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Effect of Alloxan-Diabetes on Multiple Forms of Hexokinase in Adipose Tissue and Lung

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Comparison has been made of the effect of alloxan-diabetes on the multiple forms of hexokinase (EC 2.7.1.1) in adipose tissue and lung. Types I and II hexokinase were distinguished in adipose tissue by their different stabilities to heat treatment, which made it possible to determine the activity of each form spectrophotometrically; additional confirmatory evidence was obtained from starch-gel electrophoresis. Type II hexokinase was markedly depressed in adipose tissue from alloxan-diabetic rats. Lung contained types I, II and III hexokinase, type I predominating. There was no significant change in the pattern of these multiple forms of hexokinase in lung from alloxan-diabetic rats. These results are discussed in relation to current ideas that the insulin-sensitivity of a tissue may be correlated with the content of type II hexokinase.

The presence of multiple forms of hexokinase has been reported in a wide variety of rat tissues (González, Ureta, Sanchez & Niemeyer, 1964; Moore, Chandler & Tettenhorst, 1964; Katzen, Sonderman & Nitowsky, 1965; Katzen & Schimke, 1965; Katzen, 1966*a*,*b*; Grossbard & Schimke, 1966; Walters & McLean, 1966, 1967). In all, three distinct types of hexokinase with a low K_m for glucose have been described (EC 2.7.1.1), and in addition there is in liver a fourth enzyme, glucokinase, with a high K_m for glucose (EC 2.7.1.2) (Walker, 1963; Viñuela, Salas & Sols, 1963; Walker & Rao, 1964; Salas, Salas, Viñuela & Sols, 1965).

A correlation between insulin-sensitivity of the tissue and the distribution of type II hexokinase was first observed by Katzen & Schimke (1965), who reported large amounts of type II enzyme in adipose tissue, muscle and heart, but that in brain and kidney type I hexokinase predominated. Lactating mammary gland, an insulin-sensitive tissue, has also been shown to contain large amounts of type II hexokinase (Walters & McLean, 1966, 1967). Type II hexokinase decreases in adipose tissue on starvation, a condition of decreased blood insulin (Moore *et al.* 1964; Katzen & Schimke, 1965).

Estimations of the relative amounts of types I and II hexokinase in adipose tissue by using starch-gel electrophoresis techniques have given somewhat conflicting results. McLean, Brown, Greenslade & Brew (1966) found a definite decrease in adipose tissue from diabetic rats of type II hexokinase with a medium containing β-mercaptoethanol. However, Katzen (1966*a*,*b*) found that when electrophoresis was carried out in the presence of this thiol the lowering of type II hexokinase activity was questionable. He demonstrated that type II hexokinase of adipose tissue may be separated into two distinct bands when electrophoresis is carried out in the absence of β-mercaptoethanol and EDTA; the faster-moving type IIa hexokinase disappeared in the starved animal and in the streptozotocin-diabetic animal. A similar pattern of response was found in other insulin-sensitive tissues such as heart, diaphragm and gastrocnemius muscle (Katzen, 1966*a*,*b*).

In the present work on adipose tissue the reported differences in stability of types I and II hexokinase on treatment at 45° for 1 hr. (Grossbard & Schimke, 1966), together with a spectrophotometric assay and starch-gel electrophoresis, have been used in a further attempt to estimate the relative activities of the two types of hexokinase found in adipose tissue from normal and alloxan-diabetic rats. Lung was also examined, since this tissue has, in common with adipose tissue, an active pentose phosphate pathway and a high activity of hexokinase but does not appear to be greatly influenced by insulin (Glock & McLean, 1954; J. Brown & P. McLean, unpublished work). It was found that type II hexokinase was decreased in adipose tissue but unchanged in lung from alloxan-diabetic rats.

METHODS

Materials. Commercial reagents were used with the exception of 6-phosphogluconate dehydrogenase, which was prepared as described by Glock & McLean (1953) and used in the assay of hexokinase. Glucose 6-phosphate

dehydrogenase and NADP were obtained from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). Phenazine methosulphate and nitro-blue tetrazolium were from Sigma Chemical Co. (St Louis, Mo., U.S.A.).

Animals. Male albino rats (body wt. 220–250g.) were starved for 48hr. before a subcutaneous injection of alloxan (20mg./100g. body wt.). The rats were then given 2 units of protamine–zinc–insulin/day for 5 days; no further treatment was given for the next 2 weeks, at the end of which time the rats were killed. Food and water were allowed *ad lib.* throughout the experiment.

Preparation of tissue extracts. The two epididymal fat pads from one control rat were removed and homogenized in an all-glass Potter–Elvehjem homogenizer in 7ml. of medium containing KCl (150mM), $MgCl_2$ (5mM), EDTA (5mM) and β-mercaptoethanol (10mM) and adjusted to pH7·4 with $KHCO_3$. The epididymal fat pads of the alloxan-diabetic rats were considerably smaller than those from control animals, and usually four fat pads, combined from two rats, were homogenized in 7ml. of the medium described above. This gave tissue extracts with approximately the same protein concentration in the control and alloxan-diabetic groups (see Table 1). These preparations were centrifuged at $100000g_{av.}$ for 45min. and the supernatant fraction was dialysed against the same extracting medium for 2hr. in the cold. This preparation was used for the spectrophotometric assay of hexokinase and for starch-gel electrophoresis.

Hexokinase assay. The hexokinase activities were measured spectrophotometrically essentially as described by Sharma, Manjeshwar & Weinhouse (1963) with modifications as described by McLean & Brown (1966). A unit of enzyme activity is defined as 1 μmole of glucose 6-phosphate formed/min. at 25°. Estimations of types I and II hexokinase in adipose tissue were made with a final glucose concentration of 10mM; concentrations of 0·1M were used to show that types I and II hexokinase were not inhibited by high glucose concentrations. Type III hexokinase activity has been shown to be inhibited by high glucose concentrations (Katzen & Schimke, 1965; Grossbard & Schimke, 1966). Therefore in lung measurements of hexokinase activity were made with low and high glucose concentrations (0·5mM and 0·1M respectively), the difference in activity between these being used as an estimate of type III hexokinase activity.

The rate of reduction of NADP was measured with a Unicam SP. 800 recording spectrophotometer with constant-temperature cell housing and scale-expansion accessory.

Heat treatment. A portion of the dialysed extract was heated at 45° for 1hr., the insoluble protein was removed by centrifugation and the supernatant fraction used for hexokinase assay. Grossbard & Schimke (1966) have shown that type I hexokinase loses not more than about 10% of activity when heated in the absence of glucose whereas type II hexokinase loses more than 90% of activity with this same treatment. Thus, with adipose tissue, it may be taken as a first approximation that the initial assay measures types I and II hexokinase; after heat treatment virtually only type I hexokinase remains and the difference may be taken to equal type II hexokinase. It may be calculated that if the hexokinase type II/type I quotient is greater than unity then this method underestimates the quotient, and that at values smaller than unity this quotient is overestimated.

With lung the situation is more complex, since three forms of hexokinase have been detected in the present experiments corresponding to types I, II and III hexokinase; of these type I stains most intensely on the starch gels. Heat treatment of these extracts would be expected to inactivate 90% of type II hexokinase and about half of type III enzyme (Grossbard & Schimke, 1966), as was indeed shown experimentally by using starch-gel electrophoresis. Since type III hexokinase accounts for no more than 10–20% of the total hexokinase, an error of 5–10% will be introduced into the values of type II hexokinase estimated by subtracting the values for the hexokinase activity before and after heating. If the value of type III hexokinase is derived from the inhibition studies, then corrected values for types I and II hexokinase may be calculated (see the Results section).

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 0·02M-sodium barbitone buffer, pH8·6, containing EDTA (1mM) and β-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 values are given in arbitrary units, which are comparable from one experiment to the next.

In view of the results of Katzen (1966*a*,*b*), starch-gel electrophoresis was carried out in the absence of β-mercaptoethanol and in some cases also in the absence of EDTA. The media in which the tissue extracts were prepared were also either without β-mercaptoethanol or without β-mercaptoethanol and EDTA.

RESULTS

Adipose tissue. The results of the combined use of heat treatment and spectrophotometric assays for measurement of types I and II hexokinase, as described above, were supported by starch-gel electrophoresis, which confirmed that heat treatment caused almost complete destruction of type II hexokinase in adipose tissue while type I remained unchanged. Values for type I hexokinase of adipose tissue of eight rats before and after heating were 56 ± 3 and 58 ± 4 respectively, the values being given in arbitrary units, which were proportional to the areas under the curves obtained with the Chromoscan. Type II hexokinase was 140 ± 14 units before heating and could not be detected on the starch gel after heating.

The activities of types I and II hexokinase in adipose tissue of normal and diabetic rats are given in Table 1. There is a very considerable fall in the activities of both types I and II hexokinase when considered on the basis of total activity in the two fat pads, in keeping with the known depression in glucose utilization by adipose tissue from diabetic rats.

When the results are expressed as units/mg. of protein it is clear that there is only a small decrease

Table 1. *Effect of alloxan-diabetes on hexokinase of adipose tissue*

The results are given as units/two fat pads or as milliunits/mg. of protein in the supernatant fraction. Measurements of hexokinase activity were made before and after heating at 45° for 1 hr., and the activities of types I and II calculated as described in the Methods section. The predominant types are given in parentheses. A unit is defined as the amount of enzyme catalysing the formation of 1 μmole of glucose 6-phosphate/min. at 25°. Starch-gel electrophoresis was used to separate types I and II hexokinase; the gels were stained and scanned with a Joyce–Loebl Chromoscan apparatus. The figures are for the areas under the curves given in arbitrary units. Values are given as means ± S.E.M. Fisher's P values are given; where these exceed 0·1 they are quoted as not significant (N.S.). For details of assay and electrophoresis, see the Methods section.

		Control group	Diabetic group	Fisher's P
(A)	Spectrophotometric assay			
	No. of animals	15	19	
	Wt. of 2 fat pads (g.)	2·60 ± 0·23	0·50 ± 0·07	
	Protein of 2 fat pads (mg.)	22·0 ± 1·4	9·3 ± 0·6	
	Hexokinase (unit/2 fat pads)			
	Without heating (types I + II)	0·230 ± 0·020	0·083 ± 0·10	< 0·001
	Heated (type I)	0·093 ± 0·009	0·049 ± 0·004	< 0·001
	Difference (type II)	0·137 ± 0·013	0·034 ± 0·006	< 0·001
	Hexokinase (milliunits/mg. of protein)			
	Without heating (types I + II)	10·60 ± 0·77	8·90 ± 0·72	0·10
	Heated (type I)	4·27 ± 0·31	5·25 ± 0·27	0·03
	Difference (type II)	6·33 ± 0·54	3·65 ± 0·56	0·002
	Quotient: type II/type I	1·53 ± 0·11	0·68 ± 0·09	< 0·001
(B)	Chromoscan data			
	No. of animals	8	11	
	Hexokinase (type I)	65 ± 10	50 ± 5	N.S.
	Hexokinase (type II)	140 ± 14	72 ± 8	< 0·001
	Quotient: type II/type I	2·5 ± 0·4	1·5 ± 0·2	0·050

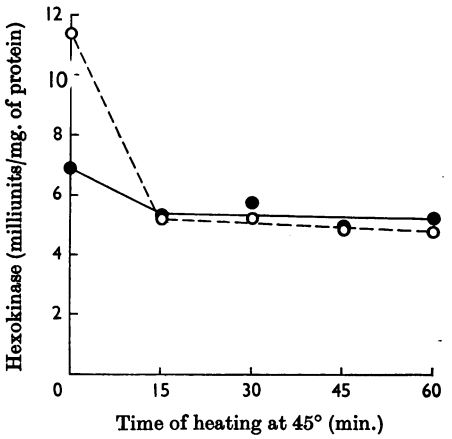

Fig. 1. Effect of heat treatment on the activities of types I and II hexokinase of adipose tissue from normal and alloxan-diabetic rats. Dialysed adipose-tissue extracts from normal and alloxan-diabetic rats were heated at 45° for periods up to 1 hr. After removal of insoluble protein the total hexokinase activity was estimated spectrophotometrically. ○, Activity of extract from normal rat; ●, activity of extract from alloxan-diabetic rat. Starch-gel electrophoresis showed that type II hexokinase was no longer detectable after heating for 1 hr. For details see the Methods section.

in the overall activity of hexokinase in diabetic rats, but that the pattern had altered in that there was a small but significant increase in type I hexokinase and a marked fall in type II hexokinase. The hexokinase type II/type I quotient falls from a control value of 1·53 to 0·68 in the diabetic group. An essentially similar pattern was found by using starch-gel electrophoresis. Here there was little or no change in the type I hexokinase of diabetic rats whereas type II falls to half the control value; once again the hexokinase type II/type I quotient fell.

A time-course of the effect of heat treatment on the hexokinase activity of adipose tissue from normal and diabetic rats is shown in Fig. 1. Essentially all the type II hexokinase is lost in the first 15 min. heating period. The stability of type I hexokinase appears to be the same in alloxan-diabetic and in control rats.

In view of the results of Katzen (1966*a*,*b*) starch-gel electrophoresis was carried out in the absence of β-mercaptoethanol and in some cases in the absence of β-mercaptoethanol and EDTA; these components were omitted from both the homogenizing medium and from the starch gel; however, under the conditions used here type II hexokinaso remained as a single band and did not divide inte types IIa and IIb as reported by Katzen (1966*a*,*b*).

Table 2. *Effect of alloxan-diabetes on hexokinase activity of lung*

The results are given as milliunits/mg. of protein in the supernatant fraction. Measurements of hexokinase activity were made before and after heating at 45° for 1 hr. The predominant types are given in parentheses. A unit is defined as the amount of enzyme catalysing the formation of 1 μmole of glucose 6-phosphate/min. at 25°. Starch-gel electrophoresis was used to separate types I, II and III hexokinase; the gels were stained and scanned with a Joyce–Loebl Chromoscan apparatus. The figures are for the areas under the curves given in arbitrary units. Values are given as means $\pm$ S.E.M. Fisher's P values are given; where these exceed 0·15 they are quoted as not significant (N.S.). For details of assay and electrophoresis, see the Methods section.

		Control group	Diabetic group	Fisher's P
(A)	Spectrophotometric assay			
	No. of animals	6	11	
	Wt. of 2 lungs (g.)	1·74 $\pm$ 0·25	1·75 $\pm$ 0·17	
	Protein of 2 lungs (mg.)	158 $\pm$ 23	150 $\pm$ 12	
	Hexokinase (milliunits/mg. of protein)			
	Without heating (types I+II+III)	6·39 $\pm$ 0·91	7·02 $\pm$ 0·47	N.S.
	Heated (types I+0·5 III)	5·27 $\pm$ 0·65	4·95 $\pm$ 0·53	N.S.
	Difference (types II+0·5 III)	1·12 $\pm$ 0·36	2·07 $\pm$ 0·46	0·12
(B)	Chromoscan data			
	No. of animals	7	13	
	Hexokinase (type I)	175 $\pm$ 17	168 $\pm$ 12	N.S.
	Hexokinase (type II)	50 $\pm$ 6	58 $\pm$ 5	N.S.
	Hexokinase (type III)	22 $\pm$ 5	37 $\pm$ 8	0·15

It is not at present clear why we were unable to obtain these two forms of type II hexokinase, but it is noteworthy that Grossbard & Schimke (1966) have reported that the removal of β-mercaptoethanol from enzyme preparations and from the starch-gel buffer system resulted in no change in the mobility or pattern of any of the enzyme types on starch gel.

Lung. Starch-gel electrophoresis of the supernatant fraction from lung homogenates revealed that there were three hexokinase bands corresponding to types I, II and III. The relative intensities of stain in these bands on the Chromoscan were 175, 50 and 22 respectively (mean values of six control rats). In four of these rats the intensities of stain in each band when heated extracts were used were 162, 5 and 11 respectively. These results correspond well with the changes found by Grossbard & Schimke (1966), using partially purified preparations of the hexokinases, that is there is a loss of 10% of type I hexokinase, 90% of type II and 50% of type III on heating for 1 hr. at 45°.

Katzen & Schimke (1965), using starch-gel electrophoresis, found that type III hexokinase stained intensely at a glucose concentration of 0·5 mM but virtually disappeared at 0·1 M. Grossbard & Schimke (1966) also reported that high glucose concentrations inhibited type III hexokinase but not types I and II. We have also shown, with tissue preparations containing only types I and II hexokinase, that no inhibition occurred even at glucose concentrations as high as 0·1 M, so that any inhibition found with lung preparations presumably arose from type III hexokinase. By using these parameters some approximation to the relative activities of the three forms of hexokinase could be obtained: these were 5·0, 0·8 and 0·6 milliunit/mg. of protein for types I, II and III hexokinase respectively. It was found that there was no significant change in the total hexokinase (types I, II and III) of lung in alloxan-diabetic rats (Table 2) and no significant shift in the pattern of the distribution of activity among the three forms of this enzyme.

DISCUSSION

The possibility that type II hexokinase is related to insulin-sensitivity of tissues was first suggested by Katzen & Schimke (1965) from a survey of the multiple forms of hexokinase in a wide range of tissues. Adipose tissue, which is sensitive to the presence of insulin both *in vivo* and *in vitro*, seemed an ideal tissue in which to test this hypothesis. The divergent results obtained with respect to the activity of type II hexokinase in adipose tissue of normal and alloxan-diabetic rats (Katzen, 1966*a*,*b*; McLean *et al.* 1966) suggest that more complex factors, in addition to the presence of insulin, are controlling the activity of the type II enzyme. Katzen (1966*a*,*b*) demonstrated that, when electrophoresis was carried out in the absence of β-mercaptoethanol and EDTA, type II hexokinase separated into two distinct bands, types IIa and IIb. Type IIa hexokinase was preferentially lost in adipose tissue of starved and diabetic animals. In view of his observations that the

lowering of type II hexokinase was questionable when electrophoresis was carried out in the presence of β-mercaptoethanol, Katzen (1966*a*,*b*) concluded that the presence of thiols *in vitro* was able, at least partially, to mask any changes in types II or IIa hexokinase due to diabetes in adipose tissue, heart, diaphragm and gastrocnemius muscle.

The present results are in agreement with our previous finding that type II hexokinase is decreased in adipose tissue from alloxan-diabetic rats (McLean *et al.* 1966), and data from the spectrophotometric assay and from starch-gel electrophoresis support this view. We were also unable to separate type II hexokinase into two bands in the absence of thiol or of thiol and EDTA. It may be of importance that different methods of inducing diabetes were used. Katzen (1966*b*) used streptozotocin whereas in the present experiments alloxan was used. It is also possible that the time-interval between the onset of diabetes and the hexokinase assay could be of importance. In our experiments considerable changes had occurred in both the weight and the protein content of the epididymal fat pads in the alloxan-diabetic rats 3 weeks after the administration of alloxan and 2 weeks after cessation of insulin treatment.

The changes observed in types I and II hexokinase in adipose tissue from alloxan-diabetic rats are closely similar to the pattern of change in lactating mammary gland from normal and alloxan-diabetic rats (Walters & McLean, 1967), i.e. the hexokinase type II/type I quotient is markedly decreased in the alloxan-diabetic rats, an increase in type I and a decrease in type II both contributing to this change. The many similarities of mammary gland and adipose tissue with respect to pathways of glucose utilization and sensitivity to insulin *in vitro* again support the contention that type II hexokinase is related to the well-established insulin response (Abraham, Cady & Chaikoff, 1957; Winegrad & Renold, 1958; Folley & Greenbaum, 1960; Katz, 1961; Wood, Katz & Landau, 1963; Ball & Jungas, 1964; Landau & Katz, 1965).

Lung was selected for a comparison of the effects of alloxan-diabetes on the multiple forms of hexokinase in another tissue because it is known to have an active pentose phosphate pathway and a high rate of lipid synthesis (Glock & McLean, 1954; Popják & Beeckmans, 1950). Previous work has shown that in lung there was a tendency for hexokinase and glucose 6-phosphate dehydrogenase to be increased in alloxan-diabetic animals, whereas the enzymes 6-phosphogluconate dehydrogenase and citrate-cleavage enzyme (ATP citrate lyase) remained unchanged, in contrast with adipose tissue, where it was found that all these enzymes decreased in activity (J. Brown & P. McLean, unpublished work). The correlation of changes in type II hexokinase accompanying insulin-sensitivity of tissues appears to be supported by the present results on lung hexokinase, where type I hexokinase is the predominant form.

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REFERENCES

Abraham, S., Cady, P. & Chaikoff, I. L. (1957). *J. biol. Chem.* **224**, 955.
Ball, E. G. & Jungas, R. L. (1964). *Recent Progr. Hormone Res.* **20**, 183.
Folley, S. J. & Greenbaum, A. L. (1960). *Brit. med. Bull.* **16**, 228.
Glock, G. E. & McLean, P. (1953). *Biochem. J.* **55**, 400.
Glock, G. E. & McLean, P. (1954). *Biochem. J.* **56**, 171.
González, C., Ureta, T., Sanchez, R. & Niemeyer, H. (1964). *Biochem. biophys. Res. Commun.* **16**, 347.
Grossbard, L. & Schimke, R. T. (1966). *J. biol. Chem.* **241**, 3546.
Katz, J. (1961). In *Radioactive Isotopen in Physiologie Diagnostik und Therapie*, p. 705. Heidelberg: Springer-Verlag.
Katzen, H. M. (1966*a*). *Abstr. 26th annu. Meet. Amer. Diabetic Association*, p. 15.
Katzen, H. M. (1966*b*). *Biochem. biophys. Res. Commun.* **24**, 531.
Katzen, H. M. & Schimke, R. T. (1965). *Proc. nat. Acad. Sci., Wash.*, **54**, 1218.
Katzen, H. M., Sonderman, D. D. & Nitowsky, H. M. (1965). *Biochem. biophys. Res. Commun.* **19**, 377.
Landau, B. R. & Katz, J. (1965). In *Handbook of Physiology vol. 5: Adipose Tissue*, p. 253. Ed. by Renold, A. E. & Cahill, G. F. Washington: American Physiology Society.
McLean, P. & Brown, J. (1966). *Biochem. J.* **98**, 874.
McLean, P., Brown, J., Greenslade, K. & Brew, K. (1966). *Biochem. biophys. Res. Commun.* **23**, 117.
Moore, R. O., Chandler, A. M. & Tettenhorst, N. (1964). *Biochem. biophys. Res. Commun.* **17**, 527.
Popják, G. & Beeckmans, M. L. (1950). *Biochem. J.* **47**, 233.
Salas, J., Salas, M., Viñuela, E. & Sols, A. (1965). *J. biol. Chem.* **240**, 1014.
Sharma, C., Manjeshwar, R. & Weinhouse, S. (1963). *J. biol. Chem.* **238**, 3840.
Viñuela, E., Salas, M. & Sols, A. (1963). *J. biol. Chem.* **238**, PC1175.
Walker, D. G. (1963). *Biochim. biophys. Acta*, **77**, 209.
Walker, D. G. & Rao, S. (1964). *Biochem. J.* **90**, 360.
Walters, E. & McLean, P. (1966). *Biochem. J.* **100**, 58P.
Walters, E. & McLean, P. (1967). *Biochem. J.* **104**, 778.
Winegrad, A. I. & Renold, A. E. (1958). *J. biol. Chem.* **223**, 273.
Wood, H. G., Katz, J. & Landau, B. R. (1963). *Biochem. Z.* **338**, 809.