THE EFFECT OF STARVATION AND DIABETES ON GLYCOLYTIC ENZYMES IN HUMAN ADIPOSE TISSUE

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SUMMARY

1. The activities of hexokinase (EC 2.7.1.1) and phosphofructokinase (EC 2.7.1.11) have been studied in homogenates of adipose tissue taken from human diabetics, fasting and control patients.

2. Three isoenzymes of hexokinase were observed with apparent $K_m$ values for glucose of $1.04 \times 10^{-5} M$, $2.6 \times 10^{-4} M$ and $2.9 \times 10^{-4} M$, respectively.

3. No change in activity of hexokinase was found in adipose tissue of untreated diabetics ($n=22$), treated diabetics ($n=13$) or non-diabetic controls. However, fasting was associated with a decrease of approx. 40% in the activity of hexokinase in adipose tissue.

4. In contrast, there was a marked decrease in the activity of phosphofructokinase in adipose tissue from untreated diabetics ($n=24$) which was restored to normal by either insulin therapy or treatment by hypoglycaemic drugs.

5. There was a negative correlation between the phosphofructokinase/hexokinase ratio in adipose tissue and the fasting blood glucose ($P=0.01$) and the 2 h blood glucose ($P=0.03$) after an oral glucose load (50 g).

6. The functional significance of the changes in enzyme activities is discussed in relation to the glucose intolerance of diabetes.

The supply of L-glycerol 3-phosphate from glucose for synthesis of glycerides in human adipose tissue shares the first five reactions of glycolysis. Two of these steps, catalysed by hexokinase (EC 2.7.1.1) and phosphofructokinase (EC 2.7.1.11) respectively are thought to be of importance for regulation of glycolysis and might therefore regulate the supply of L-glycerol 3-phosphate for glyceride biosynthesis (Randle, 1966). At least two conditions are known to decrease the lipogenic activity of the adipose cell, namely fasting and diabetes. Moreover there is indirect evidence that the activity of phosphofructokinase is decreased in adipose tissue of adult diabetics, although these experiments could not exclude an associated defect of...
hexokinase (Galton \& Wilson, 1970a). The purpose of this paper is therefore to examine the total activities of hexokinase and phosphofructokinase in diabetics and fasting patients to see if changes in glyceride biosynthesis are reflected by changes in the activity of these enzymes.

**SUBJECTS AND MATERIALS**

*Adipose tissue*

Tissue was obtained from subcutaneous and extra-peritoneal sites of patients undergoing routine abdominal surgery and the tissue was transferred to the laboratory in 0-9\% (w/v) saline solution. Such patients had fasted overnight but usually had caloric replacement during the operation in the form of dextrose (5\%) or Hartmann solution. Other specimens (200–600 mg) were obtained by needle biopsy (Diengott \& Kerpel, 1967) from the anterior abdominal wall under local anaesthesia (<1 ml of 1\% lignocaine). Previous work has shown that differences in anaesthesia do not have measurable effects on glucose metabolism (Galton, Wilson \& Kissebah, 1971).

The following groups of patients were selected for study.

1. Twenty-four uncontrolled diabetics (all females) whose ages ranged from 18 to 69 years (mean 50 years), whose weight ranged from 102 to 218 lb (mean 169 lb) and whose fasting blood sugar concentrations from 85 to 295 mg/100 ml (mean 189 mg/100 ml).
2. Eleven well-controlled diabetics (three males, eight females) whose ages ranged from 31 to 75 years (mean 53 years), whose weights ranged from 143 to 163 lb (mean 150 lb) and whose fasting blood sugar concentrations ranged from 35 to 138 mg/100 ml (mean 96 mg/100 ml).
3. Seven uncontrolled adult diabetics (one male, six females) age range 34–52 years, weight range 143–230 lb, who were biopsied before and after a week’s trial of insulin.
4. Seven non-diabetic obese patients (two males, five females) whose ages ranged from 19 to 59 years (mean 40 years), whose weights ranged from 140 to 320 lb (mean 218 lb) and who had fasted for at least 7 days (mean 8 days).
5. Nine non-diabetic obese patients (one male, eight females) whose ages ranged from 28 to 65 years (mean 40 years), whose weights ranged from 136 to 254 lb (mean 202 lb) and who were biopsied before and after a week’s fast.

The controls for these groups respectively comprised the following:

1. Twenty-five non-diabetics (five males, twenty females) operated on mainly for hysterectomy, hernia, cholecystectomy, pyloroplasty and nephrectomy, whose ages ranged from 6 to 70 years (mean 42 years).
2. Ten non-diabetics (four males and six females) whose ages ranged from 29 to 61 years (mean 37 years).
3. This group did not require controls.
4. Controls for the fasting patients comprised seven patients (one male, six females), whose ages ranged from 28 to 58 years (mean 39 years).
5. This group acted as their own controls since they were studied before and after fasting.

Tissue from all sources was treated in a similar manner, i.e. washed in 0-9\% (w/v) saline solution and then homogenized in an equal volume of buffer (150 mm-KCl; 5 mm-MgCl₂;
Glycolytic enzymes in starvation and diabetes

5 mM-EDTA; 10 mM-mercaptoethanol; 5 mM-NaF; 20 mM-tris-HCl, pH 7.9) by using a ground-glass Potter-Elvehjem tissue grinder (clearance 0.01 in) at room temperature. The homogenate was centrifuged for 5 min at 700 g and the upper fat cake and nuclear pellet were discarded. Before polyacrylamide-gel electrophoresis was performed the fat-free homogenate was centrifuged at 15000 g for 20 min to precipitate mitochondria. The supernatant was then applied to polyacrylamide-gel columns for electrophoresis.

Chemicals

Palmitic acid and D-glucose were purchased as AnalAr reagents from British Drug Houses, Poole, Dorset, U.K. The palmitic acid was checked for purity on a gas chromatograph (Pye series 104; column of siliconized Celite coated with polyethylene glycol adipate), and found to migrate as a single peak. Adenine and pyridine nucleotides, phenazine methosulphate, glucose 6-phosphate and fructose 6-phosphate were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A., acrylamide, bisacrylamide, glycine and NNN'N'-tetramethylthelyldiamine from Eastman Chemical Co. and purchased through Kodak Ltd, London, Sephadex G-200 (lot 2208) from Pharmacia, Uppsala, Sweden, nitro-blue tetrazolium chloride and iodo-3-p-nitrophenyl 5-phenyltetrazolium chloride from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Enzymes used for assays (aldolase, triose phosphate isomerase, glycerol-1-phosphate dehydrogenase, glucose 6-phosphate dehydrogenase, phosphofructokinase and hexokinase) were purchased from Boehringer Corp. (London) Ltd.

METHODS

Enzyme assays

Hexokinase (EC 2.7.1.1) was measured by two methods. One was a modification of the micro-assay of Lee & Lardy (1965), which uses phenazine methosulphate (PMS) as intermediary and 2-p-iodophenyl-3-p-nitrophyl-5-phenylmonotetrazolium (INT) as final electron acceptor from NADPH produced by the oxidation of glucose 6-phosphate with glucose 6-phosphate dehydrogenase. The incubation system contained in a final volume of 0.45 ml: tris-HCl buffer, pH 7.4, 36 mM; KCN 0.4 mM; MgCl₂ 4 mM; ATP 2.6 mM; NADP⁺ 0.4 mM; glucose varied up to 0.6 mM; PMS–INT 20–40 µg/assay tube; glucose 6-phosphate dehydrogenase 10 µg/assay tube; and 0.1 ml of enzyme preparation. The reaction was stopped after various time-intervals by adding 0.2 ml of aq. 10% (w/v) trichloroacetic acid and the insoluble formazan was extracted into 3 ml of ethyl acetate. Samples of the supernatant were then transferred to cuvettes and the extinction measured in a Unicam SP. 600 spectrophotometer at 490 nm. The assay tubes were incubated in the dark to minimize the colour change of the PMS–INT solution produced by direct light. A blank, incubated without substrate, was subtracted from the experimental tube and this corrected for the spontaneous colour change of the incubation system. On some occasions the extract of ethyl acetate was cloudy and unsuitable for measurement of extinction; the turbidity was found to disperse easily on centrifugation for 1 min at approx. 300 g. A standard curve of iodonitrophenyl tetrazolium formazan was prepared to convert extinction units into µg of formazan and this was linear over a range of extinction of 000–0.820. The tetrazolium assay conducted in the absence of ATP gave a value of 2%, in the absence of glucose 10%, in the absence of NADP⁺ 15%, and in the absence of MgCl₂ 36% of the activity with the complete system (taken as 100%). In addition the assay was linear with time up to 25 min and
linear with homogenate protein up to 320 μg. Hexokinase activity is expressed as μg of for- mazan produced/mg of protein.

Alternatively hexokinase was measured in the following system: tris–HCl buffer, pH 7.4, 70 mm; glucose 10 mm; NADP+ 0.55 mm; MgCl₂ 7.4 mm; ATP 3.7 mm; mercaptoethanol 5 mm; and glucose 6-phosphate dehydrogenase 2.5 μg/ml. The reaction was started by addition of 0.2 ml of homogenate (equivalent to 100 mg of tissue) and the change in $E_{340}$ was measured in a 10 mm light-path cell at 25°C by a recording spectrophotometer (Unicam SP. 800A, with scale-expansion accessory).

**Phosphofructokinase**

The assay system contained in 2.0 ml was: tris–HCl buffer, pH 7.4, 60 mm; MgCl₂ 6.2 mm; ATP 2.0 mm; NADH 1.0 mm; (NH₄)₂SO₄ 10 mm; fructose 6-phosphate 3.0 mm; aldolase 30 μg/ml, triose phosphate isomerase 1 μg/ml and glycerol 1-phosphate dehydrogenase 10 μg/ml. The reaction was started by addition of 0.2 ml of homogenate (equivalent to 100 mg of tissue). In the first set of experiments the measurement of initial velocities was made difficult by a variable lag-period of about 15 min which seemed to depend on the activity of the preparation; accordingly the cuvette was pre-incubated for 15 min before starting the enzyme measurement. This lag-period was not found when the solutions for the assay were made up for a second set of experiments.

The progress curve once started was usually linear with time and was linearly related to the volume of homogenate added up to 0.4 ml. Experimental samples were frequently tested at the end of the run by adding pure phosphofructokinase after which an immediate change in extinction was noted. Enzyme velocities were measured over 5 min of the linear part of the time-course. Results are expressed as nmol of coenzyme converted min⁻¹ g of tissue⁻¹ or mg of tissue protein⁻¹.

**Electrophoresis**

Polyacrylamide-gel electrophoresis was performed at 0–4°C by using 4% (w/v) polyacrylamide running gel according to the method of Davis (1964) except that in place of the spacer and sample gel a Sephadex–sucrose–tris solution was used. Electrophoresis required approx. 45 min at 2.5–3 mA/column. After electrophoresis the gels were stained for hexokinase as described by Katzen & Schimke (1965). Controls were performed for each experiment without added substrate to identify artifactual bands. After staining the gels were washed in water and placed in aq. 5% (v/v) acetic acid and then scanned with a Zeiss Absorbance Recorder (model 2).

**Statistics**

Comparisons are based on means of paired observations and the significance of the difference ($P$) was calculated by a non-parametric sign test (Siegel, 1956), since this makes no assumption about the distribution of the results.

**RESULTS**

**Hexokinase in adipose tissue**

Studies on hexokinase in human adipose tissue are complicated by the presence of isoenzymes (Galton & Jones, 1967). We confirmed previous experiments that showed that hexokinase from human adipose tissue migrates on polyacrylamide-gel electrophoresis as two
distinct bands in addition to some enzymic activity remaining at the origin. Other tissues that have been examined by this method include: adrenal, brain, stomach, ileum, colon and muscle. The bands with increasing anodal mobility are numbered I, II and III to conform to other results by investigators using starch-gel electrophoresis. This does not necessarily imply correspondence between the properties of the types of isoenzymes previously described on starch-gel electrophoresis since a different medium is used. In some experiments the authenticity of hexokinase III was in doubt since the control gel (incubated without glucose) displayed a similar band in the position of hexokinase III. However, when the protein of the gel segment corresponding to hexokinase III was eluted into tris buffer in four out of five succeeding experiments, the eluted material behaved like hexokinase in the spectrophotometric assay for this enzyme. The protein eluted from the gel segments corresponding to hexokinase I and II always behaved like hexokinase in the spectrophotometric assay so we concluded that there were at least two forms of hexokinase in human adipose tissue and sometimes a third form could be detected.

The proteins corresponding to hexokinase bands I, II and III on polyacrylamide-gel electrophoresis were eluted into tris buffer to give a four- to ten-fold degree of purification of the enzyme, and each fraction was investigated for dependence on glucose. The apparent \( K_m \) of hexokinase I for glucose was \( 1.04 \times 10^{-5} \text{M} \) and that of hexokinase II \( 2.6 \times 10^{-4} \text{M} \). In a separate experiment the apparent \( K_m \) for hexokinase III was similar to that of hexokinase II at \( 2.9 \times 10^{-4} \text{M} \). Repeat assays for hexokinase I, II and III gave the following \( K_m \) values for glucose: \( 1.5 \times 10^{-5} \text{M}, 5 \times 10^{-4} \text{M} \) and \( 3.3 \times 10^{-4} \text{M} \) respectively. Table 1 shows the maximal activity of hexokinase in adipose tissue of untreated diabetics, of insulin-requiring diabetics before and after therapy, and of well-controlled diabetics. No gross differences were noted between these groups. Table 2 presents the activity of hexokinase measured by two different methods in adipose tissue of fasting patients. In Experiment 1 patients were biopsied at the end of a week's fast and the tissue activities compared with controls during surgery on the same day. In Experiment 2 the fasted patients acted as their own controls, being biopsied before and after a fasting
period of at least 7 days. In both sets of experiments a small decrease in activity of hexokinase was observed. The activity of glucose 6-phosphate dehydrogenase was measured to see if there was a general decrease in enzymic activity as a result of fasting, but it did not appear to change.

Phosphofructokinase in adipose tissue

The activity of this enzyme in human adipose tissue was 0.08 units/g which is lower than a previously reported value of 0.11 units/g (Shonk, Koven, Majima & Boyer, 1963). However, despite the presence of fluoride in the homogenization buffer, the enzyme activity decays rapidly on standing at 4°. The decrease in activity could not be prevented by preincubation with ATP and Mg, but could be delayed by addition of fructose 6-phosphate. Reported

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean weight loss (kg)</th>
<th>Hexokinase (µg of formazan formed min⁻¹ mg of protein⁻¹)</th>
<th>Glucose 6-phosphate dehydrogenase (nmol of NADP min⁻¹ mg of protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed controls</td>
<td>—</td>
<td>23.7±2 (7)</td>
<td>—</td>
</tr>
<tr>
<td>Fasted patients</td>
<td>7±0.6</td>
<td>15.7±4 (7)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td>Fed patients</td>
<td>—</td>
<td>20.0±2 (11)</td>
<td>31.0±3 (5)</td>
</tr>
<tr>
<td>Same patients fasted</td>
<td>6±0.3</td>
<td>10.0±1 (12)</td>
<td>26.0±5 (10)</td>
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<td>0.01</td>
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Results are given as mean ± SEM of number of patients given in parentheses. The significance of the difference (P) was calculated by a non-parametric sign test (Siegel, 1956).

difference in total activity may therefore be due to instability of the enzyme. The activity of phosphofructokinase in adipose tissue of a group of untreated or uncontrolled diabetics was found to be depressed (Table 1). Results in Experiment 1 are means of two sets of assays and include the group in which a lag-period occurred. However, paired comparisons were made with control tissue under similar conditions so a mean was taken of the results from both sets of experiments. Treatment with insulin appeared to reverse the change in activity and a time-course for the restoration of activity was performed on two patients, biopsies being taken at days 0, 3 and 8 after starting insulin. The activity of phosphofructokinase appeared to be restored between days 3 and 8 after starting insulin. In addition a group of adult diabetics treated by hypoglycaemic drugs showed normal activities of phosphofructokinase, thus suggesting the reversible nature of the enzyme change (Table 1). An attempt to see if there was any relation between the severity of the diabetes and the activity of phosphofructokinase was undertaken. Twenty patients (eleven females, mean age 48 years, mean fasting blood sugar concentration 128 mg/100 ml) were biopsied for assay of enzymes at the end of an oral glucose tolerance test (50 g). Since the mean activity of hexokinase does not appear to vary between diabetics and non-diabetics the enzyme results were expressed as a phosphofructokinase/hexokinase ratio for each patient. There was a good linear relation between phosphofructo-
kinase activity and the phosphofructokinase/hexokinase ratio. The enzyme ratio was chosen so as to decrease the variability between patients and the value was then related to various parameters of the glucose tolerance test. Two such correlations are shown in Fig. 1 with their correlation coefficients and probability values. In addition the phosphofructokinase/hexokinase ratio related in a similar way to the mean glucose concentration during the glucose tolerance test at a significance level of 0.03. However there were very poor correlations between the phosphofructokinase/hexokinase ratio and the fasting and 2 h insulin values during the glucose tolerance test (P > 0.1 for fasting insulin level).

**DISCUSSION**

Hexokinase occurs in multiple forms in human adipose tissue as judged by polyacrylamide-gel electrophoresis. All types had low Michaelis constants for glucose and there was no enzyme with a high $K_m$ corresponding to the glucokinase in liver. The separate functions of these enzymes of hexokinase remain conjectural at present; it is possible that each may have a separ-
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ate metabolic role in analogy to the special function of glucokinase in liver. No evidence was found for an adaptive change in the activity of hexokinase in adipose tissue of adult diabetics, despite a decrease in glyceride biosynthesis from glucose of about 35% which occurs in this condition (Galton et al., 1971). This is in contrast with adipose tissue of rats made diabetic by alloxan or streptozotocin which shows a decrease in activity of hexokinase particularly in isoenzyme II fraction (Mclean, Brown, Walters & Greenslade, 1967). The difference may be attributed to the continuing presence of circulating plasma insulin in adult diabetics as opposed to the deficiency of insulin in the animal model. This is in accord with other evidence that insulin is required to maintain the activities of hexokinase in rat adipose tissue, possibly by affecting synthesis of the enzyme.

A state involving insulin deficiency can be induced in man by fasting, and in rats it has been observed that starvation decreases the activity of hexokinase in adipose tissue. Likewise a small decrease of about 30-50% in activity of hexokinase of adipose tissue from fasting patients was observed, whereas a marker enzyme (glucose 6-phosphate dehydrogenase) did not appear to alter, in confirmation of other studies (Shrago, Glennon & Gordon, 1967). However, it has previously been shown that fasting decreased glyceride biosynthesis from glucose in adipose tissue by about 85% in vitro (Galton & Wilson, 1970b). The magnitudes of the change in enzyme activity and metabolic pathway are so different that it is unlikely that the decrease in hexokinase activity is solely responsible for the decrease in glyceride biosynthesis from glucose during a fast.

Adult diabetes is another condition that leads to a decrease in lipogenesis from glucose and does not appear to be accompanied by a change in the total hexokinase activity of adipose tissue, although this does not exclude a decrease in activity of one of the isoenzymes of hexokinase. However, the next glycolytic control point may be affected. Thus there is a decrease in activity of phosphofructokinase in adult diabetics. This change is in keeping with previous results showing a block in hexose phosphorylation in homogenates of human adipose tissue of untreated diabetics which was tentatively attributed to a deficiency of phosphofructokinase (Galton & Wilson, 1970a). As the activity of phosphofructokinase was restored to normal either by treatment with insulin or by hypoglycaemic drugs, the change is likely to be secondary to the altered metabolic state of the adipose cell in diabetes. Phosphofructokinase is thought to exert control over glycolysis under the influence of a variety of small metabolites (ATP, ADP, AMP, phosphate and citrate); and in alloxan-diabetic rats the absolute activity of the enzyme does not alter in heart muscle at least after 2 days from initiation of diabetes (Pogson & Randle, 1966). The results in the present paper might indicate that over a long-term the amount of enzyme protein decreases in diabetes. As to the functional significance of this alteration in enzyme activity, there is evidence that intact adipose tissue of adult diabetics utilizes less glucose than tissue from non-diabetics. This has been attributed to a defect in membrane transport of glucose into the cell. However, there is the added possibility that an intracellular deficiency of a key glycolytic enzyme phosphofructokinase may also contribute to the decrease in glucose consumption by the whole cell. What effect this may have on the clearance of glucose from the blood stream after a glucose load remains conjectural at present.

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