Lack of Effect of Insulin and Blood Fractions on Glucose-ATP Phosphotransferases.* (31121)

CHAKRAVARTHI SHARMA†, ALAN GARBER‡ AND SIDNEY WEINHOUSE
Fels Research Institute, and Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pa.

Although a lack of insulin is generally assumed to underlie the many disturbances of carbohydrate metabolism in diabetic animals, the recognition that clinical diabetes may be due in part at least to insulin resistance or non-responsiveness(1-3) has prompted many efforts to establish the existence of insulin inhibitors in serum. Much evidence now exists for the presence of inhibitors of insulin action on glucose transport into muscle and adipose tissue(3-5); however, the question whether specific inhibitors of glucose phosphorylation play a role in glucose utilization is still unresolved. The long, controversial subject of direct effect of insulin and antagonists thereof on the hexokinase reaction((3) pp 131 ff) has been reopened by several new findings. Following earlier observation of Broh-Kahn and Mirsky(6), Abood and Gerard(7), Krahl and Bornstein(8) and Bornstein(9) observed that lipoprotein fractions from rat serum, under certain conditions inhibited heart hexokinase. More recently Ilyin and coworkers(10-12) observed effects of insulin on liver hexokinase *in vitro,* particularly when this activity is inhibited by glucocorticoids; and more strikingly, have found an insulin-reversible inhibition of yeast hexokinase treated *in vitro* with cortisone dissolved in a β-lipoprotein solution. Recently, Vester and Reino(13) reported an insulin stimulation of rat liver hexokinase *in vitro.*

Our recent observation of a unique glucokinase in rat liver with such distinctive properties as a high Km for glucose and requirements for glucose and/or insulin for full activity *in vivo*(14,15) (see also(16-18) for work of others) prompted us to examine the action of insulin and serum on the activity of this enzyme, as well as on the classical type of hexokinase in rat liver and heart. Although our results did not substantiate the claims of previous investigators, we nevertheless felt it worthwhile to make these results known.

Materials and methods. NADP, ATP, N-acetylglucosamine and glucose-6-P dehydrogenase were obtained from Calbiochem, Inc., New York; Na ethylenediamine tetraacetate and glycylglycine from Sigma Chemical Co., St. Louis; β-mercaptoethanol from Eastman Kodak Co.; Triton X100 from Rohm and Haas, Philadelphia; human serum protein fractions from Nutritional Biochemicals Corp., Cleveland; and the glucagon-free pork insulin, lot no. 499667, was a generous gift of the Eli Lilly Laboratories, Indianapolis.

The animals were male rats, 2 to 4 months old, obtained from Carworth Farms, New City, N. Y., and were rendered diabetic by alloxan injection as described previously(15). All were maintained on a commercial Checker diet. Serum and plasma were obtained by standard procedures after collection of the blood by decapitation and collecting the blood in an open beaker, with or without added heparin. For enzyme assay the freshly excised liver after decapitation was homogenized as described previously and centrifuged at 100,000 $\times$ *g* at 2-4°(15). Heart muscle was homogenized in the same medium in the same manner, except for addition of 0.3% Triton X100 to "solubilize" the particulate hexokinase(15), followed by short centrifugation at low speed to remove gross particles.

Assays were conducted exactly as described previously(15) using either a Zeiss PMQ II spectrophotometer or a Gilford spectrometer with automatic cuvette positioning. All assays were conducted in a total volume of 0.4 ml in quartz microcuvettes with the temperature maintained constant at 22-25°. When serum,

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† Present address: Radioisotope Laboratory, Pharmacology Dept., Seth G. S. Medical College, Parel, Bombay, India.

‡ Summer student fellow.

TABLE I. Effect of Insulin *in vitro* on Glucose-ATP Phosphotransferase Assay in Rat Liver. Conditions of the assay are described in experimental section. Each sample was obtained from a separate, normal rat.

	Treatment									
	None		Water		Insulin 300 mu/ml		Insulin 600 mu/ml		Difference, col. 5—col. 2	
Sample	Hk	Gk	Hk	Gk	Hk	Gk	Hk	Gk	Hk	Gk
1	.22	1.36	.25	1.32	.22	1.30	.19	1.38	—.06	+.06
2	.18	1.26	.22	1.30	.19	1.35	.25	1.25	+.03	—.05
3	.20	1.40	.18	1.45	.25	1.48	.21	1.37	+.03	—.08
4	.24	1.45	.23	1.45	.23	1.43	.20	1.50	—.03	+.05
5	.19	1.21	.21	1.28	.24	1.32	.26	1.36	+.05	+.08
Mean	.21	1.34	.22	1.36	.23	1.38	.20	1.37	+.02	+.06
S.D.	.02	.10	.03	.08	.02	.07	.04	.09	.05	.07

plasma, insulin or the lipoprotein preparations were added, they replaced an equal volume of the buffer solution, so as to keep the volume at 0.4 ml. The fraction V human albumin solution was prepared by dissolving 200 mg of the solid in 5 ml of the homogenization medium of pH 6.8 and stirring a few minutes. The fraction III human serum β-globulin was dissolved in the same buffer with the aid of Triton X100 at 1% concentration. This yielded a slightly turbid, but stable suspension that was satisfactory for absorbance measurements.

Insulin solutions containing 12.5 and 25 units per ml, respectively, were prepared by dissolving 5 mg of the solid in 5 and 10 ml of 0.25 M glycylglycine buffer at pH 6.8. Equal volumes of these solutions were incubated with the liver preparation for exactly 4 minutes at 30°, then 20 μl of each solution was used for hexokinase and glucokinase assay. At the same time, the enzyme preparation itself, and a control, incubated with water instead of insulin, were also assayed.

Results. To test whether insulin *in vitro* might have a stimulating effect on liver hexokinase and glucokinase, we treated liver preparations with insulin in a manner similar to the procedure used by Vester and Reino, except that the treatment with insulin was not carried out directly in the cuvettes. Instead, the preparation after 4 minutes of insulin treatment at 30° was added to the cuvette just prior to beginning of the assay. The results, shown in Table I, demonstrate that insulin has no significant effect on either hexokinase or glucokinase, even at a concentration which, according to Vester and Reino, should have enhanced the activity of "total" hexokinase 50%. Neither the treatment with water nor that with two different insulin concentrations altered the assay perceptibly.

The differences in hexokinase activity due to addition of insulin ranged from —0.04 to +0.07 unit/g, with a mean of the differences of +0.02 unit. The differences in glucokinase ranged from —0.03 to +0.15, with a mean of +0.04 unit. Obviously these small effects are well within the experimental error of the assays and are of no significance. It is evident by comparison of columns 2 and 3 that treatment with water itself does not affect either enzyme; therefore insulin is not restoring or protecting these activities, which otherwise might have decreased.

In the experiments of Table II, tissue extracts were mixed with equal volumes of normal or diabetic rat serum or plasma and were then assayed for glucose-ATP phosphotransferase activities. As observed previously (15) the activity of hexokinase in rat heart (this tissue contains no glucokinase) was slightly, but not significantly, lowered in diabetic rats. With neither tissue type did the addition of either normal or diabetic rat serum or plasma lead to a significant alteration in activity, the differences being invariably well within the relatively low experimental error. In normal rat liver, the same was true for both hexokinase and glucokinase; and in "diabetic" livers, the characteristic low glucokinase activities were not affected by similar treatment.

To reveal whether high levels of inhibitors might exert effects not evident at lower concentrations, the assays were conducted in the

TABLE II. Effect of Normal or Diabetic Rat Serum on Rat Heart and Liver Glucose-ATP Phosphotransferases.

	Tissue	No. of assays	Activity, munits/g tissue	Change in activity on serum addition, munits/g tissue: Normal	Diabetic
Normal	Rat heart	5	1800 ± 40	20 ± 40	30 ± 30
	Rat liver—Hexokinase	5	190 ± 10	50 ± 20	30 ± 30
	Glucokinase	5	1570 ±100	0 ± 50	30 ± 50
Diabetic	Rat heart	4	1600 ± 50	—20 ± 30	—40 ± 30
	Rat liver—Hexokinase	4	180 ± 20	20 ± 20	10 ± 20
	Glucokinase	4	210 ± 20	—10 ± 20	0 ± 20
				Change in activity on plasma addition: Normal	Diabetic
Normal	Rat heart	5	1670 ± 60	10 ± 30	—10 ± 40
	Rat liver—Hexokinase	5	220 ± 20	10 ± 10	0 ± 40
	Glucokinase	5	1730 ± 30	—10 ± 50	—30 ± 50
Diabetic	Rat heart	5	1460 ± 70	—20 ± 40	—40 ± 30
	Rat liver—Hexokinase	5	190 ± 10	10 ± 20	20 ± 20
	Glucokinase	5	200 ± 10	20 ± 20	—10 ± 30

presence of normal or diabetic plasma up to as much as 50% of the total assay volume. Again, as shown in Fig. 1, neither normal nor diabetic plasma nor human β-lipoprotein lower rat liver glucokinase activity; nor did diabetic plasma or β-lipoprotein inhibit rat heart hexokinase. A moderate enhancement of heart hexokinase by lipoprotein was observed; however, a protective action of protein on enzyme activity is not unusual.

Discussion. These experiments are typical of many others carried out by us demonstrating the lack of effect of insulin or rat serum or plasma, or various serum fractions on heart and liver glucose-ATP phosphotransferases. The use of a spectrophotometric procedure allowed detailed continuous examination of the course of the reaction. In all instances the rate curves were perfectly normal, and the slopes were proportional to enzyme concentration, and linear with time over the 10-minute period of measurement. There were no unusual blanks or lag periods. Consequently, we assume that under the conditions used, these substances do not affect the activities of these enzymes.

Although the role of insulin in maintaining high levels of rat liver glucokinase is well established(15,16), our results failed to confirm the report of Vester and Reino(13) that insulin stimulates the activity of this enzyme by short contact *in vitro*. It is difficult to make strict comparisons, since Vester and

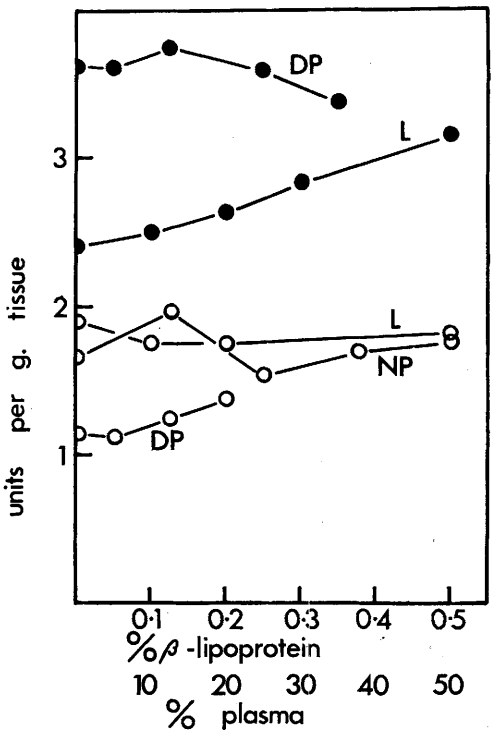

FIG. 1. Effect of varying quantities of normal and diabetic rat plasma and human serum β-lipoprotein on hexokinase and glucokinase activities. DP, diabetic rat plasma; NP, normal rat plasma; L, human β-lipoprotein. Closed circles, heart hexokinase; open circles, liver glucokinase.

Reino measured only total phosphotransferase activity, and their experiments were not carried out at optimal glucose concentration for glucokinase. Moreover, this enzyme is quite labile, particularly in the absence of a stabilizer such as β-mercaptoethanol(15).

Our failure to observe effects of normal or diabetic serum or plasma on the glucose-ATP phosphotransferase reactions speaks against the presence of inhibitors of these enzymes in the body fluids. However, they do not argue against the existence of inhibitors of glucose transport(3-5). They also do not discount possible significant indirect effects of insulin lack, mediated *via* intermediate enzyme reactions. Morgan *et al*(14) have observed a lowering of glucose phosphorylation in intact, perfused diabetic rat heart; however, our present results in confirmation of previous results(15) reveal that there is no appreciable effect of alloxan diabetes on the hexokinase activity of rat heart when assayed under optimal conditions. Recent findings of Randle and coworkers(19) indicate that the mobilization of fatty acids occurring as a result of insulin lack may set into motion a train of events leading to functional inhibition of hexokinase. The immediate effect, according to this concept, is the accumulation of glucose-6-P, owing to inhibition of phosphofructokinase caused by an increase of citrate concentration brought about by elevated levels of fatty acids or certain of their intermediary metabolites, such as long chain acyl or acetyl CoA. Such indirect effects of altered intermediary metabolism may be more important than specific serum inhibitors in causing the apparent non-responsiveness to insulin in diabetes.

Summary. Glucose-ATP phosphotransferase activities of normal and diabetic rat heart and liver were assayed in the presence of normal and diabetic rat serum and plasma and various human serum protein fractions, and in the presence of insulin. No significant alterations in the activities were observed under a variety of conditions.

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