Biochem. J. (1968) **109**, 737
Printed in Great Britain

The Effect of Anti-Insulin Serum and Alloxan-Diabetes on the Distribution and Multiple Forms of Hexokinase in Lactating Rat Mammary Gland

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(*Received 7 December* 1967)

1. The distribution and multiple forms of hexokinase activity in lactating rat mammary gland were investigated in alloxan-diabetic rats and in rats treated with anti-insulin serum. It was found that 46% of the total hexokinase of mammary-gland tissue from control rats was in the particulate fraction, but this percentage was decreased in the alloxan-diabetic rats to 11% of the total hexokinase. The hexokinase activity of the soluble fraction was not significantly altered but there was a decrease in the type II/type I quotient. 2. The early changes that occurred on insulin deprivation were studied 1hr. after administration of anti-insulin serum to lactating rats, at which time the hexokinase bound to the particulate fraction had decreased to 11% of the control value and that in the soluble fraction had increased by approx. 50%. The hexokinase type II/type I quotient in the soluble fraction was significantly decreased. These results suggested that there was a release of particulate-bound hexokinase in rats treated with anti-insulin serum.

The existence of at least four different forms of glucose–ATP phosphotransferase has now been firmly established by several groups of workers (Gonzalez, Ureta, Sanchez & Niemeyer, 1964; Katzen & Schimke, 1965; Grossbard & Schimke, 1966). Designated types I–IV in order of increasing mobility on starch-gel electrophoresis, type IV corresponds to glucokinase (EC 2.7.1.2) with a high K_m for glucose and types I–III comprise the low-K_m activity previously considered to be due to a single enzyme (EC 2.7.1.1). Katzen & Schimke (1965) and Katzen (1966), from a survey of multiple forms of hexokinase in a range of normal tissues and from changes found in starved or alloxan-diabetic rats, first suggested that there might be a correlation between hexokinase type II and the action of insulin. This observation was supported by several workers, who demonstrated that the activity of type II enzyme in adipose tissue decreased in starvation and diabetes, conditions of low blood insulin concentration (Moore, Chandler & Tettenhorst, 1964; McLean, Brown, Greenslade & Brew, 1966; Katzen, 1966). More recently Katzen (1967) has modified this view after studying a wider range of tissues and has proposed that the amount of hexokinase type I relative to that of types II and III might be an important factor in determining insulin sensitivity, a relative deficiency in type I appearing to be correlated with an insulin requirement.

The sensitivity of rat mammary-gland slices to the addition of insulin *in vitro* is different at various stages of the lactation cycle. Balmain & Folley (1951) have evidence, from studies of the respiratory quotients, that insulin does not stimulate the synthesis of fatty acids from glucose and acetate by slices from either mammary glands of pregnant rats or involuting glands, though a marked effect was found in the lactating rat mammary gland. The response to insulin as judged by the increase in $^{14}CO_2$ formation from [1-^{14}C]glucose and lipid synthesis from ^{14}C-labelled glucose by mammary-gland slices is seen mainly during lactation and there is only a small response during pregnancy (McLean, 1960).

Previous studies with the soluble fraction of mammary-gland homogenates showed that 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 quotient rose from about 1 in pregnant rats to a mid-lactation value of 3, returning to 1 on involution; the changes in type II hexokinase activity during the lactation cycle paralleled the changes in the insulin sensitivity of mammary-gland tissue (Walters & McLean, 1967). The interrelationship

of type II hexokinase with insulin sensitivity was further strengthened by the observation that alloxan-diabetes altered the relative contributions of type I and type II hexokinase in the lactating mammary gland, decreasing the type II/type I activity quotient to about 1. The capacity for glucose phosphorylation by the soluble hexokinase of the mammary gland was unchanged in the alloxan-diabetic rats (Walters & McLean, 1967). In these studies on mammary-gland metabolism only the hexokinase present in the soluble fraction of the cell was measured.

Further possibilities of control were raised by the observation by Bartley, Abraham & Chaikoff (1966) that a considerable percentage of mouse mammary-gland hexokinase activity was found in the mitochondrial fraction of the cell and by the studies by Rose & Warms (1967) and Karpatkin (1967) on factors involved in the release and binding of hexokinase to mitochondria in ascites-tumour cells and frog skeletal muscle. It was therefore decided to reinvestigate the changes occurring in mammary gland from alloxan-diabetic rats in relation to the distribution of hexokinase types I and II between the soluble and particulate fractions. Since the experiments with alloxan-diabetic rats involve a relatively long period of insulin deprivation, comparisons were made with the effects of anti-insulin serum on the activities and distribution of hexokinase types I and II in mammary gland 1 hr. after treatment of the animal.

METHODS

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.* On the fourth day of lactation diabetes was induced by the subcutaneous injection of alloxan (20 mg./100 g. body wt.) and these rats were then maintained on 2 units of protamine–zinc–insulin/day. The insulin treatment was stopped 3 days before the rats were killed, by cervical dislocation, on the eleventh day of lactation (i.e. 7 days after treatment with alloxan). The blood glucose concentrations were then 415 ± 90 mg./100 ml. of blood (mean and S.E.M. for six rats).

Anti-insulin serum was kindly supplied by Dr A. Beloff-Chain of Imperial College, London. This was prepared in guinea pigs against crystalline glucagon-free ox insulin from Burroughs Wellcome (Beckenham, Kent) as described by Mansford (1967). The potency was such that 1 ml. of undiluted serum bound approx. 4 m-units of insulin. The intravenous injection of 0·5 ml. of anti-insulin serum/100 g. body wt. under ether anaesthesia into the albino rats used here raised the blood glucose concentration to 237 ± 26 mg./100 ml. of blood after 60 min. In preliminary experiments a small group of control rats received a similar dose of serum albumin under ether anaesthesia, and no significant difference was found in the percentage of bound hexokinase. The rats were used on the eleventh day of lactation and the animals were killed 1 hr. after the injection. Blood samples were taken from the dorsal aorta immediately before the mammary gland was excised.

Preparation of extracts. A 1:10 homogenate was prepared in 0·15 M-KCl adjusted to pH 7·4 with $KHCO_3$. Whole cells and debris were removed by centrifugation at 600*g* for 10 min. and the supernatant is referred to below as 'whole homogenate'. This was dialysed for 1 hr. against the same extraction medium before use for determination of hexokinase activity of whole homogenate, i.e. of particulate plus soluble fractions. The soluble fraction was prepared by centrifuging the undialysed whole homogenate at 105000*g* for 45 min. in a Spinco model L centrifuge and then dialysing the supernatant against the extraction medium for 1 hr. The 105000*g* particulate fraction was resuspended with gentle homogenization so that the particulate fraction from 1 g. of tissue was in a final volume of 10 ml. of the medium. Comparison of homogenates prepared in 0·25 M-sucrose with those prepared in 0·15 M-KCl neutralized with $KHCO_3$ showed essentially the same pattern of distribution of the activity between the particulate and soluble fractions.

Bartley *et al.* (1966) showed that hexokinase was associated with the mitochondrial fraction in lactating mouse mammary gland. In preliminary experiments in the present work it was shown that most of the particulate hexokinase of lactating rat mammary gland could be sedimented at 12000*g* for 20 min. and was associated with the fraction containing the maximum succinoxidase activity. The succinoxidase activity was measured by the method of Slater & Planterose (1960).

Since mammary-gland homogenates contain a great deal of fat and since cleaner separation of the particulate fraction from the soluble fraction and lipid was obtained at the higher speed of centrifugation (105000*g* for 1 hr.) this procedure was adopted.

Determination of hexokinase activity. (*a*) Spectrophotometric assay. The hexokinase activity of the whole homogenates, resuspended 105000*g* particulate fractions and soluble fractions was determined essentially by the method of Sharma, Manjeshwar & Weinhouse (1963) with modifications as described by McLean & Brown (1966); this involved the use of purified glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, giving 2 equiv. of NADPH/glucose 6-phosphate formed. The final glucose concentration was 1 mM.

The hexokinase activity of lactating rat mammary gland is high and it is possible to use small amounts of the cell fractions in the determination of the enzyme activity. Normally 0·05 ml. of each cell fraction was used in the spectrophotometer cell and turbidity did not seem to cause any interference even with the whole homogenate. (The use of a double-beam spectrophotometer and scale-expansion accessory is valuable in this context.) By addition of the mammary-gland preparation to a single cuvette containing all the reagents except substrate it was possible to show that E_{340} was quite steady after 1–2 min. (with the conditions used in the present experiments no interference was observed that could be ascribed to swelling of mitochondria). In Table 1 the results for the particulate fraction are the difference between the values for the whole homogenate and the soluble fraction, and in Table 2 the values are direct measurements of the hexokinase activities of each of the fractions. Similar values for the activity of the particulate fraction were obtained irrespective of the method used. Values for three control animals in which the activity of the

particulate fraction was both estimated by difference and determined directly were 24·5±1·5 and 29·5±2·8 respectively (means±S.E.M.).

A unit of enzyme activity is defined as the amount of enzyme catalysing the formation of 1 μmole of glucose 6-phosphate/hr. at 25°. 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. 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) as described by Walters & McLean (1967).

(*b*) Assay with [1-^{14}C]glucose. To check the intracellular distribution of hexokinase by a method other than the spectrophotometric assay, which might have some interference from turbidity of the whole homogenate or from swelling of the mitochondria, a radioactive assay was used similar to that described by DiPietro (1963). This method measures the production of $^{14}CO_2$ from [1-^{14}C]glucose in the presence of an excess of ATP, $NADP^+$, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, so that hexokinase is the rate-limiting enzyme.

The incubation medium contained the normal hexokinase reaction mixture used in the spectrophotometric assay, except that the concentration of $NADP^+$ was doubled, additional components of the system were 500 μmoles of NaF, 200 μg. of phenazine methosulphate, 0·5–1·0 μc of [1-^{14}C]glucose and 0·1ml. of 1:10 whole homogenate or fraction therefrom. After incubation of the mixture for 15min. at 25° in rubber-stoppered conical flasks with centre wells, 0·3ml. of 1% $NaHCO_3$ and 0·5ml. of 0·5N-HCl were added to the outer compartment of the flask to stop the reaction and release the $^{14}CO_2$ formed. The $^{14}CO_2$ was collected in Hyamine in the centre well and was counted in a scintillation counter. The percentage of the hexokinase activity of the whole homogenate found in the soluble fraction of the cell was 54·7±3·5 by the radioactive assay and 53·8±5·5 by the spectrophotometric assay (means± S.E.M. of four determinations).

Determination of nucleic acids. DNA and RNA were determined as described by Glock & McLean (1955), but with the modified diphenylamine reagent described by Burton (1956) for DNA.

Statistical analysis. The mean values are given together with their S.E.M. values. The differences are considered significant if P is no greater than 0·05. Values greater than 0·1 are quoted as not significant.

RESULTS

Effect of alloxan-diabetes on the distribution of hexokinase. Previous work on the hexokinase activity of lactating mammary gland from alloxan-diabetic rats revealed that there was no effect on the activity of the soluble fraction hexokinase when expressed as units/whole gland. The statistically significant decrease in gland weight in the alloxan-diabetic rat actually caused a rise in the concentration of hexokinase as units/g. of tissue (Walters & McLean, 1967). The DNA content of the whole abdominal mammary gland did not change in the alloxan-diabetic rats (see Table 1), and the values therefore have been given as units/whole gland, a value that will be proportional to the activity or content/cell and that is related to the total capacity of the mammary gland for milk production. The activities in the whole homogenate and in the particulate fraction are shown in Table 1, where the values are given as units/total abdominal mammary gland in view of the decline in mammary-gland weight. The hexokinase activity of the whole homogenate significantly decreased in the alloxan-diabetic rats to a value about 55% of the control. Since, in agreement with previous results (Walters & McLean, 1967), no decrease was found in the hexokinase activity in the soluble fraction, the fall must be almost entirely in that part of the hexokinase bound to the particulate fraction. Examination of the particulate fraction showed that the

Table 1. *Comparison of hexokinase activities in mammary glands from control and alloxan-diabetic rats*

The results are given as units in the cell fractions derived from an amount of mammary-gland tissue equal to the total weight of the abdominal glands. The results are quoted as means±S.E.M. Fisher's P values are given, and are considered significant if P is less than 0·05, values greater than 0·1 being quoted as N.S. (not significant). No differentiation was made between hexokinase types I and II.

	Controls	Alloxan-diabetic	Fisher's P
No. of animals	6	6	
Blood glucose (mg./100ml.)	85±1	415±90	
Wt. of gland (g.)	7·1±0·5	4·1±0·5	0·01
DNA (mg./total gland)*	24·7±1·7	19·2±1·9	0·08
Hexokinase (units/total gland) in:			
Whole homogenate	385±50	212±36	0·004
Soluble fraction	207±29	189±27	N.S.
Particulate fraction	178±25	23±3	0·001
% of total activity in particulate fraction	46·3±2·7	10·7±4·4	0·001

* Five values only in this group.

Table 2. *Comparison of hexokinase activities in mammary glands from control rats and rats treated with anti-insulin serum*

The results are given as units/g. of tissue or fractions derived from 1 g. of tissue. The results are quoted as means ± S.E.M. Hexokinase types I and II were estimated by measuring the activity before and after heat treatment at 45° for 1 hr. (see the Methods section). Fisher's *P* values are given and are considered significant if *P* is less than 0·05.

	Controls	Anti-insulin-serum-treated	Fisher's *P*
No. of animals	5	5	
Blood glucose (mg./100 ml.)	98 ± 6	237 ± 26	0·007
(*a*) Intracellular distribution of hexokinase (types I and II)			
Hexokinase (units/g. of tissue) in:			
Soluble fraction	38·4 ± 6·2	60·3 ± 7·1	0·05
Particulate fraction	33 ± 7·0	8·0 ± 3	0·01
% of total activity in particulate fraction	46·3 ± 2·7	11·7 ± 2·2	< 0·001
(*b*) Relative activities of hexokinase types I and II in the soluble fraction			
Hexokinase (units/soluble fraction from 1 g. of tissue):			
Hexokinase type I	9·3 ± 1·3	19·3 ± 3·9	0·05
Hexokinase type II	31·0 ± 5·1	49·0 ± 7·7	0·09
Type II/type I quotient	3·8 ± 0·35	2·7 ± 0·22	0·03

proportion of bound activity fell from 46·3% in the controls to 10·7% in the alloxan-diabetic group.

It was not clear from these experiments whether the particulate fraction contained less hexokinase because of release into the soluble fraction or whether there was a specific loss of particulate hexokinase. This was examined further by studying the effect of anti-insulin serum on hexokinase activity and distribution in experiments in which measurements were made 1 hr. after treatment with the serum, at a time in which the initial pattern of change might more readily be seen, the pattern in the alloxan-diabetic rat representing the long-term adaptation.

Effect of treatment with anti-insulin serum on mammary-gland hexokinase. The results obtained in the study of mammary-gland hexokinase from lactating rats killed 1 hr. after treatment with anti-insulin serum are summarized in Table 2. The 1 hr. treatment led to no significant changes in the mammary-gland weight and therefore the enzyme activities/g. of tissue have been used for comparison. The hexokinase activity of the soluble fraction from mammary-gland tissue of rats treated with anti-insulin serum increased from a control value of 38·4 units/g. of tissue to 60·3 units/g. This somewhat surprising result was further investigated by a study of the distribution of the hexokinase activity between the particulate and soluble fractions. The hexokinase activity of whole homogenates from control rats and rats treated with anti-insulin serum showed no significant difference. Hence the increased activity observed in the soluble fraction of mammary gland from treated rats was the result of a redistribution of the enzyme in which some of the bound activity was being released into the soluble fraction. This change in distribution is sufficient to account for all the increased activity found in the soluble fraction.

Heat treatment was used to try to reveal any differentiation in the type of hexokinase released into the soluble fraction (Table 2*b*). In the mammary gland from rats treated with anti-insulin serum there was an increase in type II activity in the soluble fraction; however, there was an even greater increase in the type I activity from 9·3 units/g. of tissue in the controls to 19·3 units/g. of tissue. This differential effect results in a statistically significant lowering of the type II/type I quotient.

DISCUSSION

By using rats treated with anti-insulin serum and alloxan-diabetic rats it was possible to study the effects of short-term and long-term insulin deprivation on the pattern and distribution of hexokinase within the mammary gland. An early change is the release of hexokinase from the particulate fraction; this results in a loss of particulate hexokinase and an increase in soluble hexokinase, and there is a decrease in the type II/type I quotient of the soluble fraction. Longer-term changes, in alloxan-diabetic rats, show an almost identical distribution pattern, the percentage in the particulate fraction remaining essentially the same at 11%. There was no increase in the soluble hexokinase, but there was a decline in the hexokinase activity of the whole homogenate and a marked decrease in the hexokinase type II/type I quotient, from 2·5 in the control animals to 0·84 in the alloxan-diabetic rats (Walters & McLean, 1967), suggesting that in addition to release of hexokinase from the particu-

late fraction there was a loss of hexokinase type II from the soluble fraction.

The significance of the particulate-bound hexokinase is not yet clear. Though Rose & Warms (1967) found no differences between bound and free hexokinase with respect to apparent activity and dependence of $V_{max.}$ on pH, Li & Ch'ien (1965), working with ascites-tumour mitochondrial hexokinase, demonstrated differences in K_m for ATP and apparent K_i for glucose 6-phosphate between the bound and eluted enzymes. Karpatkin (1967), investigating the particulate and soluble hexokinase from frog skeletal muscle, found that the K_m of the particulate enzyme for ATP in the presence of Mg^{2+} was 0·28mM, which was about one-fifth of that for the soluble enzyme.

A noteworthy effect in relation to the hormonal control of hexokinase is that reported by Li & Ch'ien (1965), who have shown that compounds that inhibited mitochondrial shrinkage, such as insulin and thyroxine, also decreased the activity of the bound form of hexokinase by about 50%. To some extent this is the converse of the present results, where a higher activity of mitochondrial hexokinase was found in the presence of insulin than in the deficient state.

In adipose tissue the changes in the relative activities of hexokinase types II and I in alloxan-diabetic rats are somewhat similar to those seen in the lactating mammary gland, namely a decrease in hexokinase type II/type I quotient in the soluble fraction (McLean, Brown, Walters & Greenslade, 1967). In lung, a tissue not known to be sensitive to insulin, there was no change in the activities of hexokinase types I, II and III in alloxan-diabetic rats (McLean *et al.* 1967). It is therefore possible that the sequence of events and pattern of change found in the present work on mammary gland may be common to other insulin-sensitive tissues. Katzen (1967) has concluded that not only hexokinase type II but also types I and III are involved in the action of insulin; type I, or more precisely the absence of type I, may result in a high degree of insulin-sensitivity. The greater stability of hexokinase type I in the insulin-deficient animal is clearly seen in the mammary gland.

The present results also suggest that insulin may be of importance both in the binding of hexokinase to cell membranes, in particular to the mitochondria, and in the stability of hexokinase type II in lactating rat mammary gland, a view in keeping with current ideas on the significance of multiple forms of hexokinase to insulin action (see Katzen, 1967).

The authors are indebted to Dr A. Beloff-Chain of Imperial College, London, for the anti-insulin serum, and to Mr B. C. Teo for skilled technical assistance. Acknowledgement is also made to the Medical Research Council for a grant to purchase a Unicam SP.800 recording spectrophotometer and for a research grant. This work was in part supported by a grant to The Middlesex Hospital Medical School from the British Empire Cancer Campaign.

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