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LIVER MICROSOMAL INORGANIC PYROPHOSPHATE–GLUCOSE PHOSPHOTRANSFERASE AND GLUCOSE-6-PHOSPHATASE EFFECTS OF DIABETES AND INSULIN ADMINISTRATION ON KINETIC PARAMETERS

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SUMMARY

1 Alterations in kinetic properties of rat hepatic glucose-6-phosphatase (D-Gluc-6-*P* phosphohydrolase, EC 3 1 3 9) and associated PP_i-glucose phosphotransferase resulting from alloxan diabetes and insulin treatment of such diabetic animals have been studied

2 Maximal reaction velocity values extrapolated to infinite substrate concentrations (v_{max}) and Michaelis constant (K_m) values for all substrates were assayed with liver homogenates prepared from eight untreated normal, seven alloxan-diabetic, and seven insulin-treated diabetic animals Average enzymic activity levels (units/mg liver protein) for the various groups of animals were calculated on the basis of v_{max} values

3 All assays were carried out both in the absence of added detergent and with homogenates which had been supplemented with sodium deoxycholate to 0 20% (w/v)

4 As determined either in the presence or absence of detergent, K_m values for glucose were not affected significantly by hormonal manipulations

5 K_m values for both Glc-6-*P* and PP_i increased significantly in diabetes, based on assays carried out in the absence of detergent These elevations were completely reversed by administration of insulin to diabetic animals, and were not apparent when homogenates prepared from livers of all three groups of animals were supplemented with deoxycholate prior to assay

6 Most significantly, preliminary treatment of homogenates with the detergent markedly potentiated responses of activity levels, based on extrapolated v_{max} values, to diabetes The ratio of Glc-6-*P* phosphohydrolase activity level in diabetic animals to activity level in normal animals increased from a value of 1 5, noted in the absence of detergent, to 3 5 based on assays with deoxycholate-supplemented homogenates Even more dramatic potentiating effects of detergents on the diabetes response were noted with PP_i–glucose phosphotransferase With this activity, the ratio of v_{max}-

based activity levels in diabetic animals to v_{max}-based activity levels in normal animals increased from a statistically insignificant value of 0 90 in the absence of detergent to 4 2 when assessed in the presence of deoxycholate Administration of insulin to diabetic rats reversed this response of v_{max}-based activity level values These observations confirm our previous findings on the potentiating effect of detergent on responses of enzymic activity levels to experimental diabetes, and further, reveal that this effect persists even with substrate levels extrapolated to infinity and hence is not explainable simply on the basis of alterations in affinity of enzyme for substrates under the various experimental conditions

7 These patterns of effects of diabetes on kinetic parameters of the enzyme, as assessed both in the absence and presence of detergent supplementation of homogenates, closely resemble those previously noted in acutely fasted animals and contrast sharply with glucocorticoid-induced alterations

INTRODUCTION

Earlier studies in this laboratory and elsewhere have suggested that interesting differences exist in the modes of responses of microsomal D-Gluc-6-*P* phosphohydrolase, EC 3 1 3 9)[1–7], and PP_i-glucose phosphotransferase[1–5,8] also catalyzed by this enzyme[9–11], to glucocorticoid administration, fasting, and alloxan diabetes The preliminary treatment of enzyme preparations with detergents nearly or completely abolished the response of activities to glucocorticoid therapy[1,2,5,8], and in contrast, markedly accentuated increases in activity levels in response to the latter two experimental treatments[3–5,8] Kinetic studies have revealed that these differences in patterns of response of enzymic activities to glucocorticoid administration[1] and acute fasting[3] persist even at infinite substrate concentrations and hence are not explainable simply on the basis of alterations in Michaelis constant values induced by such treatments *in vivo*

SEGAL AND WASHKO[6], and more recently FREEDLAND AND BARNES[12] and JAKOBSSON AND DALLNER[13], have noted differences in K_m values for Glc-6-*P* phosphohydrolase in livers from normal and diabetic animals in the absence of detergents It was noted[6,13] that these differences, observed under certain conditions[13], disappeared when microsomers or homogenates were treated with various detergents With the idea in mind that differences in the extent of response of PP_i-glucose phosphotransferase activity of this same enzyme to experimental diabetes, which we previously[5,8] noted in the absence compared with presence of detergent treatment of enzyme preparations, might be a reflection of hormonally altered affinities of the enzyme for substrates, we have carried out rather thorough kinetic analysis of this activity in normal, diabetic, and insulin-treated diabetic animals Results of these studies as well as those of Glc-6-*P* phosphohydrolase activity which was studied concurreutly for comparative purposes, are reported in this paper, and patterns of response of K_m and v_{max} values, observed in the absence and presence of supplemental detergent, are compared with alterations produced in these same parameters under identical assay conditions by glucocorticoid administration[1] and fasting[3]

MATERIALS AND METHODS

Sources of most chemicals[1,9,14] and analytical procedures for measuring enzymic activities[15], protein[16], and blood sugar concentration[17] were as described previously Alloxan monohydrate, obtained from Distillation Products, Inc , Rochester, N Y , was twice recrystallized before use Young adult, male, albino rats obtained from Sprague–Dawley Inc , Madison, Wisc , utilized in all studies, were maintained on Purina Laboratory Chow and distilled water, *ad libitum*, except that water was replaced by a 10% (w/v) glucose solution for 24 h after injection of alloxan

Diabetes was produced by the intraperitoneal injection of 145 mg alloxan per kg body weight, as in previous studies[5,14] Animals weighed between 90 and 100 g at the time of alloxan treatment Diabetic animals were killed or further treated with insulin at least 12 days after alloxan injections Only rats with blood sugar levels exceeding 300 mg per 100 ml were considered diabetic One group of diabetic animals, selected at random, was injected with 8-units doses of Lilly Pork NPH Iletin Isophane insulin suspension, 60, 36, and 12 h before animals were killed Average blood sugar levels, liver and body weights, and liver protein concentrations for the various groups of animals are given in Table I

Animals were decapitated and livers were removed, weighed, and homogenized at 0° in 0 25 M sucrose as in previous studies[1–5,14] Homogenates then were appropriately diluted with this sucrose solution, and aliquots were further supplemented with aqueous deoxycholate to a final concentration of 0 20% (w/v) Previous[1,5,18] and supplementary studies revealed that this concentration of the detergent maximally activated both phosphotransferase and phosphohydrolase activities of the enzyme from both normal and diabetic animals over a broad range of protein concentrations (0 15 – 1 1 mg protein per 1 5-ml reaction mixture)

Glc-6-*P* phosphohydrolase assay mixtures (pH 6 5) contained in 1 5 ml 40 mM sodium cacodylate buffer and sodium Glc-6-*P* concentrations varying between 0 97 and 9 66 mM Phosphotransferase activity was measured at pH 5 5 in assay mixtures of 1 5 ml volume containing 40 mM sodium cacodylate buffer and (a) D-glucose concentrations maintained constant at 28 2, 37 6, 62 6, or 188 mM and sodium PP_i concentrations varying between 0 67 and 5 0 mM, or (b) sodium PP_i concentrations maintained constant at 0 67, 1 00, 1 67, or 5 00 mM and D-glucose concentrations varying from 28 2 to 188 mM Incubations were in all cases carried out for 10 min at 30 ± 0 1°, with shaking Supplementary experiments indicated that under these conditions activity was first order with respect to both time of incubation and protein concentration with all types of preparations studied

Experimental results were subjected to statistical analysis[18] and are expressed as mean values ± standard deviations of means The Student *t* test of significance[19] was applied to mean activity values for the various groups of animals, and *P* values (the probability of observed differences in mean values for normal compared with diabetic or insulin-treated diabetic animals being due solely to chance) are indicated in Table I Differences in mean values for which $P < 0\ 05$ are considered significant statistically

RESULTS

Both PP_i–glucose phosphotransferase and Glc-6-*P* phosphohydrolase activities from livers of normal, diabetic, and insulin-treated diabetic rats were subjected to kinetic analysis Activities were studied both in the absence and presence of deoxycholate supplementation (to 0 20%, w/v) of homogenates Results of representative experiments are presented, for illustrative purposes, in Figs 1A–1D Similar sets of studies were carried out, both in the absence and presence of detergent supplementation, with homogenates from eight normal, seven diabetic, and seven insulin-treated diabetic rats

Glc-6-*P* phosphohydrolase activity was measured as a function of substrate concentration, and data were plotted in conventional double-reciprocal fashion[20] (see Fig 1D) K_m and v_{max} values were calculated, respectively, as the negative reciprocals of the *x*-axis intercepts and as the reciprocals of the *y*-axis intercepts of extrapolations of such plots[21]

PP_i–glucose phosphotransferase activity in liver homogenates from all groups of animals was subjected to "two-substrate" kinetic analysis of the type previously carried out with the partially purified enzyme by ARION AND NORDLIE[22] PP_i concentrations were held at four constant levels (see experimental procedure above), and activity was measured as a function of varying glucose concentrations, and *vice versa* Primary double-reciprocal plots such as those in Figs 1A and 1B were prepared from data obtained, and secondary plots (see Fig 1C) were then constructed in which *y*-axis intercepts of the lines in the primary plots were plotted against reciprocals of concentrations of either PP_i (from Fig 1A, for example) or glucose (from Fig 1B

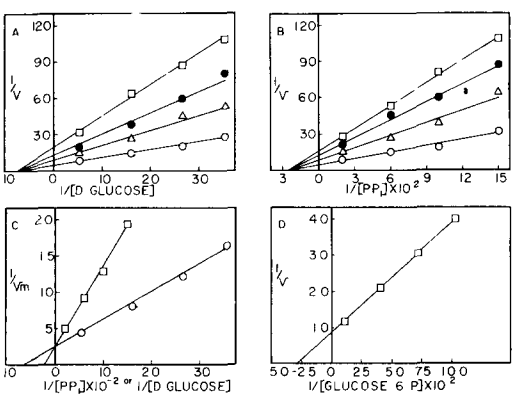

Fig 1 Representative kinetic experiments Data presented were obtained in the absence of deoxycholate supplementation with liver homogenate from a diabetic rat Reaction mixture compositions and other experimental details are given in the text A and B are primary double-reciprocal plots of data obtained with phosphotransferase activity, while C is a secondary plot[23] of *y*-axis intercepts from graphs A and B against reciprocals of molar concentrations of glucose (○) or PP_i (□) v_{max} and K_m values were calculated from these plots as described in the text In A, PP_i concentrations were held constant at 0 67 (□), 1 00 (●), 1 67 (△), or 5 00 (○) mM and initial reaction velocity, *v*, was measured as a function of glucose concentration In B, glucose was held constant at 27 4 (□), 36 5 (●), 60 8 (△), or 183 (○) mM and *v* was measured as a function of varied PP_i concentrations D describes results obtained with Glc-6-*P* phosphohydrolase activity. K_m was calculated directly as $-1/x$-axis intercept and v_{max} as $1/y$-axis intercept Average values for kinetic parameters calculated from these and like studies with enzyme preparations for normal, diabetic, and insulin-treated diabetic animals are given in Table I

TABLE I

AVERAGE K_m AND v_{max} VALUES, LIVER AND BODY WEIGHTS, LIVER PROTEIN AND BLOOD GLUCOSE CONCENTRATIONS, AND ACTIVITY RATIOS IN NORMAL, DIABETIC, AND INSULIN-TREATED DIABETIC ANIMALS

	Experimental animal					
	Normal		*Insulin-treated diabetic*		*Diabetic*	
	Deoxycholate absent	*Deoxycholate present*	*Deoxycholate absent*	*Deoxycholate present*	*Deoxycholate absent*	*Deoxycholate present*
Number of rats	8		7		7	
Body weights (g)	276.8 ± 16.7*		186.1 ± 6.7**		170.3 ± 17.1**	
Liver weights (g)	11.4 ± 0.5		12.9 ± 0.8		9.2 ± 1.0	
Liver protein concentrations (mg protein/g wet liver)	181 ± 4		167 ± 5		216 ± 5**	
Blood glucose concentrations (mg/100 ml)	100 ± 2		45 ± 3**		520 ± 22**	
Michaelis constant values***						
$K_{Glc\ 6\ P}$	2.2 ± 0.2	1.7 ± 0.1	2.0 ± 0.1	1.4 ± 0.02	3.1 ± 0.2**	0.95 ± 0.08**
K_{PP_1}	2.7 ± 0.3	1.3 ± 0.1	3.4 ± 0.2	1.0 ± 0.1	3.6 ± 0.1**	1.2 ± 0.1
K_{Glc}	127 ± 6	103 ± 3	142 ± 7	102 ± 2	136 ± 7	100 ± 4
Enzymic activity levels based on v_{max} values§						
Glc-6-*P* phosphohydrolase	0.79 ± 0.04	0.91 ± 0.04	0.70 ± 0.04	0.83 ± 0.05	1.21 ± 0.05**	3.16 ± 0.10**
PP_1–glucose phosphotransferase	0.54 ± 0.07	1.20 ± 0.08	0.54 ± 0.04	1.05 ± 0.07	0.49 ± 0.03	5.07 ± 0.47**
v_{max} (treated)/v_{max} (normal)						
Glc-6-*P* phosphohydrolase			0.89	0.91	1.54	3.49
PP_1–glucose phosphotransferase			1.00	0.88	0.90	4.23

* All values in the table are presented as mean values ± S.E.

** $P < 0.05$. P notation applies between anotated value and comparable value for normal rats.

*** Michaelis constant values are expressed as mmolar concentrations.

§ Enzymic activity values are presented in terms of 10^{-3} units of activity per mg of liver protein. 1 unit of enzymic activity is that amount catalyzing the hydrolysis (phosphohydrolase) or formation (phosphotransferase) of 1 μmole of Glc-6-*P* per min under the defined conditions.

for example)[23] Maximal reaction velocity (v_{max}) values, extrapolated to infinite concentrations of both substrates, were then determined as the reciprocals of the y-axis intercepts of such secondary plots[23] K_m values were calculated as $-1/x$-axis intercepts of extrapolations of these secondary plots[23] As in previous studies[3,22,24], K_m values for both PP_i and glucose were in all instances found to be independent of concentration of second substrate, as indicated by common points of convergence of extrapolations of primary plots on the x-axis in each experiment (see Figs 1A and 1B, for example) Average K_m values for all substrates, as well as v_{max} values, are recorded in Table I The latter values are expressed as specific enzymic activity—10 × units of activity per mg liver protein—in the table

Glc-6-P phosphohydrolase responses

Data in Table I relating to Glc-6-*P* phosphohydrolase activity confirm the original findings of SEGAL AND WASHKO[6] in that (a) K_m values for Glc-6-*P* assayed in the absence of deoxycholate were significantly (approx 50%) increased in diabetic compared with normal, control animals, and (b) activity levels based on v_{max} values were significantly higher in diabetic than in normal animals regardless of whether or not detergent treatment was employed, although the ratio of activity in diabetic animals to that in normal animals was considerably higher in the presence (ratio value 3 5) than in the absence (ratio value 1 5) of detergent This latter difference in the extent of response to diabetes noted with detergent supplementation also is apparent from the data of SEGAL AND WASHKO[6], although not discussed by these workers While the latter investigators noted no significant difference in K_m value for Glc-6-*P* with preparations from the two groups of animals as assayed in the presence of detergent, our data indicate a small, but statistically significant, decrease in the values for diabetic as compared with normal animals when determined with deoxycholate-supplemented preparations Our studies extend the interesting findings of SEGAL AND WASHKO[6] in that we have observed (see Table I) that the diabetes-induced alterations in v_{max} and K_m values are returned to essentially normal, control values by insulin treatment, this generalization holds for values determined on the basis of assays carried out both with and without detergent supplementation of homogenates JAKOBSSON AND DALLNER[13] recently have observed that repeated injections of insulin at intervals for 36 (but not 5) h after alloxan administration normalize both v_{max} and K_m values for this activity which otherwise are apparent in alloxan-treated animals

PP_i–glucose phosphotransferase responses

As with K_m values for Glc-6-*P*, the K_m for PP_i in the phosphotransferase reaction was increased significantly in diabetes, based on assays in the absence of detergent, while no significant differences in values for this parameter in preparations from diabetic compared with normal animals were apparent when deoxycholate was present Mean K_m values for glucose in diabetic and insulin-treated diabetic groups of animals were slightly, but not statistically significantly, higher than those of normal rats Most strikingly, it is apparent from these studies that activity levels, based on v_{max} values extrapolated to infinite concentrations of both substrates, did not vary significantly among the three groups of animals when assessed in the absence of detergent supplementation, but that a dramatic elevation (a more than 4-fold increase)

was apparent in diabetes when all preparations were supplemented with deoxycholate before assay Further, this large elevation in activity levels as assayed under these latter conditions was completely reversed by insulin treatment of diabetic rats

DISCUSSION

The experimental results presented in this paper confirm and extend earlier observations made both by ourselves[5] and by others[6,13] with Glc-6-*P* phosphohydrolase activity, and in this laboratory[5,8] with PP_i–glucose phosphotransferase activity * It is clear from the present studies that K_m values for PP_i, as well as for Glc-6-*P* (refs 6, 13) are elevated in diabetes, but that the differences noted with both phosphate substrates are apparent only in the absence of detergent treatment of homogenates SEGAL AND WASHKO[6] have suggested that these detergent-sensitive alterations in K_m values for the sugar phosphate ester in diabetes might be due either to enhanced action of a competitive inhibitor or to alterations in microsomal membranes in diabetes The present authors believe that the latter explanation is the more likely one, since agents affecting the lipoprotein membrane of which glucose-6-phosphatase either is an integral part or to which it is very tightly bound[25]—detergents (see, for example, refs 1, 5, 6, 8, 9, 12, 18, 26–30), organic solvents[9,30], phospholipids and phospholipases[26,32,33] and NH_4OH at high pH (ref 34)—strikingly affect catalytic properties of the various activities of the enzyme Inhibition by alloxan of glucose-6-phosphatase has been reported by BROH-KAHN *et al* [35], JAKOBSSON AND DALLNER[13] have recently shown that this compound, studied *in vitro*, somewhat activates microsomal Glc-6-*P* phosphohydrolase activity and also increases K_m for substrate with untreated liver microsomes, but not with enzyme prepared by deoxycholate treatment of microsomes We have been unable to obtain any effects *in vitro* of alloxan, in the concentration range studied by the latter workers[13], on activity levels or K_m values for either PP_i–glucose phosphotransferase or Glc-6-*P* phosphohydrolase activity of liver microsomes under the assay conditions employed in the present studies On the basis of these observations, and in view of the marked instability of alloxan in aqueous solution (see ref 36), it seems highly unlikely that alterations in kinetic parameters, which we noted with preparations from diabetic animals killed 12 or more days after administration of alloxan, could be manifestations of direct effects of this compound on the enzyme

The observed diabetes-induced alterations in K_m values (detergent absent) appear to be subordinate in significance to the noted changes in v_{max}-based activity level values The present findings confirm our earlier observations that responses of activity levels of both phosphotransferase and phosphohydrolase to diabetes are markedly enhanced when detergent treatment of enzyme preparations precedes assay

* In an earlier study[8] in which enzymic activity levels in normal and diabetic animals were measured as a function of assay mixture pH, the potentiation by deoxycholate of the diabetic response was most striking between pH 5 5 and 7 A lesser, but still significant, detergent effect was also apparent at pH 4 5 and 5 More detailed studies of the effects of various concentrations of deoxycholate and Triton X-100 on the response of phosphotransferase activity to experimental diabetes (and also to cortisone administration) were carried out at pH 5 5 (see ref 5) In investigations supplementary to the former series of studies[8], insulin treatment (5 units of protamine–zinc insulin daily for 3 days) of diabetic animals was found to return both PP_i–glucose phosphotransferase and Glc-6-*P* phosphohydrolase activity levels towards normal at all assay pH values (R C NORDLIE, J R GILSDORF AND R N HORNE, unpublished observations)

Further, it is now clear that this differential response pattern is not explainable in terms of alterations in the affinity of the enzyme for substrates, for these same patterns of variation in normal and diabetic preparations previously noted with mixtures with single, constant substrate concentrations, are still abundantly apparent from an examination of activity values extrapolated to infinite concentrations of substrates A precise mechanistic explanation for the marked differences in extents of response of activity levels to diabetes noted with and without detergent still remains to be found However, the fact that this effect is much more pronounced with phosphotransferase than with phosphohydrolase (see last two horizontal lines in Table I) correlates directly with the previously observed much greater sensitivity of the former than the latter activity to activation by both natural and synthetic detergents[1,5,6,8,9,12,18,26–30], as well as to phospholipase treatment and phospholipid supplementation[26,32,33], and suggests to the authors that conformational alterations in this membrane-bound microsomal enzyme, with concomitant differential, activity-discriminating effects on the active site, may well be involved (along with stimulated synthesis of new enzyme protein[13,37]) in the enzyme's response to diabetes KRAHL[38] previously has hypothesized in a general manner the effects of insulin on lipoprotein membranes of microsomes

The K_m values obtained for glucose are of interest in that they did not vary significantly form normal diabetic animals in which a physiological synthetic role for the phosphotransferase activity previously[13,14,24,39,40] has been suggested These kinetic parameters for the animals in various hormonal states, presented in Table I, should be of some practical use in the interpretation of results of physiological studies relating to the phosphorylative role of the enzyme, and indeed they already have been thus employed (see ref 41)

The question[5,40] of whether activity levels and kinetic parameters evaluated with, or without, detergent supplementation best reflect properties of the enzyme in the intact cell still cannot be answered with certainty However, as SEGAL AND WASHKO[6] have pointed out, alterations in K_m values for Glc-6-*P* in diabetes are not a reflection of the ultimate intrinsic properties of the enzyme, for detergents abolish these differences Extending this same argument, we suggest that the differences in activity levels in the various groups of animals noted in the presence of detergent best reflect such intrinsic catalytic properties of the enzyme *in vivo* As we[11,28,40] have pointed out previously, the precise behavior *in vivo* of this catalyst may more or less closely approach that observed with the maximally activated enzyme *in vitro*, depending in part on the intracellular concentrations of such naturally occurring detergents as long-chain fatty acyl CoA esters and lysolecithins

Finally, a comparison of the responses of both phosphotransferase and phosphohydrolase activities of the enzyme to acute fasting[3,4], glucocorticoid treatment[1], and diabetes and insulin administration can now be made from a strictly kinetic point of view That is, it is now possible to distinguish clearly the effects of such treatments on the affinity of enzyme for substrates from effects directly on velocities Variations in the enzyme in diabetes closely resemble those previously noted in acutely (48 h) fasted rats[3], and markedly contrast with responses of the enzyme following glucocorticoid administration[1] The latter treatment was without effect on K_m values and produced elevations in v_{max} activity values which were nearly or completely abolished by detergent action[1] In contrast, both diabetes (this study) and fasting[3] produced

detergent-abrogated increases in K_m values for phosphate substrates and detergent-potentiated increases in activity levels based on v_{max} values. Since these effects in the diabetic animal were reversed by insulin administration (see Table I), it appears likely that the depression in levels of insulin in blood of the hypoglycemic, fasting rat[42] may also be the triggering mechanism producing the enzymic responses noted previously[3,4] in such animals.

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