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Multiple Forms of Glucose–Adenosine Triphosphate Phosphotransferase in Rat Mammary Gland

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Measurements have been made of the total hexokinase activity and of the relative amounts of types I and II hexokinase in rat mammary gland and at different stages of the lactation cycle. The total hexokinase activity increased during lactation, that of type II increasing to a greater extent than that of type I; the type II/type I activity ratio rose from a pregnancy value of about 1 to a mid-lactation value of 3, returning to 1 on involution. The changes in type II hexokinase activity during the lactation cycle parallel the changes in the insulin sensitivity of mammary-gland tissue. A study of the effect of alloxan-diabetes on mammary-gland hexokinase during the mid-lactation period revealed that, although the total glucose-phosphorylating capacity of the mammary gland was almost unchanged, the relative contributions of type I and type II hexokinases altered, decreasing the type II/type I activity ratio to about 1.

Evidence has accumulated for the existence of multiple forms of glucose–ATP phosphotransferase. Gonzales, Ureta, Sanchez & Niemeyer (1964) separated four different forms of glucose–ATP phosphotransferase from liver by chromatography on DEAE-cellulose columns. They demonstrated that there were three enzymes with a low K_m for glucose, termed hexokinases (EC 2.7.1.1), and one with a high K_m for glucose, this being equivalent to the glucokinase (EC 2.7.1.2) previously shown to be present in liver (Viñuela, Salas & Sols, 1963; Walker, 1963; Sharma, Manjeshwar & Weinhouse, 1963). Katzen & Schimke (1965) also distinguished four hexokinases by vertical starch-gel electrophoresis; these they designated types I–IV in order of increasing mobility, the fastest-moving type IV corresponding to the high-K_m glucokinase. Grossbard & Schimke (1966) have isolated and purified the three low-K_m hexokinases chromatographically and have characterized their kinetic and physical properties.

Having investigated a wide range of tissues Katzen & Schimke (1965) observed that the presence of the type II enzyme appeared to be correlated with the insulin-sensitivity of the tissue. Fat pad, muscle and heart, which contain large amounts of type II enzyme, are highly insulin-sensitive, whereas brain and kidney contain largely type I enzyme and are insensitive to insulin. Further, type II hexokinase was shown to decrease in adipose tissue during starvation (Moore, Chandler & Tettenhorst, 1964) and in alloxan-diabetes (McLean, Brown, Greenslade & Brew, 1966), both associated with a decreased concentration of blood insulin.

This correlation between type II hexokinase and insulin was extended by Katzen (1966), who demonstrated that type II enzyme exists in fat pad as two electrophoretically distinct bands when assayed in the absence of mercaptoethanol and EDTA, the faster-moving type IIa disappearing in the starved animal and in streptozotocin-diabetic animals.

It has been demonstrated (McLean, 1958; Baldwin & Milligan, 1966) that there is a marked increase in hexokinase activity during lactation and this is followed by a sharp fall during the period of declining lactation and involution. Abraham, Cady & Chaikoff (1957) showed that both the oxidation of ^{14}C-labelled glucose and the incorporation of labelled glucose into lipids by lactating mammary gland are stimulated *in vitro* by the presence of insulin in the incubation medium. In contrast, only a small or negligible stimulation of glucose utilization was obtained when insulin was added *in vitro* to mammary-gland slices from pregnant rats (McLean, 1960).

In the present work the relative activities of hexokinase types I and II in mammary gland at different stages of the lactation cycle were determined and an attempt was made to correlate these activities with the insulin-sensitive and insulin-insensitive phases of the lactation cycle. In

addition, the effect of alloxan-diabetes on the hexokinase activity of lactating rat mammary gland was measured.

A preliminary account of part of this work has been published (Walters & McLean, 1966).

METHODS

Materials. Commercial reagents were used with the exception of 6-phosphogluconate dehydrogenase, which was a partially purified preparation from liver, prepared as described by Glock & McLean (1953) and used in the assay of hexokinase.

Animals. Primiparous albino rats of the Wistar strain were used, the litters in all cases being reduced to eight pups. Throughout the experimental period rats were allowed food and water *ad lib.* The litters were weighed daily and the litter weights used as an index of milk production. On the fourth day of lactation, diabetes was induced by the subcutaneous injection of alloxan (20mg./100g. body wt.); these rats were then maintained on 2 units of protamine–zinc–insulin/day. At this stage of the experiment the average weight gain of the whole litters of both control and insulin-treated diabetic mothers was 13g./day. The insulin treatment was stopped 3 days before the rats were killed by cervical dislocation on the ninth or tenth day of lactation, i.e. 5 or 6 days after treatment with alloxan. At this stage the litters of diabetic mothers had ceased to show any gain in weight. The blood sugar concentrations of the mothers were 433 ± 90mg./100ml. compared with $83 \pm 0{\cdot}5$mg./100ml. for the control mothers (means ±S.E.M. for five rats in each group).

Preparation of tissue extracts. The abdominal mammary glands were removed and passed through an ice-cold stainless-steel tissue press (purchased from Climpex Ltd., London, N.W. 7; made to the design of Porterfield, 1960) and then homogenized in twice the weight by volume of 150mM-KCl–5mM-$MgCl_2$–5mM-EDTA–10mM-mercaptoethanol medium adjusted to pH7·4 with $KHCO_3$. The homogenate was centrifuged at 105000*g* for 45min. in a Spinco model L centrifuge. The supernatant fraction was dialysed for 1hr. against the same extraction medium and was used for determination of hexokinase activity, for starch-gel electrophoresis and for protein determination. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Hexokinase activity. This was estimated essentially by the method of Sharma *et al.* (1963) with modifications as described by McLean & Brown (1966). The final glucose concentration was 1·0mM. A unit of enzyme activity is defined as 1μmole of glucose 6-phosphate formed/hr. at 25°. The values of μmoles of NADPH produced were divided by 2 to convert them into moles of glucose 6-phosphate formed, since the assay system contained excess of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, as described by McLean & Brown (1966). Hexokinase types I and II were distinguished by their different stabilities to heat treatment at 45° for 1hr. in the absence of glucose (Grossbard & Schimke, 1966). Type I enzyme was very stable and there was only a small loss of activity, whereas type II enzyme lost over 90% of its activity with the same treatment. The rate of reduction of $NADP^+$ was measured with a Unicam SP.800 recording spectrophotometer with a constant-temperature cell housing and scale-expansion accessory.

Starch-gel electrophoresis. Horizontal starch-gel electrophoresis was carried out at 4° for 16hr. with 10% (w/v) starch gels. The gel buffer consisted of 20mM-sodium barbitone buffer, pH8·6, containing EDTA (1mM) and mercaptoethanol (5mM). The electrode buffer was 60mM-sodium barbitone buffer, pH8·6, containing EDTA (1·7mM) and mercaptoethanol (5mM). The extract was applied to the gel with a pad of Whatman no. 17 filter paper. The gels were sliced and stained as described by Katzen & Schimke (1965). A Joyce–Loebl Chromoscan was used for measurement of the intensity of staining of the hexokinase bands; the gels were either scanned directly or photographed and the photographs scanned by reflectance.

RESULTS AND DISCUSSION

Hexokinase activities at different stages of the lactation cycle. The measurements of the total activity of hexokinase in mammary gland during pregnancy, lactation and involution followed a similar pattern to that shown by McLean (1958) with a different and less direct technique and also by Baldwin & Milligan (1966) with essentially the same method as that used in the present work. From a value of 150 units/total gland weight in pregnancy the activity rose sharply as soon as lactation started, reaching a maximum of 330 units/total gland weight by the twelfth day of lactation. The activity fell during late lactation, returning to 120 units/total gland in involution (see Fig. 1). Preliminary experiments have shown that about one-third of the total hexokinase activity of rat mammary-gland tissue is in the mitochondrial

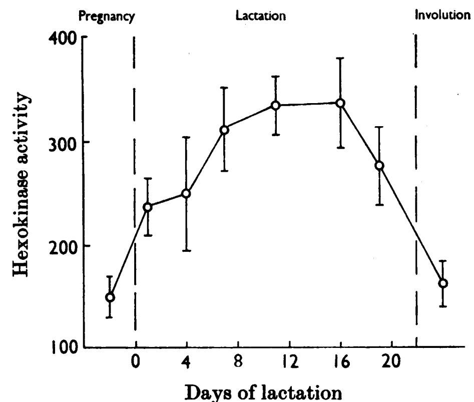

Fig. 1. Hexokinase activity of rat mammary gland during pregnancy, lactation and mammary involution. The results are given as units/total abdominal mammary-gland weight, 1 unit being defined as the amount catalysing the formation of 1μmole of glucose 6-phosphate/hr. at 25°. The vertical lines represent the S.E.M. values; each group contained six animals.

Table 1. *Comparison of the relative amounts of type I and type II hexokinase of mammary gland at different stages of the lactation cycle*

Spectrophotometric values are given as units of hexokinase activity/g. of tissue. Types I and II hexokinase were distinguished by heating the extract at 45° for 1 hr. Integral values, in arbitrary units, were obtained from scans of starch gels developed and stained for the presence of hexokinase. The areas under the peaks were measured with a Joyce–Loebl Chromoscan. For details see the Methods section.

		Hexokinase					
		Spectrophotometric values			Integral values		
Stage of lactation	Wt. of gland (g.)	Type I	Type II	Type II/ type I	Type I	Type II	Type II/ type I
Pregnancy	6·7	9·1	6·6	0·7	71	78	1·1
1st day	7·4	9·7	8·0	0·8	95	101	1·1
4th day	6·8	8·9	16·1	1·8	100	142	1·4
7th day	8·8	7·9	20·3	2·8	83	268	3·2
12th day	8·9	8·2	24·8	3·0	92	250	2·7
18th day	6·0	8·5	26·0	3·1	98	287	3·0
Involuted	8·5	8·3	7·3	0·9	96	67	0·7

fraction. A similar situation has been demonstrated in lactating mice by Bartley, Abraham & Chaikoff (1966). It would therefore be of interest to know how, if at all, the mitochondrial hexokinase contributes to the observed changes in supernatant activity during the lactation cycle.

In the present experiments the results are expressed as activity in the total weight of the two abdominal glands, since the main point of interest seemed to be the total capacity of the gland to phosphorylate glucose in different conditions. There are many difficulties in choosing the method of expressing the results because of the milk content of the gland (see Greenbaum & Slater, 1957*a*), the changing cell population with a large content of adipose cells, up to 30% in early lactation but declining to 10% at mid-lactation (Weinberg, Pastan, William & Field, 1961; Wren, DeLauder & Bitman, 1965), and the cell division occurring at parturition (Greenbaum & Slater, 1957*b*; Baldwin & Milligan, 1966). It has been shown that the total DNA of the abdominal mammary glands remains almost constant from the first to the eighteenth day of lactation (Greenbaum & Slater, 1957*b*; Munford, 1964; Baldwin & Milligan, 1966). Thus hexokinase activity/total gland during lactation is also representative of the pattern of change/unit of DNA.

Starch-gel electrophoresis of mammary-gland hexokinases. The result of starch-gel electrophoresis showed that mammary-gland tissue contains two types of hexokinase corresponding to the types I and II described by Katzen & Schimke (1965). In mammary-gland tissue from pregnant rats the bands of the two types are of approximately equal intensity and this pattern is maintained in early lactation. However, as lactation proceeds there is a marked increase in type II enzyme, reaching a maximum by the seventh day. At the end of lactation and during involution there is a decrease in type II enzyme and the overall pattern reverts to that shown in the mammary gland from pregnant rats.

The results given in Table 1 are the integrals of the areas under the curves from the Chromoscan recordings; there was an increase in the type II/type I activity ratio of hexokinase from late pregnancy to a maximum value at mid-lactation. However, by loading different volumes of extract on to the gel it was apparent that this method yielded only semiquantitative data, one factor being, presumably, the maximum intensity of stain that a given area of gel can exhibit, so that increases in enzyme activity over and above a certain quantity would not produce equivalent increases in reduced dye. The occurrence of artifacts during the electrophoresis or staining period seemed possible, particularly in view of the rather complex nature of the developing medium. Fildes & Parr (1963) and Fitch & Parr (1966) have developed techniques with agar gel on thin films of developer applied by brush to the surface of the gel to overcome difficulties due to diffusion of substrates.

Starch-gel electrophoretograms of mammary-gland extracts, when stained for 6-phosphogluconate dehydrogenase and glucose 6-phosphate dehydrogenase, revealed a disturbing coincidence of these enzymes with hexokinase types I and II. Glucose 6-phosphate dehydrogenase forms an intensely stained band in the region of type II hexokinase; 6-phosphogluconate-dehydrogenase activity appears as a diffuse band across the area from type I to type II hexokinase.

The responses of glucose 6-phosphate dehydro-

genase and 6-phosphogluconate dehydrogenase to hormonal stimulus are known to parallel changes in hexokinase in a variety of tissues such as adipose tissue, liver and adrenal (Brown, McLean & Greenbaum, 1966) as well as in mammary gland (McLean, 1958; Baldwin & Milligan, 1966), where the activities of all three enzymes increase rapidly at the onset of lactation and decline with involution.

The juxtaposition of these observations led to the following tests. First, glucose 6-phosphate dehydrogenase was omitted from the staining mixture and it was found that a band still appeared in the hexokinase type II position, presumably from the endogenous glucose 6-phosphate dehydrogenase in that area of the gel using the glucose 6-phosphate formed by hexokinase type II. Hexokinase type I did not appear under these conditions. Sufficient time must therefore be given in the developing medium to allow for the slower development of type I hexokinase.

Secondly, the overlapping of type I hexokinase with 6-phosphogluconate dehydrogenase might lead to an apparently high value for the former enzyme, since 2 equiv. of NADPH could be produced. However, the high glucose 6-phosphate-dehydrogenase and 6-phosphogluconate-dehydrogenase activities compared with that of hexokinase in mammary gland make the hexokinase activity the rate-limiting step in the development of stain intensity.

Repeated observations of duplicate samples stained for hexokinase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase showed that the bands of hexokinase activity were never defined by the bands of glucose 6-phosphate dehydrogenase or 6-phosphogluconate dehydrogenase, and it seemed likely that the apparent hexokinase bands were true indications of hexokinase activity. Further, by cutting the gel above type I hexokinase and below glucose 6-phosphate dehydrogenase before staining, a hexokinase band of equal intensity to that shown on an intact gel was observed, thus confirming that there was no artifact produced by diffusion of substrates through the gel.

When EDTA was omitted from the buffer mixture used for starch-gel electrophoresis the glucose 6-phosphate dehydrogenase migrated faster than hexokinase type II. Comparison of the intensity of the bands for hexokinase type II in the two situations, with and without EDTA, showed little or no difference.

From the present work it would seem that the bands did, in fact, represent the true hexokinase activity, but the necessity of confirming these results by another more quantitative method seemed clear.

Differentiation of type I and type II hexokinase by heat treatment. Grossbard & Schimke (1966) demonstrated that at 45° type II hexokinase preparations lose 90% of their activity within 60min., but type I loses only 10% of its activity in this time. These authors have also shown that the K_m is $4{\cdot}5\times10^{-5}$–$4{\cdot}9\times10^{-5}$M for type I hexokinase and 23×10^{-5}–28×10^{-5}M for type II hexokinase.

The activities of hexokinase type I and type II and of type I alone, and the K_m values of the mixtures of enzymes and of type I hexokinase, were determined by estimations on the extracts of mammary gland before and after treatment at 45° for 1hr. Losses on heating were taken as 100% and zero for type II and I respectively. It can be shown that this approximation results in a small underestimation of the type II/type I activity ratio when this is greater than 1.

The results in Table 1 show that, whereas type I hexokinase activity remains relatively constant throughout the lactation cycle, that of type II enzyme increases markedly during lactation and is low in mammary tissue from pregnant rats and during involution. The type II/type I activity ratio also reflects the increase in type II hexokinase activity. Values obtained by this method, heat treatment of the mammary-gland extracts, were compared with the values obtained with starch-gel electrophoresis, and good agreement was found (Table 1).

The apparent overall K_m of hexokinase types I and II for glucose increased from a typical value in pregnancy of $7{\cdot}6\times10^{-5}$M to 12×10^{-5}M at mid-lactation, and decreased to $6{\cdot}5\times10^{-5}$M during mammary involution. Heated extracts yielded values of $3{\cdot}8\times10^{-5}$–$5{\cdot}0\times10^{-5}$M, typical of type I hexokinase.

Effect of alloxan-diabetes on mammary-gland hexokinase. The effect of alloxan-diabetes was studied at a relatively short time (3 days) after cessation of insulin treatment. At this time it was clear that lactation had almost ceased, since the gain in weight of the litter was extremely small. Measurements of the overall activity of hexokinase (type I plus type II) revealed an increase in the activity/g. of tissue in the mammary glands from the alloxan-diabetic rats as compared with those from control rats (Table 2). This difference is probably due to the absence of retained milk from the mammary glands from diabetic animals, since the total phosphorylating capacity of the tissue was not significantly different from the control value.

Heat treatment at 45° for 1hr. was again used to distinguish between the contributions of type I and type II hexokinase to the total glucose-phosphorylating activity (Table 2). The type II/type I activity ratio was markedly decreased from $2{\cdot}5\pm0{\cdot}14$ in the control rats to $0{\cdot}84\pm0{\cdot}17$ in the

Table 2. *Hexokinase activity of mammary gland from normal and alloxan-diabetic rats*

The values are given as μmoles of glucose 6-phosphate formed/hr. at 25° per g. of milk-containing tissue or per total weight of the abdominal mammary glands. The results are the means ± S.E.M. Fisher's P values are given; where P is greater than 0·1 the values are quoted as not significant (N.S.). The rats were killed on the ninth or tenth day of lactation. The alloxan-diabetic rats had received 20mg. of alloxan/100g. body wt. on the fourth day of lactation and then 2 units of protamine–zinc–insulin/day until 3 days before being killed. For further details see the Methods section. Hexokinase types I and II were determined by the heat-treatment procedure.

	Control rats	Alloxan-diabetic rats	P
No. of animals	7	7	
Body wt. (g.)	236 ± 9	203 ± 15	
Wt. of gland (g.)	6·90 ± 0·46	4·07 ± 0·46	0·01
Hexokinase types I+II			
Units/g. of tissue	23·3 ± 2·8	35·3 ± 4·0	0·033
Total units	165 ± 24	143 ± 21	N.S.
Hexokinase type I			
Units/g. of tissue	6·6 ± 0·7	19·0 ± 1·9	0·01
Total units	46·6 ± 5·8	77·7 ± 12	0·035
Hexokinase type II			
Units/g. of tissue	16·6 ± 2·2	16·3 ± 3·3	N.S.
Total units	119 ± 19	65·0 ± 15	0·046
Type II/type I ratio	2·5 ± 0·14	0·84 ± 0·17	0·01
$10^5 \times K_m$ (M)	11·8 ± 2·3	5·0 ± 0·2	0·018

alloxan-diabetic rats; an increase in type I hexokinase and a decrease in the type II enzyme both contribute to the fall in this ratio.

These results, in part, support the view that type II hexokinase is associated with the insulin-sensitivity of tissues. The increase in type II enzyme during lactation (the insulin-sensitive phase of the lactation cycle) and the decreased contribution of type II enzyme to the total glucose-phosphorylating activity in diabetes are in accord with this view. There is evidence that a high concentration of glucose leads to a higher concentration of type II hexokinase. Katzen, Soderman & Nitowsky (1965) showed that cultures of liver cells grown at high glucose concentrations, compared with cultures grown in low glucose concentrations, had an increase of approximately tenfold in the type II hexokinase but not in the type I enzyme. If the transport mechanism in mammary gland is sensitive to insulin and if insulin controls glucose entry into the cell in the way that is seen in adipose tissue (Crofford & Renold, 1965*a*,*b*; Renold, Gonet, Crofford & Vecchio, 1966), then insulin, by promoting glucose entry, would be associated with a raised type II hexokinase activity.

In addition to the changes in type II hexokinase activity there is a highly significant increase in type I hexokinase activity in alloxan-diabetic rats and in lactation, that in the latter case being obvious only on a total gland basis. Since type I hexokinase has a lower K_m for glucose it would seem that a change in favour of this enzyme would lead to a more efficient system for glucose phosphorylation, which seems paradoxical in alloxan-diabetic rats; the utilization of glucose by alloxan-diabetic rats is known to be decreased. It has yet to be resolved whether the multiple hexokinases represent a family of different enzymes with a similar function or are multiple forms of the same enzyme with different sub-unit composition (Katzen *et al.* 1965; Grossbard & Schimke, 1966). Since the present results show that, though the total glucose-phosphorylating activity of mammary-gland extracts is unchanged in diabetes, there is a fall in type II activity and an accompanying rise in type I activity, the possibility arises that these two types may be interconvertible and that insulin may play a part in the conversion.

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