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HEXOKINASE OF WHITE ADIPOSE TISSUE

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SUMMARY

Apparent Michaelis constants for glucose and fructose of white adipose tissue hexokinase have been estimated by determining the rate of hexose C-1 oxidation through the hexose monophosphate shunt with added glucose-6-*P* dehydrogenase and $NADP^+$. The values obtained are: fructose, 3 mM; glucose, 0.07 mM.

It is likely that a single enzyme catalyzes the phosphorylation of glucose and fructose since a summation of reaction rates is not observed in the presence of both sugars. Glucose competitively inhibits fructose phosphorylation although the presence of fructose has little or no effect on glucose phosphorylation. This observation is in keeping with the relative affinities of the enzyme for the two hexoses.

The enzyme displays a specific requirement for ATP and is activated both by magnesium and manganese ions.

Under the experimental conditions described, glucose phosphorylation rates of between 30 and 60 μmoles/min/g of adipose tissue nitrogen were observed.

INTRODUCTION

Several glycolytic enzymes have been measured in extracts of white adipose tissue of the rat[1,2]. Rud[3] has determined the level of hexokinase activity in human subcutaneous adipose tissue and white adipose tissue from mice. However, there has been no detailed report concerning the properties of the hexokinase in the epididymal fat pad of the rat, although the capacity of this tissue for the rapid conversion of glucose to various intermediates for fat synthesis implies an active phosphorylating mechanism[4]. Moreover, Jeanrenaud and Renold[5] and Leonards and Landau[6] have shown that the intact epididymal fat pad of the rat is sensitive to the glucose concentration of the medium, metabolizing increasing amounts of this sugar as its concentration is raised up to rather high levels. In this regard, adipose tissue is similar to rat liver and it was of interest to see whether, as in rat liver, this phenomenon could be explained by a low affinity of glucokinase for its substrate[7]. Accordingly, the following experiments were designed to measure the affinity of adipose hexokinase for glucose and fructose. Other properties of this enzyme will also be reported. A preliminary estimation of the Michaelis constant for glucose of adipose tissue hexokinase has been published[7].

MATERIALS AND METHODS

Nucleoside 5'-triphosphates were obtained from the Pabst Laboratories, $NADP^+$ from the Sigma Chemical Company, and glucose-6-*P* dehydrogenase and hexose phosphates from the California Corporation for Biochemical Research. [^{14}C]glucose and fructose were obtained from the New England Nuclear Corporation and were diluted to 33 μC/milliatom of carbon before use. All solutions were prepared with distilled, deionized water, deionization being accomplished by passage through a column of MB-3 mixed bed resin (Rohm and Haas). Unlabeled, C.P. fructose was obtained from Pfanstiehl Laboratories and was used without further purification.

Preparation of the extract

White rats weighing between 200 and 250 g and fed *ad libitum* were used as a source of adipose tissue. Epididymal and perinephric adipose tissue was dissected immediately after decapitation of the rat and was homogenized in one or two volumes of the following salt solution: 0.15 M potassium chloride, 0.005 M magnesium chloride, 0.005 M disodium EDTA (pH 7.5). It was found that homogenization at 0° caused the fat to solidify and to trap a significant portion of the aqueous phase even after high speed centrifugation. Moreover, the occluded liquid was found to possess enzyme activities two to three-fold higher than the main bulk of extract. For this reason, extracts were prepared by homogenizing the tissue at room temperature for a few minutes followed by low speed centrifugation of the emulsion, also at room temperature, to separate the liquified fat. This procedure yielded extracts richer in protein and hexokinase activity than those obtained by extraction in the cold. Recentrifugation of the aqueous (bottom) layer at 100 000 × *g* for 30 min did not measurably change the hexokinase activity of the solution. The high speed centrifugation step was therefore omitted. The crude extracts contained traces of ATP and appreciable amounts of glucose (on the order of 60 μg/ml as determined with glucose oxidase): these were removed by overnight dialysis in the cold against a large volume of the solution used for extraction of the tissue.

Measurement of glucose phosphorylation

The conversion of glucose to glucose-6-*P* was determined by measuring the reduction of $NADP^+$ in the presence of exogenous glucose 6-*P* dehydrogenase as described previously for liver extracts[8]. Because of the high endogenous 6-phosphogluconic dehydrogenase activity of the adipose extracts, each molecule of glucose-6-*P* oxidized yielded 2 molecules of NADPH and one molecule of CO_2 from C-1. These reactions were followed either by measuring the formation of NADPH spectrophotometrically, or by determining the yield of $^{14}CO_2$ from [1-^{14}C]glucose. The latter method is more suitable for precise measurements since endogenous glucose-6-*P* formation does not interfere.

In the spectrophotometric assay the test cuvette contained the following substances at the designated concentrations in a total volume of 0.40 ml (pH 7.5): glycylglycine, 44 mM; $NADP^+$, 0.75 mM; $MgCl_2$, 7.5 mM; ATP, 3 mM; glucose-6-*P*

dehydrogenase, 0.07 Racker Unit*; glucose, 50 mM, and adipose extract, usually 0.05 ml.

The enzyme extract was added last to initiate the reaction and the linear increase in absorbancy was recorded at 0.5-min intervals, the first reading being taken 30 sec after the time of mixing. The reaction was carried out at room temperature, 20°–25°. In the assay of dialyzed extracts a control cell, containing no added glucose, commonly showed a reaction rate of less than one-twentieth that of the glucose stimulated reaction. When undialyzed extracts were assayed it was necessary to withhold the addition of ATP in order to obtain a comparable control, since sufficient endogenous glucose is present in the extract to give an appreciable reaction.

In the isotopic assay, the reaction medium was the same as described above except that the volume of medium for each test was increased to 4.0 ml, the concentrations of reactants remaining the same. Incubation was carried out in 25-ml Erlenmeyer flasks fitted with rubber caps and small plastic wells suspended halfway down in the flasks and attached to the caps by means of wire. The wells contained 0.2 ml of 10 M sodium hydroxide for absorption of the carbon dioxide. The adipose extract (0.5 ml) was added last, and the flasks were capped and placed in a Dubnoff shaker at 28° for 15 min. The reaction was terminated by the injection of 0.3 ml of 10% sulfuric acid through the rubber cap with a syringe. Shaking was continued for 30 min to allow for complete absorption of the CO_2 which was then isolated with "carrier" carbonate, converted to barium carbonate, plated and counted as previously described[9]. Specific activities were corrected for traces of acid-labile ^{14}C in the glucose substrate.

Measurement of fructose phosphorylation

Assuming that the product of fructose phosphorylation in adipose tissue is fructose-6-*P*, this intermediate must be quantitatively converted to glucose-6-*P* in order to measure the reaction rate with the present system**. The rate of the phosphohexoseisomerase reaction in these extracts at saturating levels of fructose-6-*P* was found to be quite high, at least 10-fold higher than the rate of the hexokinase reaction (see also WEBER *et al.*[2]). However, in the presence of ATP one might expect some fructose-6-*P* to be lost by phosphorylation to the diphosphate. To investigate the extent of the phosphofructokinase reaction in the extracts, glucose-6-*P* was added with and without ATP, and after a suitable incubation the amount of glucose-6-*P* remaining was determined with glucose-6-*P* dehydrogenase. It was found that ATP did not measurably stimulate the disappearance of glucose-6-*P*. Thus phosphofructokinase was found to be inactive in these preparations. PASSONNEAU AND LOWRY have recently described the lability of phosphofructokinase and the precautionary measures which must be taken to prevent its inactivation within a few minutes after extraction[10]. Accordingly, it becomes reasonable to assume that under the experimental conditions which have been described, C-1 of fructose-6-*P* is con-

* A unit of activity as proposed by Racker is that amount of enzyme which will catalyze the oxidation of 1 μmole of glucose-6-*P* per min.

** The possibility that fructose is phosphorylated to fructose-1-*P* as in liver[11] was eliminated by the observation that these extracts do not convert fructose-1-*P* to glucose-6-*P*, whereas fructose is readily converted to glucose-6-*P*.

verted to carbon dioxide by the combined action of phosphohexoseisomerase and the enzymes of the direct oxidative pathway. Hence, fructose phosphorylation was determined exactly as described for glucose except that uniformly labeled fructose was used as substrate since [1-^{14}C]fructose was unavailable.

RESULTS

The nitrogen content of adipose tissue taken from male rats fed *ad libitum* and weighing between 200 and 250 g was about 1.7 mg/g of tissue (fresh weight) as determined by the Kjeldahl procedure. Water content of the fresh tissue varied between 5 and 9% as estimated by heating preweighed samples at 130° to constant weight. Other estimates of the chemical composition of adipose tissue have been reported by BABINEAU AND PAGE[12]. The hexokinase activity of the tissue, as determined in extracts, was fairly constant and corresponded to the phosphorylation of between 0.05 and 0.1 μmole of glucose/min/g of tissue, or between 30 and 60 μmoles/min/g

TABLE I

GLUCOSE PHOSPHORYLATION BY AN UNDIALYZED EXTRACT OF WHITE APIDOSE TISSUE

Each test cell contained in addition to those substances designated below, 0.3 μmole $NADP^+$, 3 μmoles $MgCl_2$, 17 μmoles glycylglycine (pH 7.5) in a total volume of 0.40 ml. Glucose, 40 μmoles; glucose-6-*P*, 0.4 μmole; ATP and glucose-6-*P* dehydrogenase (GDH) were added as indicated. Adipose extract was added to each cell in 0.05-ml portions with the exception of the last experiment where 0.02 ml was added. The reaction rate was linear for at least 5 min.

Additions	*NADPH formed per minute (mμmoles)*	
	Per cell	*Per gram tissue**
Glucose, ATP, GDH	3.4	136
Glucose, ATP	2.8	112
ATP, GDH	1.7	68
Glucose, GDH	0.2	8
Glucose-6-*P*	10.3	412
Glucose, ATP, GDH	1.3	130

* Fresh weight, animals fed *ad libitum*.

of tissue nitrogen. The rate of fructose phosphorylation was found to be about the same as that for glucose, or a little higher.

The data recorded in Table I define some of the properties of the assay system. By comparing lines 1 and 3 it can be seen that there is sufficient glucose in 0.05 ml of undialyzed extract to give a reaction rate of about one-half that obtained in the presence of excess glucose. From a glucose determination on the extract the final glucose concentration in the cell was estimated to be $5 \cdot 10^{-5}$ M. The formation of glucose-6-*P* is considerably reduced by withholding ATP (line 4) and a comparable control can be obtained by withholding glucose from a dialyzed preparation. One does not observe maximum rates in the absence of a large excess of glucose-6-*P* dehydrogenase (line 2) even though the endogenous hexosemonophosphate shunt

enzymes have a high capacity for oxidizing added glucose-6-*P* (line 5). The data in the last line demonstrate that the reaction rate is proportional to the volume of adipose extract assayed between 0.02 and 0.05 ml.

The affinity of adipose tissue hexokinase for glucose was estimated using the isotopic assay procedure, and a typical LINEWEAVER-BURK plot is shown in Fig. 1.

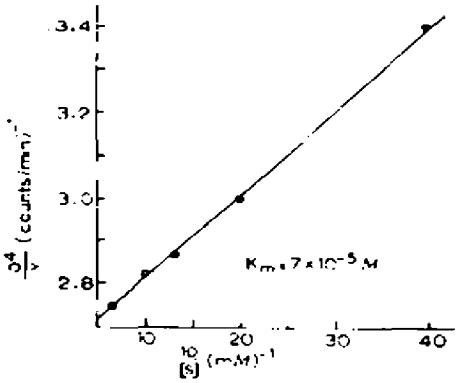

Fig. 1. LINEWEAVER-BURK plot. Abscis: glucose concentration.

The reaction is expressed as counts/min of barium carbonate at "infinite thickness". The data are consistent with an apparent Michaelis constant for glucose of 0.07 mM. Values as high as 0.10 mM have been obtained with a greater scatter of points. The experiment was repeated using fructose (Fig. 2) and this sugar was found to have a much lesser affinity for the enzyme with an apparent MICHAELIS constant on the order of 3 mM.

Competition experiments with glucose and fructose suggested that a single hexokinase phosphorylates both sugars. In a typical experiment in which NADPH formation was measured, glucose and fructose together (each at a final concentration of 0.05 M) yielded an increase in absorbancy per minute of 0.056; glucose alone (0.1 M), 0.060; fructose alone (0.1 M), 0.080. It is evident that the total reaction

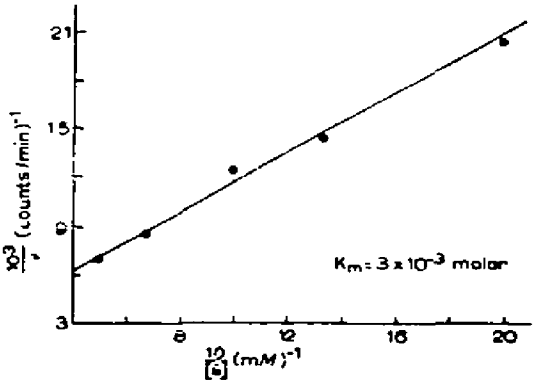

Fig. 2. LINEWEAVER-BURK plot. Abscis: fructose concentration.

rate in the presence of saturating concentrations of both sugars together lies near the rates observed with each sugar separately, and a summation of rates is not obtained. These observations constitute at least suggestive evidence that we are dealing here with a single phosphorylating enzyme rather than two enzymes.

In order to learn whether one sugar is preferentially phosphorylated when both are available, the experiment whose results are outlined in Table II was carried out.

TABLE II

COMPETITION BETWEEN GLUCOSE AND FRUCTOSE FOR PHOSPHORYLATION BY ADIPOSE HEXOKINASE

The phosphorylation rate of each sugar alone and in combination was determined by the isotopic assay method described in the experimental section. The radioactivity in the CO_2 arising from C-1 of the labeled hexose is given in column 3 as counts/min per standard planchet. For further details see text.

	Concentration of unlabeled hexose (mM)	Counts/min	Per cent inhibition of phosphorylation	
			Glucose	Fructose
4 mM [1-^{14}C]glucose	0	2660	0	—
	2.0	2870	0	—
+ fructose	20.0	2830	0	—
4 mM [1-^{14}C]fructose	0	566	—	0
	0.2	209	—	63
+ glucose	2.0	16	—	97

The phosphorylation rate of the labeled hexose was estimated by measuring the incorporation of C-1 into the respiratory CO_2 as described earlier. The labeled CO_2 was isolated with 0.7 mmole of unlabeled sodium carbonate and counted as barium carbonate. The counts/min obtained, corrected for background and zero time controls, are shown in column 3 of Table II. It is apparent from the data in the first 3 lines of the table that glucose phosphorylation is not inhibited by fructose, even when the latter sugar is present at 10 times the concentration of the former. On the other hand, glucose even at low concentrations is seen to dramatically inhibit fructose phosphorylation (bottom 3 lines). These results are consistant with the observed apparent Michaelis constants for the two sugars.

Neither ITP nor UTP can replace ATP for the phosphorylation of glucose or fructose. CTP and GTP were also found to be inactive when tested with glucose, but these compounds were not tested with fructose.

In keeping with the metal ion requirements of other hexokinases, the adipose enzyme is stimulated by magnesium ions, and also by manganese ions.

DISCUSSION

The results of the present work demonstrate that adipose tissue hexokinase is similar to the brain enzyme in its affinity for fructose, although it has a somewhat lower affinity for glucose. Thus, SOLS AND CRANE[13] have reported the following constants for brain hexokinase: K_m fructose, 1.6 mM; glucose, 0.008 mM. The corresponding

constants here estimated for adipose hexokinase are: fructose, 3 mM; glucose, 0.07 mM. Further similarities between the two enzymes are seen in the marked inhibition of fructose phosphorylation in the presence of glucose[8].

In estimates of the affinity of adipose tissue hexokinase for fructose it is possible that even after dialysis of the extract sufficient glucose may remain to effectively inhibit fructose phosphorylation. In this event, the presence of traces of glucose would be expected to produce an apparent increase in the measured K'_m (fructose) by a factor of $(1 + i/K_i)$, where i is the effective glucose concentration and K_i is the inhibitor constant. If K_i is sufficiently small, then the fructose constant herein reported may be high by a factor of 2 or 3. More precise measurements of the K_m for fructose and the kinetics of fructose phosphorylation must await purification of the enzyme.

The high affinity of adipose tissue hexokinase for glucose is not surprising in view of the fact that this tissue is extremely active metabolically and is capable of rapidly converting glucose to glyceride-glycerol and glycogen[14]. However, as was pointed out earlier, the intact, isolated epididymal fat pad of the rat metabolizes increasing amounts of glucose with increasing glucose concentration of the medium. In other words, maximum glucose utilization is not observed at "physiological" concentration *in vitro*. This effect cannot be ascribed to a limiting reaction with the hexokinase since it has been demonstrated at glucose concentrations far above that required to saturate the enzyme. The phenomenon in question is more likely due to other factors, such as transport across the cell membrane as has been described by PARK and others for heart muscle[15]. Also, there remains the possibility that an accumulation of glucose-6-*P* may inhibit glucose phosphorylation as was demonstrated for brain hexokinase[16]. Product inhibition by glucose-6-*P* was not investigated in the present work; nevertheless, possible interference from this source was circumvented by carrying out the hexokinase assay under conditions which prevent the accumulation of this intermediate.

RUD has studied the hexokinase activity extractable from adipose tissue of mice and humans by a method similar to ours[3]. His values for normal grey mice correspond to the phosphorylation (at 24°) of 500 μmoles glucose/min/g nitrogen; for human subcutaneous adipose tissue, 70 μmoles/min/g nitrogen. Although the level of this enzyme in human adipose tissue is comparable to what has been found in the epididymal fat pad of the rat, the hexokinase activity of mouse adipose tissue is seen to be about 10-fold higher, probably reflecting the rapid metabolism of this animal.

Estimates of the soluble hexokinase activity of rat liver, using the present assay method, lie in the vicinity of 0.40 μmole of glucose phosphorylated/min/g (fresh weight) of tissue[7]. Assuming a liver nitrogen content of approx. 3%, the hexokinase activity per gram of nitrogen is 13 μmoles/min. By comparison it has been seen that adipose tissue extracts phosphorylate at least 30 μmoles of glucose/g nitrogen/min. When compared on this basis, the level of hexokinase activity of adipose tissue is at least of the same order of magnitude as that of liver. WEBER *et al.*[2] have determined the activities of several other enzymes of the glycolytic pathway from rat adipose tissue and have compared them with their counterparts in liver. Their results, to which the present hexokinase data may be added, generally indicate that the levels of many of these enzymes in the two tissues are similar. If

extractable tissue enzyme activities may be taken as some indication of metabolic activity, then the high metabolic activity of adipose tissue is reemphasized.

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