present in fraction C, is a possible explanation of the equal activation by both nucleotides. Atebrin and riboflavin inhibit many flavoprotein enzymes (Mahler, 1955). Thus the reversible inhibitions produced by these compounds provide supplementary evidence for the participation of a flavin in the biological reduction of folic acid. No indication could be obtained that fraction B was a flavin enzyme.

SUMMARY

1. A soluble enzyme system which reduces folic acid to tetrahydrofolic acid has been extracted from acetone-dried sheep liver. The component that reduces folic acid to dihydrofolic acid has been partially separated from that which reduces dihydrofolic acid to tetrahydrofolic acid.

2. The step that converts folic acid into dihydrofolic acid is mediated by a flavin enzyme in which either FMN or FAD can function as the active moiety.

3. Aminopterin preferentially inhibits the reduction of dihydrofolic acid to tetrahydrofolic acid.

4. It is concluded (*a*) that the conversion of folic acid into tetrahydrofolic acid is brought about by a two-step reduction, in which dihydrofolic acid is an intermediate, and (*b*) that each step is catalysed by a distinct enzyme.

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REFERENCES

Braganca, B. M. & Kenkare, U. W. (1959). *Nature, Lond.*, **184**, 1488.
Bratton, A. C. & Marshall, E. K. (1939). *J. biol. Chem.* **128**, 537.
Futterman, S. (1957). *J. biol. Chem.* **228**, 1031.
Futterman, S. & Silverman, M. (1957). *J. biol. Chem.* **224**, 31.
Horecker, B. L. & Kornberg, A. (1948). *J. biol. Chem.* **175**, 385.
Kalckar, H. M. (1947). *J. biol. Chem.* **167**, 461.
Kaplan, N. O. & Lipmann, F. (1948). *J. biol. Chem.* **37**, 174.
Keilin, D. & Hartree, E. F. (1938). *Proc. Roy. Soc.* B, **124**, 397.
Mahler, H. R. (1955). In *Methods in Enzymology*, vol. 2, p. 688. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Mahler, H. R. (1956). *Advanc. Enzymol.* **17**, 233.
Mueller, G. C. & Miller, J. A. (1950). *J. biol. Chem.* **185**, 145.
Negelein, E. & Brömel, H. (1939). *Biochem. Z.* **300**, 225.
Nicholas, D. J. D. & Nason, A. (1954). *J. biol. Chem.* **211**, 183.
Ochoa, S. (1955). In *Methods in Enzymology*, vol. 1, p. 699. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Osborn, M. J., Freeman, M. & Huennekens, F. M. (1958). *Proc. Soc. exp. Biol., N.Y.*, **97**, 429.
Osborn, M. J. & Huennekens, F. M. (1958). *J. biol. Chem.* **233**, 969.
Peters, J. M. & Greenberg, D. M. (1958). *J. Amer. chem. Soc.* **80**, 6679.
Zakrzewski, S. F. & Nichol, C. A. (1960). *J. biol. Chem.* **235**, 2984.

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Transport and Phosphorylation of Sugars in Adipose Tissue

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A wide interest is now centred on white adipose tissue as a major target of insulin action. The primary effect of insulin appears to be a stimulation of the transformation of extracellular glucose into intracellular glucose 6-phosphate (Jeanrenaud & Renold, 1959), although evidence has also been reported pointing to some further effect on metabolic patterns (Leonards & Landau, 1960). Insulin also stimulates the utilization of mannose and fructose by the epididymal fat pad (Ball & Cooper, 1960; Wood, Leboeuf, Renold & Cahill, 1961). Attempts to ascertain whether a mechanism of sugar transport occurs in adipose tissue have been hindered by the lack of definite information on the enzyme(s) involved in the phosphorylation of sugars in this tissue (Ball & Cooper, 1960; Wood *et al.* 1961).

The present paper reports the identification of a hexokinase in the adipose tissue of the rat and the study of its substrate specificity. The uptake of metabolizable hexoses by the intact tissue has a substrate specificity different from that of its hexokinase, in a way that indicates the occurrence of a stereospecific transport step before phos-

phorylation. The relationship between hexokinase activity and hexose uptake suggests that insulin activates the rate of transport without affecting the hexokinase.

EXPERIMENTAL

Materials

Epididymal fat pads of male albino rats weighing 150–250 g. were used throughout these studies. For enzymic assays the pads were homogenized with 3–39 vol. (as indicated) of mM-EDTA, pH 7·0, in a Kontes dual grinder. For the experiments with intact tissue the animals were starved for 12 hr. in a darkened room and handled in the manner described by Winegrad & Renold (1958).

Mannoheptulose was obtained from Dr E. Simon. Other sugars were obtained as described by Sols, de la Fuente, Villar-Palasí & Asensio (1958). ATP, ADP, glucose 6-phosphate and mannose 6-phosphate were supplied by the Sigma Chemical Co., and Glucostat by the Worthington Biochemical Corp. Glucagon-free insulin, from the Eli Lilly Research Laboratories, was obtained from Professor J. L. R.-Candela.

The phosphofructokinase preparation was obtained as described by Taylor (1951) and freed from hexokinase activity by the heating procedure described by Crane & Sols (1953).

An enzyme unit is defined as the amount that can transform 1 μmole of substrate/min. at 30°.

Methods

Enzyme assays. Assays of hexokinase in homogenates were carried out with a modification of the glucose-oxidase method (Sols *et al.* 1958) as follows. In a test tube were put 0·1 ml. of a substrate mixture of 2 mM-glucose and 30 mM-ATP–$MgCl_2$ in 20 mM-potassium phosphate buffer, pH 7·5 (containing 2 μg. of bromothymol blue/ml.); 0·05 ml. of 0·5 M-NaF; 0·05 ml. of a phosphofructokinase preparation containing about 65 units/ml.; and 0·1 or 0·2 ml. of adipose-tissue homogenate, prepared with 29 or 39 vol. of EDTA as described. Incubations were carried out at 30° for 0, 15, 30 or 60 min. The reaction was stopped by the addition of 1·0 ml. of 20 mM-EDTA, pH 7·0. The mixture was cooled and centrifuged, and 1·0 ml. was taken (without removing the floating fat layer formed) for the evaluation of residual glucose [by the addition of 1·0 ml. of a glucose-oxidase reagent containing (per ml.): 2·5 mg. of glucose oxidase, 100 μg. of peroxidase and 200 μg. of *o*-dianisidine in a medium of 50 mM-tris–10 mM-EDTA–50 mM-potassium phosphate–0·2% Triton X-100, pH 7·0]. The extinction at 420 mμ was measured immediately after the addition of the glucose-oxidase reagent and after 2 hr. at 30°. The difference between both values was referred to a standard with 0·1 ml. of substrate mixture, 0·9 ml. of 20 mM-EDTA and 1·0 ml. of the glucose-oxidase reagent read against a reagent blank.

When the hexokinase assays were carried out in crude extracts (see below), the centrifuging step in the above procedure was omitted. When this method was used to observe the effect on glucose phosphorylation of sugars that are marginal substrates of the glucose oxidase, colour development was stopped after 10 min. by the addition of 0·1 ml. of 40% (w/v) KOH; in this way the interferences in the final colour were less than 1%.

Hexokinase activity in extracts was also studied with the photometric-indicator and the spectrophotometric methods of Crane & Sols (1955).

Adenosine-triphosphatase activity was assayed by estimation of the inorganic phosphate (Fiske & Subbarow, 1925) liberated at pH 7·0 (0·12 M-tris buffer) from 4 mM-ATP–$MgCl_2$. Glucose phosphate isomerase was assayed by the borate method of Sols & de la Fuente (1961). Mannose phosphate isomerase was assayed in crude extracts by following spectrophotometrically the reduction of 0·15 mM-NADP in the presence of 0·3 mM-mannose 6-phosphate and 30 mM-tris, pH 7·5 (the extracts have excess of glucose phosphate isomerase and glucose 6-phosphate dehydrogenase).

Protein. Protein was estimated as described by Lowry, Rosebrough, Farr & Randall (1951).

Hexose uptake. The uptake of hexoses by intact tissue was observed either directly by estimation of the disappearance of sugar from the medium or indirectly by manometric observation of the net increase in CO_2 production. The experiments on the disappearance of sugar were carried out by incubation of fat pads for 5 hr. at 37°, with shaking, in open test tubes containing 15 μmoles of hexose in 0·5–5 ml. of Krebs & Henseleit (1932) bicarbonate buffer, containing 5 milliunits of insulin/ml. At the end of this incubation cold 0·9% NaCl was added to give a final volume of 15 ml. The tubes were mixed by inversion, and the residual sugar was estimated in samples of the medium. Glucose was estimated with glucose oxidase (Sols & de la Fuente, 1957), fructose with cysteine–carbazole (Dische & Borenfreund, 1951) and mannose by the method of Somogyi (1952).

The utilization of hexoses as measured by their effect on total gas exchange in the presence of insulin (5 milliunits/ml.) was studied by the method of Ball, Martin & Cooper (1959). The hexose was added from a side arm. To compare the rates with different metabolizable hexoses, the experiment was started with one hexose, and then, after establishment of a steady state, an excess of another hexose was added from a second side arm. This procedure served to circumvent the variability among pads. A similar approach was used to measure the inhibition of hexose utilization by non-metabolizable analogues.

Preparation of hexokinase

Pooled epididymal fat pads were homogenized with 3 vol. of mM-EDTA, by treatment for 3 min. in a Waring Blendor. The homogenates were filtered through gauze. The clarified homogenates were centrifuged at 8000*g* for 30 min. After removal of the fat layer a crude extract was obtained containing about 70% of the enzyme accompanied by adenosine-triphosphatase activity. The crude extract was further centrifuged at 100 000*g* for 1 hr. The supernatant from the high-speed centrifuging was essentially free of adenosine triphosphatase but still contained about 60% of the hexokinase. Glucose phosphate isomerase and glucose 6-phosphate dehydrogenase were still present in excess over the hexokinase. The hexokinase was concentrated by precipitating it with ammonium sulphate between 33 and 66% saturation in the presence of 10 mM-EDTA, pH 7·0, dissolving the precipitate in a small volume of mM-EDTA and dialysing the solution against mM-EDTA. The concentrated enzyme was stored at −20°. It could be freeze-dried without loss of activity. All operations were carried out at 0–4°.

RESULTS

Enzymic activities in homogenates and extracts of adipose tissue

Phosphorylation of sugars. Fresh homogenates of epididymal fat pads phosphorylated glucose at a rate of about 0·6 μmole/g./min. at 30°, as measured with the glucose-oxidase method. The reaction was not linear with time. The fact that the decrease in rate, as shown in Fig. 1, was related to the extent of phosphorylation rather than to the length of incubation indicates that the enzyme is stable in the assay conditions and suggests inhibition by accumulated glucose 6-phosphate (cf. Crane & Sols, 1953). This interpretation is supported by the fact that the reaction approaches linearity if either an excess of phosphofructokinase is added or if 2-deoxyglucose is substituted for glucose as substrate. The phosphorylation of glucose by homogenates could be inhibited, in decreasing order of efficiency, by mannose, *N*-acetylglucosamine and fructose, as observed with the glucose-oxidase method (at initial inhibitor:substrate ratios of 2, 30 and 100, the inhibition of glucose phosphorylation was 45, 78 and 42% respectively). These results suggest that the phosphorylation of glucose is carried out by a common hexokinase.

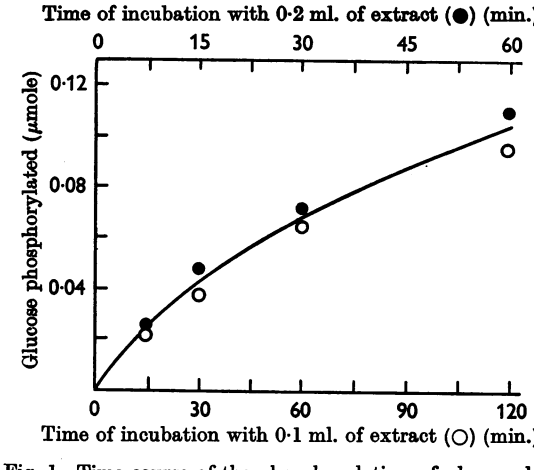

Fig. 1. Time-course of the phosphorylation of glucose by a fresh extract of rat-epididymal adipose tissue. The glucose-oxidase method as described in the text was used with 0·1 ml. (○) or 0·2 ml. (●) of extract.

In crude extracts hexokinase activity could also be measured by the spectrophotometric method, which gave values similar to those of the glucose-oxidase method. The substitution of glucose 1-phosphate for glucose plus ATP–magnesium chloride as substrate in the conditions of the spectrophotometric method greatly decreased the rate of NADP reduction. These observations support the view that the primary product of glucose phosphorylation by homogenates of adipose tissue is the 6-phosphate.

No action on galactose could be detected with the spectrophotometric method in the presence of 0·1 mM-UDP-glucose and glucose 1,6-diphosphate. Adenosine triphosphatase prevents the use of the photometric-indicator method with crude extracts but not with the supernatants from high-speed centrifuging. Phosphorylation of fructose by the latter could be strongly inhibited by *N*-acetylglucosamine, which suggests the absence of any specific fructokinase.

These results suggest that the white adipose tissue has a hexokinase as the only enzyme able to phosphorylate hexoses.

Effects of starvation and insulin. Tissue variability was high, but most cases gave values between 0·4 and 1·0 unit/g. wet wt., with an average value of 0·6. Starving of the rats for up to 3 days caused a decrease in the rate of glucose phosphorylation/mg. of protein as compared with control fed rats. This decrease was proportionately less than that of the wet weight of the tissue, but highly significant (Table 1). Incubation of epididymal fat pads with 5 mM-glucose in the presence or absence of insulin did not reveal any effect of the hormone on the hexokinase activity of the homogenates (Table 2).

Other related enzymes. Crude extracts were found to contain, in addition to adenosine triphosphatase (about 1·4 units/g.), glucose 6-phosphate

Table 1. *Effect of starvation on the hexokinase activity of rat adipose tissue*

Hexokinase assays were carried out in homogenates of epididymal fat pads, taken from fed rats and from rats starved for 3 days (the numbers of animals are given in parentheses), with the glucose-oxidase method as described in the Experimental section. The results are given as means ± S.E.M.

	Wet wt. of tissue (mg.)	Hexokinase (milliunits/g. wet wt.)	Hexokinase (milliunits/mg. of protein)
Fed rats (11)	223±15	620±20	76±9
Starved rats (8)	32±3	1720±200	25±1·7
Significance of difference (*P*)			< 0·001

dehydrogenase (about 2·0 units/g.) and glucose phosphate isomerase (about 8·0 units/g.). These findings are in agreement with the observations of Weber, Barnejee & Ashmore (1960). Mannose phosphate isomerase was also present (average value 0·3 unit/g.).

Properties of the hexokinase of adipose tissue

The phosphorylation of 2-deoxyglucose by the crude extract was inhibited 53% by the addition of glucose 6-phosphate at a final concentration of 0·75 mM. The same degree of inhibition was also observed with glucose as substrate.

With the spectrophotometric method, half the maximal rate was obtained at a concentration of ATP of 0·5 mM, in the presence of 0·5 mM-magnesium chloride. Assuming the substrate to be a ATP–magnesium chloride complex, its Michaelis constant, K_m, would be about 0·3 mM, slightly higher than that of brain hexokinase. The enzyme activity was inhibited by added ADP–magnesium chloride, in a way apparently similar to the brain hexokinase (Sols & Crane, 1954*a*).

The Q_{10}° in the 30–40° range is 1·4.

Substrate specificity. To ascertain whether a stereospecific transport was involved in the uptake of glucose by adipose tissue, it was important to have a detailed knowledge of the substrate specificity of its hexokinase. Twelve compounds structurally related to glucose were chosen for this study from among those known to be substrates and competitive inhibitors of the hexokinases of brain (Sols & Crane, 1954*b*) and yeast (Sols *et al.* 1958). The preparation free of adenosine triphosphatase was used throughout.

Relative phosphorylation rates were studied with the photometric-indicator method. Interference by the buffering capacity of glucosamine was circumvented as described by Ruiz-Amil & Sols (1961). The photometric-indicator method was also used to determine the K_m for fructose and for 1,5-anhydro-D-glucitol, and the K_i for *N*-acetylglucosamine. The K_m for fructose thus obtained served as a basis for the determination of the K_m for glucose from the inhibition of the phosphorylation of the latter by fructose with the glucose-oxidase method. The K_m (or K_i) for other compounds relative to that of glucose was studied with the same method, except for 2-deoxyglucose, the K_m for which was determined, with the photometric-indicator method, on the basis of the inhibition of its phosphorylation by *N*-acetylglucosamine. The results are summarized in Table 3.

Table 2. *Hexokinase activity of rat-epididymal fat pads incubated with or without insulin*

Epididymal fat pads from rats starved for 12 hr. (the numbers of animals are given in parentheses) were incubated in 4·0 ml. of Krebs–Henseleit bicarbonate buffer in the presence of 5 mM-glucose, with or without 20 milliunits of insulin, for 60 min. at 37°. After incubation, the pads were washed three times with mM-EDTA and homogenized with 29 vol. (w/v) of mM-EDTA, pH 7·0. Hexokinase assays were carried out as described in the Experimental section. The results are given as means ± S.E.M.

	Hexokinase	
	(milliunits/g. wet wt.)	(milliunits/mg. of protein)
Without insulin (8)	260 ± 40	55 ± 6·0
With insulin (8)	240 ± 40	66 ± 9·2

Table 3. *Substrate specificity of the hexokinase of adipose tissue*

Assays were carried out as described in the text. Where no relative maximal rate is given, this indicates an undetectable rate (< 0·05 at a concentration of 20 mM). The phosphorylation coefficient is defined as

$$\frac{V\text{ (substrate)}}{V\text{ (glucose)}} \times \frac{K_m\text{ (glucose)}}{K_m\text{ (substrate)}}.$$

This coefficient (Sols & Crane, 1954*b*) is an expression of the relative suitability of substrates when compared either at a concentration low enough for first-order kinetics or in mixtures.

Modified at C atom no.	Compound	K_m (mM)	Relative maximal rate	Phosphorylation coefficient
—	Glucose	0·03	1·0	1·0
1	1,5-Anhydro-D-glucitol	50	0·5	3×10^{-4}
1,2	Mannoheptulose	0·2	—	< 0·01
1,2	Fructose	3	1·5	0·015
2	Mannose	0·05	1·6	1·0
2	2-Deoxyglucose	0·03	1·5	1·5
2	Glucosamine	0·5	0·7	0·04
2	Glucosone	0·01	~0·5	~1·5
2	*N*-Acetylglucosamine	0·13	—	< 0·01
2	2-*C*-Hydroxymethylglucose	3	—	$< 5 \times 10^{-4}$
4	Galactose	> 100	—	$< 1 \times 10^{-4}$
6	Xylose	4	—	$< 4 \times 10^{-4}$
6,2	Lyxose	5	—	$< 3 \times 10^{-4}$

Uptake of metabolizable sugars by intact adipose tissue

A maximally stimulating dose of insulin (5 milli-units/ml.) was used throughout these studies to increase sugar uptake and make possible the use of the manometric method.

A series of potential substrates was assayed with the manometric method at concentrations of from 5 to 50 mM. Only glucose, mannose and fructose were readily utilized. In addition, glucosone gave an activity of about 5 % of that with glucose at a concentration of 5 mM. Galactose, at concentrations up to 50 mM, was consistently shown to be not utilized, if the contaminating glucose impurity is taken into account. Neither glucose 1-phosphate nor glucose 6-phosphate was appreciably utilized.

The effect of substrate concentration on the rate of utilization was studied with the manometric method, testing successively two concentrations of each sugar on the same fat pad, as illustrated in Fig. 2. By appropriate variation of the lower concentration, the following apparent K_m values were obtained: for glucose, 1 mM, for mannose, 3 mM, and for fructose, 20 mM. To obtain the relative maximal rates, the same principle of making comparisons in a single pad was used. Fig. 3 gives a typical result. The average results of maximal relative rates for glucose, mannose and fructose were 1·0, 0·6 and 1·0 respectively.

To ascertain whether the net increase in the production of carbon dioxide was indeed a faithful measure of the rate of hexose utilization, the above results were checked by determining the disappearance of substrate, as described in the Experimental section. The results were in agreement with the above values. The maximal rate of glucose utilization was 0·25 ± 0·026 μmole/g. fresh wt./min. at 37°.

Effect of non-metabolizable analogues on hexose utilization by intact adipose tissue

The above results on glucose, mannose and fructose utilization by intact adipose tissue suggested a limiting transport. In an attempt to demonstrate more marked differences between the specificities of intact tissue and its hexokinase, the glucose analogues with a good affinity for the

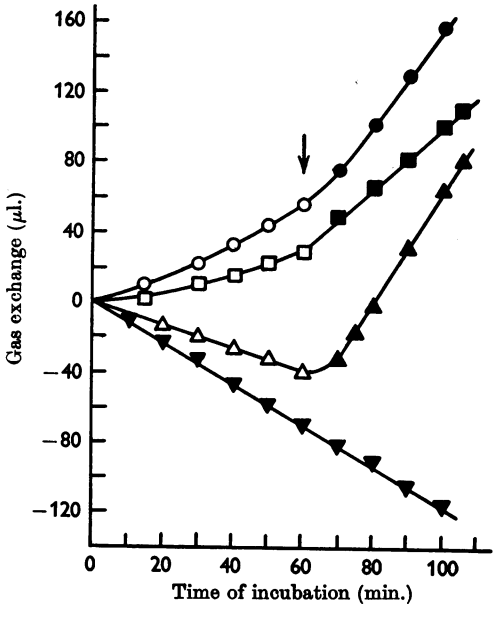

Fig. 2. Effect of a change in concentration on the rates of utilization of glucose, mannose and fructose by rat-epididymal fat pads. The manometric method as described in the text was used, with two pads weighing 500 ± 50 mg. per vessel. ▼, Control without sugar; ○, ●, glucose; □, ■, mannose; △, ▲, fructose. Each sugar was added from a side arm to give a final concentration of 3 mM at 10 min. (○, □, △). At 60 min. more sugar was added from a second side arm to give a final concentration of 0·2 M (●, ■, ▲).

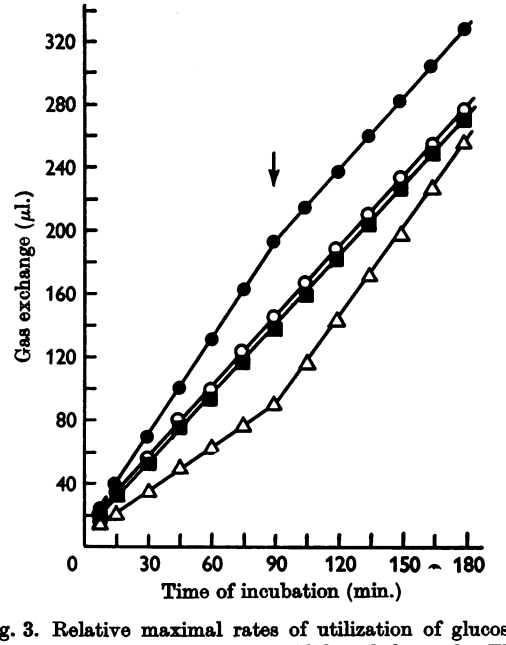

Fig. 3. Relative maximal rates of utilization of glucose, mannose and fructose by rat-epididymal fat pads. The manometric method as described in the text was used, with one pad weighing 350 ± 50 mg. per vessel. The second sugar (as indicated below) was added from a side arm at 90 min. ●, Glucose (30 mM) alone and plus mannose (0·3 M); ■, fructose (0·3 M) alone and plus glucose (0·1 M); △, mannose (30 mM) alone and plus glucose (0·3 M); ○, control of glucose (30 mM and 0·4 M). There was a burst of CO_2 evolution within the first 5 min. after the addition of the second sugar, which is not plotted in the Figure.

Table 4. *Effects of non-physiological substrates and competitive inhibitors of hexokinase on the utilization of 25 mM-fructose by intact adipose tissue*

Assays were carried out as described in the text. The percentage inhibitions of fructose utilization expected for hexokinase were calculated on the basis of the K_m values (Table 3) and concentration ratios.

Compound added	Amount added (μmoles/ml.)	Inhibition of fructose utilization (%)	
		Found	Expected for hexokinase
Glucosone	5	80	98
Glucosamine	8	64	66
2-Deoxyglucose	5	92	95
2-*C*-Hydroxymethylglucose	50	65	66
Xylose	50	31	61
Lyxose	80	35	66
Mannoheptulose	30	53	94
N-Acetylglucosamine	50	0*	97

* Within 5% sensitivity.

hexokinase were tested for their ability to inhibit hexose utilization by the intact tissue. Fructose was chosen as the experimental substrate because its higher K_m makes it easier to detect competitive inhibitions. The results are listed in Table 4. The degree of inhibition by glucosone, glucosamine and 2-deoxyglucose is of little significance, since they are good substrates of hexokinase and their phosphorylation products may contribute to the inhibition of hexose metabolism. The competitive inhibitors of hexokinase do not have this limitation. The marked inability of *N*-acetylglucosamine (which is neither phosphorylated nor metabolized) to inhibit hexose utilization by intact tissue is particularly significant, since other competitive inhibitors of hexokinase, especially 2-*C*-hydroxymethylglucose, do inhibit hexose utilization by intact tissue.

Phlorrhizin inhibited the utilization of 25 mM-fructose 55 and 72% at concentrations of 1 and 2 mM respectively. At these concentrations it did not inhibit the hexokinase activity in homogenates.

DISCUSSION

The hexokinase of rat-epididymal adipose tissue has a pattern of substrate specificity and inhibition by products that is typical of animal hexokinases (cf. Crane & Sols, 1955). Despite the broad parallelism, there are some differences in specificity between the hexokinase of rat adipose tissue and that of calf brain. The hexokinase of adipose tissue phosphorylates mannose and 2-deoxyglucose faster than glucose. The same has been reported for the hexokinases in crude extracts of a sea urchin (Krahl, Keltch, Walters & Clowes, 1954) and of the honey-bee (Sols, Cadenas & Alvarado, 1960) [but not with purified honey-bee hexokinase (Ruiz-Amil, 1962)]. Extracts of perirenal and mesenteric adipose tissue showed similar apparent affinities for glucose, mannose, fructose and *N*-acetylglucosamine as that of the epididymal fat pad. Accordingly, it is likely that the enzyme here described is common to white adipose tissue irrespective of localization.

The relative efficiencies of intact adipose tissue with glucose, mannose and fructose show quantitative differences with respect to the relative efficiencies of its hexokinase to phosphorylate these sugars. These differences suggest the occurrence of a stereospecific step before hexokinase in the utilization of hexoses by intact tissue. Nevertheless, the differences are not very considerable and are open to question since (*a*) a supply of ATP could prevent fructose utilization from exceeding that of glucose, and (*b*) the dependence of mannose utilization on mannose phosphate isomerase (which, kinetically, is potentially limiting) could prevent mannose utilization from reaching the glucose rate.

The above uncertainties with respect to the involvement of a transport process before phosphorylation seem to be removed by the results obtained with competitive inhibitors of the hexokinase. Since some of them can easily inhibit hexose utilization by intact adipose tissue, the complete inability of *N*-acetylglucosamine to do so can only be due to the existence of a stereospecific transport, for which *N*-acetylglucosamine is inert, in a tissue otherwise virtually impermeable to sugars.

The limited ability of mannoheptulose to inhibit fructose utilization by intact adipose tissue supports the conclusion of a transport step with a specificity different from that of the hexokinase. The fact that Chernick, Scow, Simon & Stricker (1962) did not obtain any significant inhibition of glucose utilization by mannoheptulose is consistent with our results, since the K_m of adipose tissue for glucose is considerably smaller than that for fructose. Xylose and lyxose are not markedly discriminated between by the intact tissue.

Ascites-tumour cells have been shown to be impermeable to *N*-acetylglucosamine but permeable to lyxose (Crane, Field & Cori, 1957). *N*-Acetylglucosamine does not inhibit fructolysis by intact cells whereas lyxose does so (Yushok, 1958), although the former is a much stronger inhibitor than the latter of the hexokinase of the tumour (McComb & Yushok, 1959), as happens also with adipose tissue and its hexokinase. This parallelism between adipose tissue and ascites tumour supports the conclusion that, if *N*-acetylglucosamine is unable to inhibit fructose utilization by intact tissue, it must be because of the requirement of a stereospecific transport for which it is not a substrate. In contrast, in liver slices, which seem to be freely permeable to hexoses (Cahill, Ashmore, Earle & Zottu, 1958), *N*-acetylglucosamine competitively inhibits glucose utilization (Spiro, 1958).

The hexokinase activity of adipose tissue is greater than that required to account for the maximal rate of glucose utilization under insulin stimulation. On prolonged starvation there is a significant decrease in the hexokinase content of the epididymal fat pad despite the apparent increase per unit of fresh weight. Nevertheless, the lack of effect of insulin in the hexokinase content of fat pads suggests that there is no immediate relationship between hexokinase activity and insulin. The primary effect of insulin would then be to activate a limiting transport rather than the phosphorylation of sugar. Our results do not support the conclusions of MacLeod, Brown & Lynn (1960) of a stimulation of hexokinase by insulin.

SUMMARY

1. Phosphorylation of glucose, mannose and fructose by homogenates of rat-epididymal adipose tissue is carried out by a hexokinase. Its substrate specificity is broadly similar to that of brain hexokinase, although it can phosphorylate mannose and 2-deoxyglucose faster than glucose. The enzyme is inhibited by glucose 6-phosphate and ADP.

2. Mannose phosphate-isomerase activity has also been identified in adipose tissue. In contrast with the glucose phosphate isomerase it does not appear to be in excess over the hexokinase.

3. The relative efficiencies of utilization of glucose, mannose and fructose by intact tissue do not parallel those of phosphorylation by its hexokinase. Moreover, hexose utilization by intact adipose tissue can be strongly inhibited by certain competitive inhibitors of the hexokinase, especially 2-*C*-hydroxymethylglucose, but not by *N*-acetylglucosamine. These results indicate the occurrence in adipose tissue of a stereospecific transport of sugars before their phosphorylation.

4. The relationships between hexokinase content and hexose uptake in various conditions suggest that insulin activates the rate of transport without affecting the hexokinase.

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REFERENCES

Ball, E. G. & Cooper, O. (1960). *J. biol. Chem.* **235**, 584.
Ball, E. G., Martin, D. B. & Cooper, O. (1959). *J. biol. Chem.* **234**, 774.
Cahill, G. F., jun., Ashmore, J., Earle, A. S. & Zottu, S. (1958). *Amer. J. Physiol.* **192**, 491.
Chernick, S. S., Scow, R. O., Simon, E. & Stricker, F. A. (1962). *Proc. Soc. exp. Biol., N.Y.*, **109**, 589.
Crane, R. K., Field, R. A. & Cori, C. F. (1957). *J. biol. Chem.* **224**, 649.
Crane, R. K. & Sols, A. (1953). *J. biol. Chem.* **203**, 273.
Crane, R. K. & Sols, A. (1955). In *Methods in Enzymology*, vol. 2, p. 277. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Dische, Z. & Borenfreund, E. (1951). *J. biol. Chem.* **192**, 583.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Jeanrenaud, B. & Renold, A. B. (1959). *J. biol. Chem.* **234**, 3082.
Krahl, M. E., Keltch, A. K., Walters, C. P. & Clowes, G. H. (1954). *J. gen. Physiol.* **38**, 31.
Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
Leonards, J. R. & Landau, B. R. (1960). *Arch. Biochem. Biophys.* **91**, 194.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
MacLeod, R. M., Brown, R. & Lynn, W. S. (1960). *J. clin. Invest.* **39**, 1008.
McComb, R. B. & Yushok, W. D. (1959). *Biochim. biophys. Acta*, **34**, 515.
Ruiz-Amil, M. (1962). *J. Insect Physiol.* **8**, 259.
Ruiz-Amil, M. & Sols, A. (1961). *Biochem. Z.* **334**, 168.
Sols, A., Cadenas, E. & Alvarado, F. (1960). *Science*, **131**, 297.
Sols, A. & Crane, R. K. (1954*a*). *J. biol. Chem.* **206**, 925.
Sols, A. & Crane, R. K. (1954*b*). *J. biol. Chem.* **210**, 581.
Sols, A. & de la Fuente, G. (1957). *Rev. esp. Fisiol.* **13**, 231.
Sols, A. & de la Fuente, G. (1961). *Meth. med. Res.* **9**, 302.
Sols, A., de la Fuente, G., Villar-Palasí, C. & Asensio, C. (1958). *Biochim. biophys. Acta*, **30**, 92.
Somogyi, M. (1952). *J. biol. Chem.* **195**, 19.
Spiro, R. G. (1958). *J. biol. Chem.* **233**, 546.
Taylor, J. F. (1951). In *Phosphorus Metabolism*, vol. 1, p. 104. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins Press.
Weber, G., Barnejee, G. & Ashmore, J. (1960). *Biochem. biophys. Res. Commun.* **3**, 182.
Winegrad, A. I. & Renold, A. E. (1958). *J. biol. Chem.* **233**, 267.
Wood, F. G., jun., Leboeuf, B., Renold, A. E. & Cahill, G. F., jun. (1961). *J. biol. Chem.* **236**, 18.
Yushok, W. D. (1958). *Cancer Res.* **18**, suppl., 379.