of glucose by adipose tissue has been shown to be constant during the 60–240 min. period of incubation (see Table 1) it seemed reasonable to use this result to define a suitable concentration of glucose in the medium for incubating fat pads for the assay of metabolic intermediates.

These results are given in Figs. 1 and 2, which show the relationship between the rate of glucose uptake and the glucose concentration in the medium for fat pads from normal rats compared in the absence and in the presence of insulin (Fig. 1), and for fat pads from normal and diabetic rats compared in the presence of insulin (Fig. 2). In each case raising the glucose concentration in the medium led to an increase in the rate of glucose uptake, which approached a maximum at the highest glucose concentration. Plots of the reciprocal of glucose

Fig. 1. Uptake of glucose by the rat epididymal fat pad as a function of the glucose concentration: the effect of insulin. ●, Control; ○, insulin-treated. Each point is the mean glucose uptake $\pm$ s.e.m. for five fat pads. The pads were preincubated for 160 min., transferred to fresh medium and glucose uptakes measured during 90 min. of incubation. Media contained albumin (4 mg./ml.), penicillin (200 units/ml.) and streptomycin (0.22 mg./ml.). The concentration of insulin when added was 0.5 milliunit/ml. The initial glucose concentrations and volumes of media were 0.25 and 0.33 mg./ml. (in 2 ml.), 0.50 and 0.75 mg./ml. (in 1.5 ml.) and 1.0 and 2.0 mg./ml. (in 1 ml.) in the absence of insulin. In experiments with the hormone the volumes were (for the concentrations given) 9, 7, 5, 4, 3 and 2 ml. The glucose concentrations given in the plot were the average of initial and final concentrations. Rats were starved for 16–18 hr.

uptake rate ($1/v$) against the reciprocal of glucose concentration ($1/s$) were linear (not shown). The calculated intercept on the $1/v$ axis indicated that the $V_{\max}$ of glucose uptake was 0.77 mg./g. wet wt. of tissue/hr. for adipose tissue from normal rats incubated without insulin. Insulin increased the $V_{\max}$ to 1.94 mg./g. wet wt. of tissue/hr. In adipose tissue from alloxan-diabetic rats the $V_{\max}$ was decreased (in the presence of insulin) to about half the normal control. From the calculated intercept on the $1/s$ axis in reciprocal plots K_u was calculated (where K_u is the glucose concentration yielding half-maximal rates of glucose uptake). In a number of experiments K_u varied from 29 to 44 mg./100 ml. Insulin or alloxan-diabetes had no significant effect on the calculated values for K_u . Representative values for K_u and $V_{\max}$ are shown in Table 3. In comparing adipose tissue from normal and alloxan-diabetic rats (in Fig. 2 and Tables 1 and 3) results have been expressed in terms of tissue DNA and not wet weight. This is considered to provide a more

Fig. 2. Uptake of glucose by the rat epididymal fat pad as a function of the glucose concentration: the effect of alloxan-diabetes. ●, Alloxan-diabetic; ○, normal. The media contained insulin (0.5 milliunit/ml.). Other details are as given in Fig. 1.

accurate basis for comparison because of the altered lipid content of adipose tissue in alloxan-diabetes.

For the assay of metabolites in adipose tissue a glucose concentration of 3 mg./ml. was chosen. At this concentration the glucose uptake is near maximum. The concentration of insulin used (10 milliunits/ml.) was shown in a number of experiments to be much greater than the minimum concentration required for a maximum effect (0.1–1.0 milliunit/ml.).

Assay of metabolic intermediates in adipose tissue

Recovery of metabolic intermediates during extraction and assay. The following points of technique were found to be important in order to obtain good recoveries. Rapid freezing of the tissue with the tissue clamp of Wollenberger *et al.* (1960) was found to be desirable in experiments in which the glucose content of the tissue was measured. The recovery of glucose from the tissue was significantly greater from pads frozen in the tissue clamp than from pads frozen on solid carbon dioxide. This indicates that the metabolism of glucose in adipose tissue is arrested at a significantly greater rate when the clamp is used. Use of ether during initial extraction was found to be essential for clean separation of the phases. Addition of buffer and careful neutralization before freeze-drying also appears to be desirable.

Table 3. *Effect of insulin and alloxan-diabetes on the kinetic constants for glucose uptake in rat epididymal adipose tissue*

K_u is the concentration of glucose yielding half-maximal rates of glucose uptake. V_{max} is the maximum rate of glucose uptake. The values were calculated from the intercepts of the linear plot of (glucose uptake) $^{-1}$ against (glucose concentration) $^{-1}$. Conditions of incubation were as given in Figs. 1 and 2. V_{max} is given in terms of g. wet wt. of tissue in Expt. 1 and in terms of mg. of pad DNA in Expt. 2.

Expt. no.	Rat	Insulin (milliunit/ml.)	K_u (mg. of glucose/100 ml.)	V_{max} (mg. of glucose/mg. of pad DNA or g. wet wt. of tissue/hr.)
1	Normal	0	29	0.77
	Normal	0.5	32	1.94
2	Normal	0.5	43	13.69
	Diabetic	0.5	44	7.41

Table 4. *Recoveries of citrate and glucose 6-phosphate added to epididymal fat pads before the extraction of metabolites*

Fat pads from 12 rats were frozen with the tissue clamp of Wollenberger *et al.* (1960), broken into small pieces and thoroughly mixed. Samples (approx. 2g.) were extracted with or without the addition of citrate or glucose 6-phosphate in the amounts shown. Other details are given in the Methods section. The quantity in the first column is the mean of three assays.

Metabolite	Quantity of metabolite (μ moles)			Recovery (%)
	Amount in pad before additions	Added to pad	Total measured in pad	
Citrate	17	10	28	110
		20	36	95
		30	47	100
		40	50	83
		50	65	94
Glucose 6-phosphate	14.6	6.1	20.1	90
		12.2	26.6	98
		18.3	31.1	90
		24.4	37.1	92
		30.5	40.6	86

The results of detailed recovery experiments for citrate and glucose 6-phosphate added at the time of extraction are given in Table 4. The quantities of glucose 6-phosphate and citrate added varied from 50 to 300% of that already present in the tissue. The recoveries were in the range 83–110%. In further experiments (not shown) the recovery of ATP was in excess of 90%.

Concentrations of adenine nucleotides in rat epididymal fat pad. The results of these experiments are given in Table 5. The concentrations of adenine nucleotides were measured in pads from normal and diabetic rats incubated for 1 hr. in medium containing glucose (3mg./ml.). The effects of albumin, insulin and adrenaline were investigated with pads from normal rats. The concentrations were calculated per g. wet wt. of tissue and per mg. of tissue DNA. Neither alloxan-diabetes nor additions of albumin, insulin or adrenaline *in vitro* had any

significant effect on the concentrations of adenine nucleotides. The mean concentrations in those experiments in which all three adenine nucleotides were assayed (Expts. 3–6) were: ATP, 65 μ moles/g. wet wt. of tissue or 356 μ moles/mg. of tissue DNA; ADP, 47 μ moles/g. wet wt. of tissue or 300 μ moles/mg. of tissue DNA; AMP, 10.2 μ moles/g. wet wt. of tissue or 63 μ moles/mg. of tissue DNA. The mean ADP/ATP/AMP concentration proportions were 10:7.5:1.6.

Concentrations of glucose 6-phosphate, L-glycerol 3-phosphate and citrate in epididymal fat pad and the lactate/pyruvate concentration ratio in the medium. The concentrations of these intermediates expressed both per g. wet wt. of tissue and per mg. of tissue DNA are given in Table 6. Qualitatively similar changes were seen with either method of calculating concentrations.

The major changes were as follows. In pads from

Table 5. *Effects of insulin, albumin, alloxan-diabetes and adrenaline on the concentrations of adenine nucleotides in the rat epididymal fat pad*

Pads were incubated for 1 hr. in medium containing glucose (3 mg./ml.) and where added albumin (20 mg./ml.), insulin (10 milliunits/ml.) and adrenaline (5 μ g./ml.). Rats were fed up to the time of use. Results are given as means $\pm$ s.e.m.

Expt. no.	Ret	Insulin	Adrenaline	Albumin	No. of groups of pads	Total no. of pads	Concn. (m μ moles/g. wet wt. of tissue)				ATP/ADP/AMP concn. proportions	Concn. (m μ moles/mg. of pad DNA)		
							ATP	ADP	AMP	AMP		ATP	ADP	AMP
1	Normal	—	—	—	4	24	51 $\pm$ 3.7	41 $\pm$ 5.6	—	10:8:—	370 $\pm$ 26	320 $\pm$ 28	—	
	Normal	+	—	—	4	24	53 $\pm$ 4.5	42 $\pm$ 6.2	—	10:8:—	410 $\pm$ 40	310 $\pm$ 33	—	
2	Normal	+	—	—	4	24	83 $\pm$ 4.5	54 $\pm$ 4.0	—	10:6.5:—	420 $\pm$ 21	280 $\pm$ 20	—	
	Alloxan-diabetic	+	—	—	4	24	74 $\pm$ 4.3	49 $\pm$ 3.5	—	10:6.6:—	385 $\pm$ 19	260 $\pm$ 30	—	
3	Normal	+	—	+	5	20	62 $\pm$ 4.4	50 $\pm$ 8.0	8.6 $\pm$ 1.1	10:8.1:1.4	298 $\pm$ 21	240 $\pm$ 38	41 $\pm$ 4	
	Alloxan-diabetic	+	—	+	5	20	73 $\pm$ 4.5	49 $\pm$ 4.0	6.7 $\pm$ 1.4	10:6.7:0.9	308 $\pm$ 19	207 $\pm$ 17	28 $\pm$ 6	
4	Normal	+	—	—	10	30	71 $\pm$ 4.2	53 $\pm$ 2.7	16 $\pm$ 3.8	10:7.5:2.3	410 $\pm$ 25	310 $\pm$ 14	92 $\pm$ 20	
	Normal	+	—	+	10	30	70 $\pm$ 3.4	53 $\pm$ 2.7	15 $\pm$ 2.7	10:7.5:2.2	450 $\pm$ 33	330 $\pm$ 14	100 $\pm$ 17	
5	Normal	+	—	+	4	12	70 $\pm$ 2.3	36 $\pm$ 1.6	9 $\pm$ 1.9	10:5.1:1.3	—	—	—	
	Normal	—	—	+	4	12	71 $\pm$ 3.8	37 $\pm$ 2.3	10 $\pm$ 1.1	10:5.2:1.4	—	—	—	
6	Normal	+	—	+	4	12	57 $\pm$ 1.8	44 $\pm$ 1.8	7.6 $\pm$ 0.9	10:7.7:1.3	410 $\pm$ 13	317 $\pm$ 13	55 $\pm$ 6	
	Normal	+	+	+	4	12	50 $\pm$ 3.7	52 $\pm$ 7.0	8.7 $\pm$ 0.8	10:10.4:1.7	379 $\pm$ 28	394 $\pm$ 53	66 $\pm$ 6	

Table 6. *Effects of insulin, albumin, alloxan-diabetes and adrenaline on the concentrations of glucose 6-phosphate, L-glycerol 3-phosphate and citrate in the rat epididymal fat pad*

For Expts. 1-6 the details are as given in Table 5 and the results were obtained with the same extracts. In Expt. 7 adrenaline was added after 30 min. of incubation and the pads were removed and frozen after a further 10 min. of incubation. An equivalent volume of solvent for adrenaline (20 μ l. of 0.1 N-HCl) was added to control flasks. Results are given as means $\pm$ S.E.M.

Expt. no.	Rat	Insulin	Adrenaline	Albumin	No. of groups	Total no. of pads	Concn. (m μ moles/g. wet wt. of tissue)				Concn. (m μ moles/mg. of pad DNA)				Lactate/pyruvate concn. ratio in medium
							Glucose 6-phosphate	L-Glycerol 3-phosphate	Citrate		Glucose 6-phosphate	L-Glycerol 3-phosphate	Citrate		
1	Normal	-	-	-	4	24	2.8 $\pm$ 0.17	1.6 $\pm$ 0.45	5.7 $\pm$ 0.45		22 $\pm$ 1.9	12 $\pm$ 3	42 $\pm$ 4		2.7 $\pm$ 0.2
	Normal	+	-	-	4	24	6.8 $\pm$ 0.35*	10.8 $\pm$ 1.3*	6.6 $\pm$ 0.7		51 $\pm$ 2.1*	78 $\pm$ 14*	49 $\pm$ 5		4.8 $\pm$ 0.1*
2	Normal	+	-	-	4	24	10.2 $\pm$ 0.52	21.0 $\pm$ 5.0	5.3 $\pm$ 0.5		52 $\pm$ 2	106 $\pm$ 26	28 $\pm$ 3		6.0 $\pm$ 0.3
	Alloxan-diabetic	+	-	-	4	24	7.5 $\pm$ 0.42*	12.2 $\pm$ 2.5	9.6 $\pm$ 0.3*		39 $\pm$ 2.2*	66 $\pm$ 14	49 $\pm$ 3*		4.5 $\pm$ 0.2*
3	Normal	+	-	+	5	20	9.6 $\pm$ 0.6	26.2 $\pm$ 4.5	8.1 $\pm$ 0.7		46 $\pm$ 3	126 $\pm$ 21	39 $\pm$ 3		7.4 $\pm$ 0.2
	Alloxan-diabetic	+	-	+	5	20	6.7 $\pm$ 0.6*	32.8 $\pm$ 4.2	11.7 $\pm$ 2.0		28 $\pm$ 2.5*	138 $\pm$ 18	50 $\pm$ 8		7.1 $\pm$ 0.4
4	Normal	+	-	-	4	12	7.8 $\pm$ 0.50	13.5 $\pm$ 1.6	-		44 $\pm$ 2	96 $\pm$ 9	-		6.7 $\pm$ 1.0
	Normal	+	-	+	4	12	7.4 $\pm$ 0.40	17.3 $\pm$ 2.2	-		41 $\pm$ 1	75 $\pm$ 5	-		8.3 $\pm$ 0.4
5	Normal	-	-	+	4	12	3.0 $\pm$ 0.22	3.3 $\pm$ 0.3	8.1 $\pm$ 0.34		-	-	-		2.7 $\pm$ 0.1
	Normal	+	-	+	4	12	7.7 $\pm$ 0.20*	14.9 $\pm$ 1.0*	8.1 $\pm$ 0.45		-	-	-		5.1 $\pm$ 0.1*
6	Normal	+	-	+	4	12	8.0 $\pm$ 0.4	11.9 $\pm$ 0.6	9.5 $\pm$ 0.8		57 $\pm$ 3	86 $\pm$ 4	68 $\pm$ 6		5.4 $\pm$ 0.5
	Normal	+	+	+	4	12	5.7 $\pm$ 0.6*	2.3 $\pm$ 0.6*	23 $\pm$ 2.0*		43 $\pm$ 4	17 $\pm$ 3*	174 $\pm$ 15*		2.9 $\pm$ 0.3*
7	Normal	+	-	+	4	12	10.8 $\pm$ 0.4	15.9 $\pm$ 0.9	6.2 $\pm$ 0.9		-	-	-		-
	Normal	+	+	+	4	12	5.4 $\pm$ 0.3*	4.4 $\pm$ 0.4*	17.0 $\pm$ 1.1*		-	-	-		-

* $P < 0.02$ when compared with appropriate control.

Table 7. *Fluid spaces of the rat epididymal fat pad incubated in vitro*

The glucose concentrations in the medium were 3 mg./ml. in Expts. 1-4. In Expt. 5, pooled results are given for measurements made at glucose concentrations in the medium of 0.33, 0.75 and 2.00 mg./ml. (between which there was no significant difference). Other concentrations were D-sorbitol (0.1 mg./ml. and 0.5 μ C/ml.), urea (0.1 mg./ml. and 0.1 μ C/ml.), inulin (2 mg./ml.), [3 H]water (5.0 μ C/ml.) and albumin (4 mg./ml. in Expt. 5, 20 mg./ml. in Expts. 1, 3 and 4, and omitted from Expt. 2). There was no significant difference between the paired values for total water, extracellular water and intracellular water unless indicated. Rats were fed up to the time of experiment in Expts. 1, 2 and 5 and starved for 16-18 hr. in Expts. 3 and 4. Results are given as means $\pm$ s.e.m.

Expt. no.	Period of incubation (min.)	Rat	Insulin (milliunits/ml.)	Adrenaline (μ g./ml.)	Marker	No. of pads	Space (ml./100 g. wet wt. of tissue)			
							Total water	Extracellular water	Intracellular water	
1	60	Normal	0	0	[3 H]Water	6	21.6 $\pm$ 1.5	20.5 $\pm$ 1.6	1.1 $\pm$ 0.6	
		Normal	10	0	[14 C]Sorbitol	6	19.6 $\pm$ 1.3	17.9 $\pm$ 1.6	1.7 $\pm$ 0.7	
2	60	Normal	0	0	[14 C]Urea	6	24.8 $\pm$ 1.0	23.4 $\pm$ 1.1	1.4 $\pm$ 0.5	
		Normal	10	0	Inulin	6	23.7 $\pm$ 0.6	22.8 $\pm$ 0.7	0.9 $\pm$ 0.4	
3	60	Normal	10	0	[3 H]Water	6	22.3 $\pm$ 1.2	20.4 $\pm$ 1.3	1.9 $\pm$ 0.3	
		Normal	10	5	[14 C]Sorbitol	6	20.1 $\pm$ 0.9	18.4 $\pm$ 1.1	1.7 $\pm$ 0.4	
4	60	Normal	10	0	[3 H]Water	8	18.7 $\pm$ 0.9	18.0 $\pm$ 1.1	0.7 $\pm$ 0.3	
		Alloxan-diabetic	10	0	[14 C]Sorbitol	7	22.2 $\pm$ 0.8*	21.6 $\pm$ 0.9*	0.6 $\pm$ 0.2	
5	120	Normal	0	0	Drying	15	31.1 $\pm$ 1.3	28.7 $\pm$ 1.2	2.4 $\pm$ 0.7	
		Normal	0.5	0	[14 C]Sorbitol	15	30.5 $\pm$ 1.4	29.4 $\pm$ 1.1	1.1 $\pm$ 0.7	
					Overall mean ...	... 81			1.4 $\pm$ 0.18	

* 0.01 < P < 0.02.

normal rats insulin increased the concentrations of glucose 6-phosphate and L-glycerol 3-phosphate substantially without changing that of citrate (Expts. 1 and 5). In pads from alloxan-diabetic rats the concentration of glucose 6-phosphate was diminished but that of glycerol phosphate was not changed significantly. The concentration of citrate was increased but the change only achieved statistical significance in the absence of albumin (compare Expts. 2 and 3). In pads from normal rats incubated with insulin, adrenaline diminished the concentrations of glucose 6-phosphate and glycerol phosphate and increased that of citrate after 10 or 60 min. of incubation (Expts. 6 and 7). Addition of albumin (which serves to remove free fatty acids formed by hydrolysis of fat-pad glycerides) had no detectable effect on the concentration of glucose 6-phosphate in pads from normal or alloxan-diabetic rats (compare Expts. 1-5) or on that of citrate (compare Expts. 1, 2, 3 and 5).

The effects of insulin or alloxan-diabetes on glucose 6-phosphate concentration and glucose uptake were directly correlated. Both were increased by insulin and diminished by alloxan-diabetes. Adrenaline, on the other hand, which was found by Flatt & Ball (1964) to increase glucose uptake under these conditions, lowered the concentration of glucose 6-phosphate.

The lactate/pyruvate concentration ratio in the medium given in Table 6 was measured to obtain an estimate of the cytoplasmic NADH_2/NAD concentration ratio. This ratio is likely to be one of the factors governing the glycerol phosphate concentration, the other being the concentration of dihydroxyacetone phosphate. The concentration of the latter in adipose-tissue extracts was too low for assay. The effects of alloxan-diabetes, adrenaline or

insulin on glycerol phosphate concentration paralleled their effects on the lactate/pyruvate concentration ratio.

Volume of intracellular water in rat epididymal adipose tissue and the intracellular concentrations of metabolites. The results of measurements of the volume of intracellular water in epididymal fat pad are given in Table 7, and the intracellular concentrations of metabolites (in $\mu\text{moles/ml.}$) calculated with these data in Table 8. Although it is not known whether metabolites are uniformly distributed in intracellular water these calculations appeared to be of value in interpreting effects of insulin and other agents on metabolite concentrations. Moreover, such measurements provide a basis for the comparison of concentrations in adipose tissue with those in other tissues such as muscle on account of the high concentration of lipid in adipose tissue.

The volume of intracellular water in adipose tissue has been calculated as the difference (total water - extracellular water) by using three different methods: (^3H]water space - [^{14}C]sorbitol space), (^{14}C]urea space - inulin space) and (weight loss on drying - [^{14}C]sorbitol space). Each method yielded similar results and no significant changes were seen with insulin, alloxan-diabetes or adrenaline. The overall mean for the volume of intracellular water was $1.4 \pm 0.18 \text{ ml./100 g. wet wt. of tissue}$ (mean $\pm$ s.e.m. for ten experimental groups and 81 fat pads). This value is somewhat lower than that reported by Crofford & Renold (1965a). The results also show that the total water and extracellular water in $\text{ml./100 g. wet wt. of tissue}$ are increased in alloxan-diabetes. Since it is well known that fat pads are hyperaemic in alloxan-diabetes and show loss of lipid this change was not unexpected.

In an attempt to obtain independent confirma-

Table 8. *Calculated intracellular concentrations of metabolites in the epididymal fat pad, perfused rat heart and isolated rat diaphragm*

Results for fat pad were derived from mean values with insulin in Expts. 4-6. Results for heart were derived from measurements with insulin and albumin from Newsholme & Randle (1964) and Garland & Randle (1964). Results for diaphragm were measured in intact diaphragms incubated for 60 min. with glucose (2.5 mg./ml.) and insulin (100 milliunits/ml.).

	Concn. in intracellular water (mm)		
	Fat pad	Rat heart	Rat diaphragm
ATP	4.6	10.5	8.0
ADP	3.3	2	0.62
AMP	0.7	0.26	—
Glucose 6-phosphate	0.57	0.60	0.56
L-Glycerol 3-phosphate	1.3	0.56	0.22
Citrate	0.6	0.56	—
Glycogen glucose	55	46	44
ATP/ADP/AMP concn. proportions	10:7.2:1.5	10:1.9:0.25	10:0.8:—
Lactate/pyruvate concn. ratio	5.7	4.2	—

tion for the calculated volume of intracellular water we have measured the concentration of K^+ in adipose tissue and corrected for K^+ in extracellular water by using the concentration of Na^+ in the tissue for calculation of the latter. Eight fat pads were incubated for 2 hr. in pairs in 10 ml. of medium containing albumin (4 mg./ml.) and glucose (0.5 mg./ml.) and then media and pads were analysed for K^+ and Na^+ as described in the Methods section. The intracellular K^+ concentration in m-equiv./100 g. wet wt. of tissue (0.153 ± 0.033) was calculated from:

$$\text{intracellular } [K^+] = [K^+]_t - [K^+]_m [Na^+]_t$$

where $[K^+]_t$ is the total K^+ concentration in the pad (0.298 ± 0.064 m-equiv./100 g. wet wt. of tissue), $[K^+]_m$ is the K^+ concentration in the medium (5.9 m-equiv./l.) and $[Na^+]_t$ is the sodium space of the pad (24.6 ± 2.2 ml./100 g. wet wt. of tissue). The sodium space was calculated from the Na^+ concentrations in the medium (143 m-equiv./l.) and in the pad (3.52 m-equiv./100 g. wet wt. of tissue). The relationship between the intracellular concentration of K^+ (in m-equiv./l.) and the intracellular volume (in ml./100 g. wet wt. of pad) was such that their product was 153. The intracellular concentration of K^+ corresponding to the intracellular volume of 1.4 ml./100 g. wet wt. of tissue that we have found is thus 110 m-equiv./l. This is consistent with the values calculated for the K^+ concentration in a number of other rat tissues (see Wilde, 1962).

The calculated intracellular concentrations of the various metabolites in adipose tissue are compared with those in rat heart and diaphragm muscles in Table 8. Consideration is given to these values in the Discussion section.

DISCUSSION

Concentrations of metabolites in rat epididymal adipose tissue

Extraction and assay. The major difficulties in extraction and assay of metabolites in adipose tissue stem from the very small volume of intracellular water and the high concentration of lipid. These difficulties have been obviated in the present study by concentrating extracts by freeze-drying, through use of fluorimetric methods of assay and by extraction with a mixture of ether and water. Judged on the basis of recovery experiments satisfactory assays of a limited number of metabolites were achieved. Attempts to assay a number of other metabolites of interest from the standpoint of metabolism control were unsuccessful because the concentrations were too low. These included fructose 6-phosphate, fructose 1,6-diphosphate, dihydroxyacetone phosphate and 6-phosphogluconate. The concentrations of metabolites that have been measured in these

experiments were whole-tissue concentrations, and though the possibility of 'compartmentation' has not been overlooked the results nevertheless suggest some conclusions about metabolism control in the tissue.

Concentration of adenine nucleotides. The concentration of ATP in rat epididymal adipose tissue was about one-half of that in rat heart or rat diaphragm muscles (Table 8). The concentrations of ADP and AMP, on the other hand, were much higher in adipose tissue both in absolute terms and in relation to the concentration of ATP. It seems unlikely that this difference is due to technical shortcomings in view of the rapid freezing and extraction methods employed. The cause for this difference is not apparent but it may have interesting consequences for the control of adipose-tissue PFK.* Adipose-tissue PFK, like that of muscle, is inhibited by increasing concentrations of ATP and activated by ADP and AMP (Denton & Randle, 1966). The ATP/ADP/AMP concentration proportions found would thus favour an active form of the enzyme. The role of PFK and glycolysis in adipose tissue is likely to be different from that in muscle. In adipose tissue the role of glycolysis may be principally concerned with the provision of acetyl-CoA and glycerol phosphate for lipogenesis. In muscle, on the other hand, glycolysis has been envisaged as providing ATP under conditions where its provision by respiration is inadequate. The differences in ATP/ADP/AMP concentration proportions noted could contribute to an explanation for the different role of the enzyme in the two tissues.

Concentration of other metabolites. The concentration of glycerol phosphate in adipose tissue was greater than in heart or diaphragm muscles. This difference could be correlated with the predominant role of adipose tissue in lipogenesis but, as mentioned below, such a high concentration of glycerol phosphate does not appear to be necessary for high rates of glyceride synthesis. The concentrations of glucose 6-phosphate and citrate in adipose tissue and in heart and diaphragm muscles were similar. The concentration of glycogen in adipose tissue was similar to that of muscle once corrections had been applied for the small volume of intracellular water. The lactate/pyruvate concentration ratio in the medium was similar for adipose tissue and for heart.

Effects of insulin, alloxan-diabetes and adrenaline

Glucose uptake. Glucose uptake in rat epididymal adipose tissue showed saturation kinetics. The K_m was 29–44 mg./100 ml. and was unchanged by insulin or alloxan-diabetes. The V_{max} (0.77 mg./g. wet wt. of tissue/hr. for normal pads without insulin)

* Abbreviation: PFK, phosphofructokinase.

was increased by insulin and lowered by alloxan-diabetes. These values for K_u and V_{max} are comparable with those reported by Froesch & Ginsberg (1962) for the metabolism of radioactive glucose and by Hernandez & Sols (1963) for net gas exchange. They differ somewhat from those reported by Baker & Rutter (1964).

Three processes may limit glucose uptake in adipose tissue, namely extracellular diffusion, membrane transport and intracellular phosphorylation by hexokinase. In the experiments reported here extracellular diffusion did not appear to be a major limiting process. In particular the concentration of glucose in extracellular water was very similar to that of the medium (Table 9). It was not possible in these experiments to distinguish between glucose transport and glucose phosphorylation as rate-limiting reactions. In other tissues the extent to which each limits glucose uptake has been distinguished by measurement of the intracellular glucose concentration, but this was not possible in these experiments with adipose tissue because of the very small intracellular volume. The calculated values of K_u and V_{max} may thus be composite constants for the sequential processes of glucose transport and phosphorylation. They do not permit conclusions about the possible effects of insulin on K_m and V_{max} of glucose transport for which evidence has been obtained by Crofford & Renold (1965b).

Glucose 6-phosphate concentration. Insulin increased the glucose 6-phosphate concentration in adipose tissue, as in muscle (cf. Newsholme & Randle, 1961, 1964). The hormone thus increases the flow of glucose into glucose 6-phosphate relative to loss of the latter through intracellular pathways

of metabolism until a new steady-state concentration is attained. The observation is compatible with the idea that a major effect of the hormone is to accelerate glucose transport (Hernandez & Sols, 1963). In alloxan-diabetes the concentration of glucose 6-phosphate in fat pads incubated with insulin was diminished relative to the control. This is in contradistinction to heart muscle, where the glucose 6-phosphate concentration is increased in alloxan-diabetes (Newsholme & Randle, 1964). Since glucose uptake by adipose tissue is decreased in alloxan-diabetes the change in glucose 6-phosphate concentration suggests either a diminished rate of transport of glucose (in spite of the presence of insulin) or a diminution of glucose phosphorylation due to factors other than inhibition of hexokinase by accumulation of glucose 6-phosphate, e.g. to a diminution in hexokinase concentration. Hernandez & Sols (1963) find that the hexokinase concentration of adipose tissue is diminished in the related condition of starvation.

Adrenaline lowered the concentration of glucose 6-phosphate in fat pads incubated with insulin. Under comparable conditions Flatt & Ball (1964) found that adrenaline accelerated glucose uptake and metabolism of glucose 6-phosphate by glycolysis and oxidation through the pentose phosphate cycle. The fall in glucose 6-phosphate concentration in adipose tissue with adrenaline is in contradistinction to the increase that adrenaline causes in muscle (Hegnauer & Cori, 1934). It thus appears that adrenaline accelerates glucose 6-phosphate breakdown in adipose tissue. Activation of adipose-tissue PFK by 3',5'-(cyclic)-AMP may be important for this effect (see Denton & Randle, 1965, and below).

Glycerol phosphate concentration. These measurements call into question the idea that the flow of glucose to glycerol phosphate may determine the rates of esterification of fatty acids in adipose tissue. Adrenaline, which Flatt & Ball (1964) find increases glucose uptake and fatty acid esterification rate, caused a substantial fall in glycerol phosphate concentration. Moreover, alloxan-diabetes, which lowered glucose uptake by fat pads incubated with albumin and insulin, did not lower the glycerol phosphate concentration.

Citrate concentration. The concentration of citrate in adipose tissue was of interest for a number of reasons. In lipogenesis citrate is thought to be involved in the transfer of acetyl-CoA from mitochondrial to cytoplasmic compartments (through citrate synthase and citrate-cleavage enzyme) and in the activation of acetyl-CoA carboxylase, and it may have a role in hydrogen transfer (see Bartley, Abraham & Chaikoff, 1965; Matsahushi, Matsahushi & Lynen, 1964; Kornacker & Ball, 1965). In rat heart muscle a role for citrate as a regulator of PFK

Table 9. *Glucose space in relation to extracellular space in the rat epididymal fat pad incubated in vitro*

Fat pads were incubated for 2 hr. in medium containing albumin (4mg./ml.) and [14 C]sorbitol (0.5mg./ml. and 0.05 μ C/ml.) and frozen with the tissue clamp of Wollenberger *et al.* (1960). Further details are given in the Methods section. None of the observed differences in (glucose space—sorbitol space) are significantly different from zero or from one another. Results are given as means $\pm$ s.e.m. of five observations.

Additions		Glucose space— sorbitol space (ml./100g. wet wt. of tissue)
Glucose (mg./ml.)	Insulin (milliunit/ml.)	
0.33	0	-1.9 ± 1.3
	0.5	-0.7 ± 1.1
0.75	0	-0.7 ± 0.7
	0.5	-0.9 ± 0.9
2.00	0	-1.1 ± 1.3
	0.5	-0.7 ± 0.8

has been proposed (Garland, Randle & Newsholme, 1963; Pogson & Randle, 1966) and citrate has been shown to be an inhibitor of adipose-tissue PFK (Denton & Randle, 1965). Since glycolysis and PFK may participate in lipogenesis by providing acetyl-CoA and glycerol phosphate the relationships between citrate concentration and rates of lipogenesis are likely to be complex.

The major changes in citrate concentration were with adrenaline and alloxan-diabetes. Adrenaline, which was found by Flatt & Ball (1964) to accelerate glycolysis, increased the concentration of citrate. The apparent anomaly of increased citrate concentration (inhibitor of PFK) and accelerated glycolysis (requiring PFK activation) may be explained by the increase in 3',5'-(cyclic)-AMP concentration that adrenaline induces in adipose tissue (Butcher, Ho, Meng & Sutherland 1965). 3',5'-(Cyclic)-AMP is particularly potent at overcoming citrate inhibition of adipose-tissue PFK and activating the enzyme (Denton & Randle, 1966). Citrate concentration was also increased in the pad in alloxan-diabetes, but whether this led to inhibition of PFK in the pad, as in muscle, could not be ascertained. This requires the demonstration of hexose monophosphate accumulation coupled with diminution in the rate of glycolysis and in fructose 1,6-diphosphate concentration (Newsholme & Randle, 1964). In the fat pad alloxan-diabetes diminished the concentration of glucose 6-phosphate but the concentrations of fructose 6-phosphate and fructose 1,6-diphosphate were too low for assay.

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