THE EFFECT OF DIABETES AND INSULIN *IN VIVO* AND *IN VITRO* ON A LOW K_m FORM OF HEXOKINASE FROM VARIOUS RAT TISSUES

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Previous studies suggested that one of the recently discovered multiple forms of mammalian hexokinase, designated type II (K_m for glucose of 10^{-4}M) may have a relationship to the action of insulin (Katzen and Schimke, 1965). The activity of this type II hexokinase is subject to nutritional control in cell culture and rat liver (Katzen, Soderman and Nitowsky, 1965) and epididymal fat pad (Katzen and Schimke, 1965). Moreover, an interesting correlation was noted between the tissue distribution of type II and the insulin sensitivity of the tissue (Katzen and Schimke, 1965). Type II hexokinase itself was reported to exist in fat pad as two electrophoretically distinguishable forms when assayed in the absence of mercaptoethanol (M.E.) and EDTA. In the present study an effect of diabetes and treatment with insulin *in vivo* on one of the two forms of type II in various insulin-sensitive tissues of the rat is demonstrated, as well as an *in vitro* effect of the hormone on this enzyme in diabetic rat heart. In addition, the ability of a thiol to convert one of the forms of type II to the other and thereby mask the effect of insulin is reported.

Of the two forms of type II hexokinase activity in fat pad, the one with the faster mobility (hereafter designated type IIa*; the slower one is designated IIb, cf. Fig. 1) preferentially and distinctly disappeared on fasting the animal (Katzen and Schimke, 1965). However, with M.E. and EDTA

*Since it has not been established whether types II and IIa (which have identical mobilities) constitute identical proteins, they will be distinguished by their method of assay, i.e., type II as the form observed in the presence- and type IIa as the form with identical mobility observed in the absence- of M.E.

added to the assay system, a diminution in the activity of type II* could barely be observed. In order to relate these findings to a reduced insulin secretion known to be associated with fasting, these experiments are now extended to the drug-induced diabetic animal. In Figure 1 it is seen that

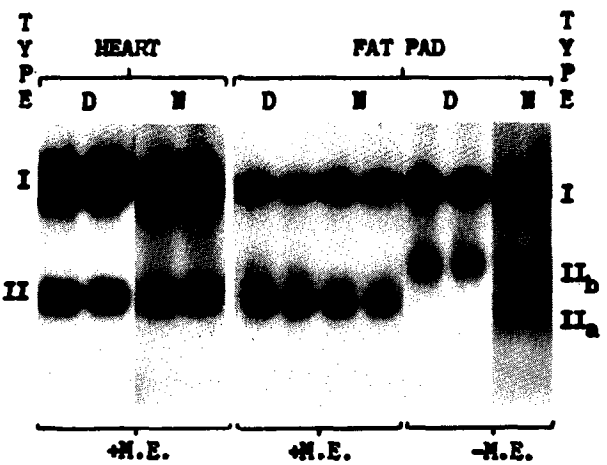

Figure 1. Effect of diabetes on fat pad and heart hexokinases assayed in the presence and absence of 10^{-3} M M.E. in the starch gel electrophoretic assay procedure of Katzen and Schimke (1965). Tissues from 150 g normal (N) and diabetic (D) rats were homogenized with equal volume of 0.1 M Tris buffer, pH 7.5, containing 2.7 x 10^{-3} M EDTA, and after centrifugation at 100,000 x g, resultant supernatants were assayed in duplicate. Gels were stained in 0.1 M glucose-containing developer solution.

type IIa disappeared in the supernatant fraction of crude fat pad homogenates from streptozotocin-diabetic rats fed ad libitum. Injection (i.v.) of the drug streptozotocin results in a highly specific defect of degranulation of the animals' pancreatic β-cells and leads to symptoms typical of those manifested in insulin-deficient animals (Rakieten et al., 1963, Arison et al., 1966, and Glitzer**). It has the advantage over alloxan of being more specific and of exhibiting no generalized cytotoxicity. It is to be noted in Figure 1, however, that in the presence of M.E. in the assay, the faintly stained fat pad type IIb is readily converted to an intensely-stained type II,

**Glitzer, M., personal communication.

and the conclusion of a lowering of type II activity due to diabetes is questionable. Although previous experiments implicated EDTA in this interconversion (Katzen and Schimke, 1965), it is now found that addition of only a thiol, such as M.E. or GSH, is required.

To further evaluate the significance of these observations to the action of insulin, other insulin-sensitive tissues such as heart, diaphragm and gastrocnemius muscles were examined. As expected, when assays were conducted in the presence of M.E., no effect of diabetes was apparent in these tissues; a typical example is seen in Figure 1 with heart muscle. However, when assays were conducted in the absence of added thiol (Fig. 2),

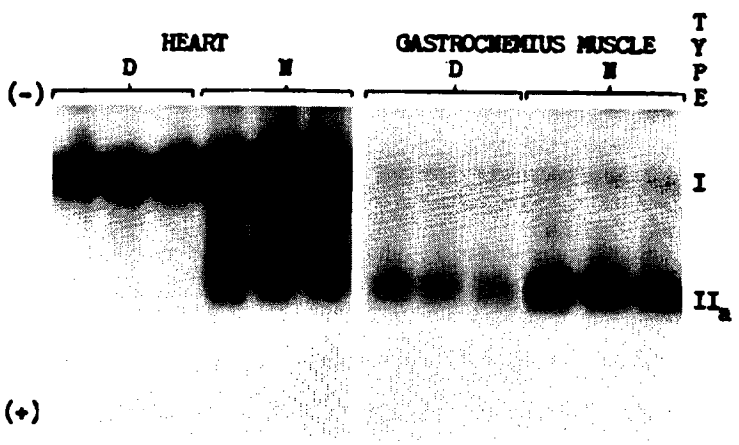

Figure 2. Effect of diabetes (D) on muscle hexokinase activities as compared to normal (N) tissue activities. Assayed in triplicate in absence of M.E. in assay described in Figure 1.

it was readily apparent that IIa activity was virtually absent in the extracts of diabetic rat heart and considerably diminished in diabetic gastrocnemius muscle extracts relative to type I (apparently unaffected by the diabetic state) and compared to the normal tissue type IIa level. Although not visible in Figure 2, type IIb, previously seen in fat pad, was observed as a faint band in electrophoretic assays of heart, gastrocnemius, and diaphragm

muscles. Soluble diaphragm muscle hexokinase activity, now found to consist primarily of type II, with very slight amounts of type I, responds in a manner virtually identical to that seen with gastrocnemius muscle in Figure 2. Although the results indicate the effectiveness of a thiol in at least partially masking the changes in type II or IIa due to diabetes, McLean et al. (1966) utilizing assays only conducted in the presence of M.E. nevertheless recently reported a decrease in fat pad type II hexokinase activity attributable to alloxan diabetes.

A study of the time-course of in vivo restoration of IIa in soluble extracts after administration of insulin to streptozotocin-diabetic rats was conducted according to the usual electrophoretic assay. An initial rise in activity in fat pad extracts is observable in this assay in less than 45 minutes after a single i.v. injection of 4 i.u. of insulin. In diabetic gastrocnemius and heart muscle extracts this initial observable effect occurs in less than 30 minutes. Complete restoration of activity in these tissues requires approximately one hour. The duration of insulin effect on IIa activity is about 2.5 hours in the fat pad and less than an hour in the two types of muscle. This effect of insulin is associated with more tissues—and is exceedingly more rapid—than that observed for liver "glucokinase" (type IV); with this latter form of hexokinase, the action of insulin has been shown to be coupled to a stimulation of new protein (enzyme) synthesis (Sharma et al., 1964, and Sols et al., 1964).

Of considerable significance is the observation of an in vitro effect of insulin on the uncut isolated diabetic rat heart (Fig. 3). Here it is seen that within a 30 minute incubation time, 10^{-6}M insulin is capable of restoring IIa activity relative to type I. In order to test the possibility that insulin and GSH were acting on the enzyme by different mechanisms, the effect of GSH plus insulin was investigated (Fig. 3). Under these circumstances, there appeared to be no additive effect. This result, however, may be attributable to the ability of GSH to reductively inactivate insulin

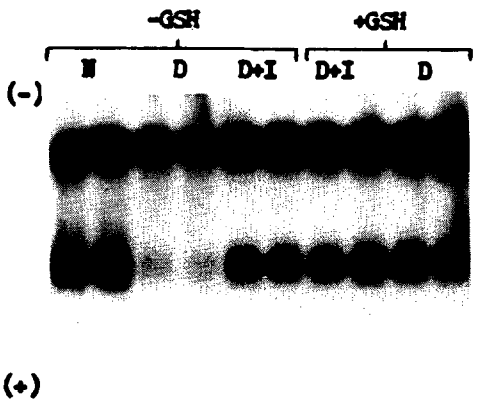

Figure 3. In vitro effect of insulin (I) and/or GSH on heart muscle hexokinases in normal (N) and diabetic (D) rats. Uncut, isolated hearts, 3 per 25 ml stoppered vessel, were incubated with shaking in 0.1 M Tris buffer, pH 7.5, in presence and absence of insulin and/or GSH, 10^{-6}M and 10^{-3}M, respectively, for 30 minutes at 37°C. Assays conducted in duplicate, according to Figure 1.

(Katzen and Stetten, 1962). The addition of GSH, however, masks the effect of insulin. Thus, this action of GSH is analogous to that of M.E. in the in vivo studies.

In most previous studies of hexokinase activity, various versions of the original assay of Slein et al. (1950) have been employed. In the assay or isolation procedures a thiol has usually been included for enzyme stabilization purposes. The present findings of a masking or mimmicking of insulin action by a thiol and the fact that hexokinase exists in multiple forms, not in a single form, must be considered as possible factors in an explanation for the time-honored controversy of insulin action on hexokinase. It is speculated from the present evidence that insulin action may be associated with the several forms of type II hexokinase via a thiol-disulfide interchange reaction between the hormone and a form of the enzyme.

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